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Biotechnology of Natural Products

Functional characterization of *Fra a 1* genes in strawberry plants

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For

My parents

My husband

My son

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Publications

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Franz-Oberdorf K, Eberlein B, Edelmann K, Hücherig S, Besbes F, Darsow U, Ring J, Schwab W (2016) Fra a 1.02 is the most potent isoform of the Bet v 1-like allergen in strawberry fruit. *Journal of Agricultural and Food Chemistry* 64:3688−3696.

Posters

Besbes F, Schwab W. Phosphorylation of *Fra a 1* genes. International symposium of DGG, BHGL & WeGa, Freising, 24.-28. February 2015.

Besbes F, Schwab W. The *Fra a* gene family in *Fragaria* × *ananassa*. The conference "Plant Biology for the Next Generation" and the International Symposium of the SFB924 "Molecular mechanisms regulating yield and yield stability in plants", Freising, 18.-20. September 2013.

Besbes F, Schwab W. 3D structural elucidation of *Fra a* allergens by NMR analysis. 1st student retreat of SFB924, Königsdorf, 29.-31. October 2012.

Table of Contents

Abbreviations

ABA	abscisic acid
ANS	1-anilino-8-naphthalene sulfonate
AP	Alkaline Phosphatase
APS	ammonium persulfate
ATP	adenosine-5'-triphosphate
bp	base pair
BCIP	5-bromo-4-chloro-3-indolyl phosphate
Bet v 1	*Betula verrucosa*
BLAST	Basic Local Alignment Search Tool
BSA	bovine serum albumin
C	catechin
$CaCl_2$	Calcium chloride
CCR3	C-C chemokine receptor type 3
cDNA	complementary DNA
CIP	calf intestinal Alkaline phosphatase
cNL	complementary nanolever
Ct	threshold cycle
CTAB	cetyltrimethylammonium bromide
$C_2H_3KO_2$	potassium acetate
dATP	deoxyadenosine triphosphate
DEPC	diethyl pyrocarbonate
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid

DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
dpi	days post infection
DTT	dithiothreitol
EC	epicatechin
EDTA	ethylenediaminetetraacetic acid
ET	ethylene
E. coli	*Escherichia coli*
e.g.,	exempli gratia, for example
FaAP	*Fragaria* × *ananassa*-Associated Protein
GFP	Green Fluorescent Protein
Gibberellic acid	GA
Hawaii 4	HW4
HCl	hydrochloride
Hectare	ha
HF buffer	High Fidelity DNA polymerase buffer
HHDP	galloylbis-hexahydroxydiphenoyl
Hour(s)	h
hpi	Hours post inoculation
HPLC	high performance liquid chromatography
IAA	indole acetic acid
IgE	immunoglobulin E
IgG	immunoglobulin G
IMAC	immobilized metal affinity chromatography
IPTG	isopropyl-β-D-thiogalactopyranoside

JA	Jasmonic acid
Kb	kilo-base pair
KCl	potassium chloride
Kcal	kilocalorie
K_D	dissociation constant
kDa	kilodalton
K_{off}	dissociation rate
KOH	Potassium hydroxide
k_{on}	association rate
LB	Luria-Bertani
LC-UV-ESI-MS	liquid chromatography ultraviolet electro-spray ionization mass spectrometry
Liter	l
Mal d 1	*Malus domestica*
MdAP	Mal d 1-Associated Protein
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulfate
Minute(s)	min
MMLV-RT	moloney murine leukemia virus-reverse transcriptase
$MnCl_2$	manganese (II) chloride
MOPS	3-(N-morpholino)propanesulfonic acid
mQ water	milli-Q, ultrapure and deionized water
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MS medium	Murashige and Skoog Basal Salt Mixture

MW	molecular weight
MYB	myeloblastosis
m/z	mass-to-charge ratio
NaCl	sodium chloride
NaOAc	sodium acetate
Na_2HPO_4	disodium phosphate
NBT	nitroblue tetrazolium
NCS	(S)-norcoclaurine synthase
NDSB	3-[benzyl(dimethyl)ammonio]propane-1 sulfonate
NMR	nuclear magnetic resonance
nsLTPs	nonspecific lipid transfer proteins
OD	optical density
Orange G	disodium 7-hydroxy-8-[(E)-phenyldiazenyl]-1,3- naphthalenedisulfonate
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PDA	Potato dextrose agar
pH	potential of hydrogen
PMSF	phenylmethylsulfonyl fluoride
PR-10	pathogenesis-related proteins-10
PVDF	polyvinylidene fluoride
PVP	polyvinylpyrrolidone
qPCR	quantitative real-time PCR
RAE	retinol activity equivalents

RbCl	Rubidium chloride
Reine des Vallées	RdV
Relative concentration	rel. conc.
Relative expression	rel. exp.
RNA	ribonucleic acid
RNase	ribonuclease activity
RNA-seq	RNA-sequencing
rpm	revolutions per minute
RT	reverse transcription
SA	Salicylic acid
SD	standard deviation
SDS	sodium dodecyl sulfate
Second	sec
SOC	super optimal broth with catabolite repression
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
TEMED	Tetramethylethylenediamine
TFBI	transformation buffer I
TFBII	transformation buffer II
Tm	melting temperature
Tris	tris(hydroxymethyl)aminomethane
U	unit
UV	ultraviolet
V	voltage
Verticillium dahliae	*V. dahliae*

VSP	vegetative storage protein
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
yeast-two-hybrid	Y2H
Yellow Wonder	YW
λ–PP	Lambda phosphatase

Table 1. One and three-Letter code of Amino acids.

Amino Acid	3-Letter	1-Letter
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid or aspartate	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid or glutamate	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Writing guideline:

Symbols for gene names are denoted in italic (e.g., *Fra a 1.06*) while symbols for protein names are not italicized (e.g., Fra a 1.06).

Zusammenfassung

Die Erdbeere, eine kleine Frucht mit antioxidativen und entzündungshemmenden Eigenschaften, gehört zur Gattung Fragaria in der Familie Rosaceae. Das putative Erdbeer-Allergen Fra a 1 ist homolog zum Haupt-Birkenpollenallergen Bet v 1 und zählt zur PR-10 Familie. Die Bildung der PR-10-Proteine wird durch Hormone sowie biotischen und abiotischen Stress reguliert, während die Expression von *Fra a 1* vermutlich mit der Flavonoidbiosynthese verbunden ist. Das Fra a 1 Allergen akkumuliert in roten reifen Früchten von *Fragaria* × *ananassa*, wohingegen farblose, weißfrüchtige Erdbeeren eines mutierten Genotyps, die von durch Allergien betroffene Personen toleriert werden, praktisch frei von dem Erdbeerallergen sind. In der Genomsequenz der Walderdbeere (*Fragaria vesca*), welche 2010 publiziert wurde konnten 21 paraloge *Fra a 1* Gene identifiziert werden. In der vorliegenden Arbeit wurden verschiedene *Fra a 1* Isoformen kloniert, heterolog in *Escherichia coli* exprimiert und funktionell charakterisiert.

Die Expression der 21 *Fra a 1* Gene wurde in verschiedenen Gewebeteilen (Wurzel, Sprossachse, Blatt, junges Blatt, Blüte und Frucht) mittels quantitativer PCR bestimmt. Es konnte eine große Variabilität der Transkriptmengen der 21 Isoformen in den untersuchten *Fragaria* × *ananassa* Geweben beobachtet werden. Dreizehn *Fra a 1* Gene wurden bevorzugt in Wurzeln transkribiert, drei in Sprossachsen, zwei in roten Früchten, zwei in Blättern und nur ein *Fra a 1* Gen wurde primär in der Blüte exprimiert.

In der aktuellen Arbeit wurden die Isoformen Fra a 1.04-1.08 näher untersucht. Eine bei ähnlichen Proteinen gezeigte Ribonukleaseaktivität konnte durch Inkubation der Proteine mit RNA bei verschiedenen pH-Werten für vier Stunden überprüft werden. Sowohl ein in-Lösung RNase Assay als auch eine in-Gel RNase Bestimmung mit den 5 Proteinen zeigte, dass nur Fra a 1.06 fähig war, RNA zu hydrolysieren.

Die Phosphorylierung der Fra a 1.04-1.08 wurde mit verschiedenen Methoden untersucht. Zunächst wurde ein Western-blot der fünf rekombinanten Proteine unter Verwendung eines primären Antikörpers, der gegen Phospho-Serin, -Threonin und -Tyrosin gerichtet war, durchgeführt. Phosphorylierte Proteine wurden mit einem Anti-Maus-IgG, konjugiert mit alkalischer Phosphatase als sekundären Antikörper, sichtbar gemacht. Mit Ausnahme von Fra a 1.06 zeigten die restlichen 4 rekombinanten Proteine Fra a 1.04, Fra a 1.05, Fra a 1.07 und Fra a 1.08 eine Bande von 18 kDa, die eine Phosphorylierung beweist. Dieser Befund wurde mit einem Pro-Q Diamond Gel und einem Chemilumineszenz-Assay bestätigt.

Aufgrund der Beobachtung, dass die Fra a 1.04, Fra a 1.05, Fra a 1.07 und Fra a 1.08 Proteine phosphoryliert vorliegen und nur das unphosphorylierte Fra a 1.06 RNase-Aktivität besitzt, wurde untersucht, ob die Dephosphorylierung die Ribonuklease-Aktivität beeinflusst. Nach Dephosphorylierung zeigten die 4 rekombinanten Proteine ebenfalls Ribonuklease-Aktivität.

Um interagierende Proteinpartner für Fra a 1 Proteine zu bestimmen, wurde die SwitchSENSE-Plattform von Dynamic Biosensors eingesetzt. Als potentieller Bindungspartner wurde das FaAP (Fra a 1-assoziiertes Protein)-Protein, fusioniert an das grün fluoreszierende Protein (GFP) untersucht. FaAP ist orthologue zu MdAP, welches als Bindungspartner des Apfel- (*Malus domestica*) Allergens Mal d 1 bereits beschrieben wurde. Es konnte gezeigt werden, dass die untersuchten Fra a 1 Proteine an FaAP-GFP binden können. Fra a 1.04, Fra a 1.05, Fra a 1.06, Fra a 1.07 und Fra a 1.08 binden an FaAP mit K_D von 27.5 nM, 2,181 nM, 72.05 nM, 51.5 nM bzw. 53.1 nM.

Darüber hinaus wurde die Expression der 21 *Fra a 1* Isoformen in *in vitro* Erdbeerpflanzen (*F.* × *ananassa*) vor und nach Infektion mit *Verticillium dahliae* bestimmt. Die Transkriptmengen der 21 Isoformen waren in den verschiednen Geweben nach *Verticillium dahliae* Infektion sehr variabel. Vier *Fra a 1* Gene wurden in Blättern stark induziert, während acht bevorzugt in Wurzeln nach Infektion exprimiert wurden. Eine gleichzeitige induzierte Expression in Blättern und Wurzeln wurde für fünf *Fra a 1* Gene gezeigt. Interessanterweise wurden zwei Isoformen in allen drei Geweben (Blätter, Wurzeln und Sprossachse) nach Infektion exprimiert und für weitere zwei wurde keine Induktion nachgewiesen.

Gewebe, die für qPCR verwendet wurden, wurden ebenfalls mittels LC-UV-ESI-MS analysiert. Es konnte eine Akkumulation von Catechin und Epicatechin in Wurzeln und der Sprossachse festgestellt werden, während Kaffeesäure und Zitronensäure nur in Wurzeln nachgewiesen wurden. Darüber hinaus wurden auch verschiedene Phytohormone wie Abcisinsäure (ABA), Salicylsäure (SA), Jasmonsäure (JA), Gibberellinsäure (GA) und Indolessigsäure (IAA) in Blättern und Sprossachse bestimmt. IAA und JA waren die einzigen Hormone, die in einer späten Phase, in Wurzeln festgestellt wurden.

Aufgrund des unterschiedlichen Expressionsmusters der *Fra a 1* Gene in den Gewebeteilen der Erdbeerpflanze, der verschiedenen Phosphorylierungsgrade der Fra a 1 Proteine und unterschiedlichen Bindungskräfte der Allergene an einen neu identifizierten Proteinpartner

sowie der Induktion mehrerer *Fra a 1* Gene nach Infektion von Erdbeerpflanzen mit *Verticillium* konnte die Beteiligung der PR-10 Gene an der Pathogenabwehr eindeutig bestätigt werden, Somit liefert die vorliegende Arbeit erste molekulare Hinweise zur Funktion der *Fra a 1* Gene in der Erdbeerpflanze.

Summary

Strawberry, a small fruit crop, belongs to the genus Fragaria in the Rosaceae family. The putative allergen Fra a 1 is homologous to the birch pollen allergen Bet v 1 and belongs to the PR-10 family. The expression of PR-10 proteins is regulated by hormones, biotic and abiotic stress, whereas the expression of Fra a 1 is directly linked to flavonoid biosynthesis. The Fra a 1 allergen is synthesized by red ripe fruits of Fragaria × ananassa while a colorless (white) strawberry variety of a mutant genotype, which is tolerated by individuals affected by allergy, was found to be virtually free from the strawberry allergen. In 2010, the genome sequence of the woodland strawberry (Fragaria vesca) has been published and 21 Fra a 1 paralogous genes were identified. In the current work, Fra a 1 isoforms were cloned, heterologously expressed in Escherichia coli and functionally characterized.

In order to evaluate the expression of the 21 Fra a 1 isoforms in different tissues (root, stem, leaf, young leaf, flower and fruit), quantitative real-time PCR analyses were performed. A huge variation in transcript levels between the 21 isoforms was observed in all investigated *Fragaria* × *ananassa* tissues: thirteen members were expressed in roots, three in stems, two in red fruits, two in leaves and only one *Fra a 1* in flowers.

Despite numerous researches and investigations, the role of PR-10 is still not well understood and requires further studies. In the current work, functional characterizations of Fra a 1.04-1.08 were explored. The ribonuclease activity was checked by incubating the proteins in the presence of total RNA at different pH for four hours. Using an in-solution RNase activity assay, complete hydrolysis of RNA was only observed for Fra a 1.06, at pH 7.4 after few minutes of incubation. An in-gel RNA degradation assay was also performed by incubating the five proteins with yeast torula RNA in polyacrylamide gel. Both assays indicated that only Fra a 1.06 was capable of hydrolyzing RNA.

Phosphorylation of the five Fra a 1 proteins was evaluated using different methods. A Western blot analysis of the five recombinant proteins was performed using a primary antibody raised against phosphoserine/ -threonine/ -tyrosine and an anti-mouse IgG conjugated to alkaline phosphatase as a secondary antibody. Except for Fra a 1.06, the remaining four recombinant proteins Fra a 1.04, Fra a 1.05, Fra a 1.07 and Fra a 1.08 showed a band of 18 kDa proving their phosphorylation. This observation was confirmed with a Pro-Q Diamond gel and a chemiluminescent detection kit.

Taking into consideration that Fra a 1.04, Fra a 1.05, Fra a 1.07 and Fra a 1.08 proteins are phosphorylated and that only Fra a 1.06 possesses the RNase activity, it was logically consistent to investigate whether the dephosphorylation is influencing the ribonuclease activity. Using an in-solution RNase activity as well as in-gel RNase activity, results demonstrated that the four recombinant proteins dephosphorylated with phosphatases exhibited ribonucleolytic activity against total RNA.

Moreover, in order to determine interacting protein partners for Fra a 1 proteins, the SwitchSENSE platform of Dynamic Biosensors was used. FaAP (Fra a 1-associated protein) fused to green fluorescent protein (GFP) was tested as potential binding partner as FaAP is orthologue to MdAP, which has been identified as interaction partner of the major apple (*Malus domestica*) allergen Mal d 1. This work provided evidence that Fra a 1 proteins are able to bind to FaAP-GFP. Fra a 1.04, Fra a 1.05, Fra a 1.06, Fra a 1.07 and Fra a 1.08 bind to FaAP with K_D of 27.5 nM, 2,181 nM, 72.05 nM, 51.5 nM, and 53.1 nM, respectively.

Besides, the expression pattern of the 21 *Fra a 1* isoforms was examined in *in vitro* strawberry plants (*F.* × *ananassa*) infected with *Verticillium dahliae*. The expression pattern of the 21 isoforms infected with *Verticillium dahliae* showed a wide range of response. Four *Fra a 1* genes were highly induced in leaves while eight members were significantly up regulated in roots. A simultaneous induced expression in leaves and roots was detected for five *Fra a 1* genes. Interestingly, two isoforms were expressed upon infection in all three tissues (leaves, roots and stems) while no induction was detected for two other members.

Tissues used for qPCR were also analyzed by LC-UV-ESI-MS. An accumulation upon infection in infected roots and stems was detected for catechin and epicatechin while caffeic acid and citric acid were observed only in infected roots. Further compounds were also identified such as glutathione and tryptophan. Furthermore, the implication of some phytohormones on strawberry *in vitro* plants defense toward *Verticillium* infection was also tested. Abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA), gibberellic acid (GA) and indole acetic acid (IAA) were all detected in infected leaves and stems upon infection. IAA and JA were the unique hormones to be ascertained in infected roots at late stages.

Overall, the variable expression levels of *Fra a 1* genes in different tissues of the strawberry plants, the diverse phosphorylation pattern of the Fra a 1 proteins and various binding forces of the allergens to a newly identified protein partner as well as the induction of several *Fra a 1* genes upon infection of strawberry plants with *Verticillium* confirm the role of *PR-10* genes

in the defense against pathogens, Thus, the presented work provides first molecular clues on the function of *Fra a 1* genes in strawberry plants.

1 Introduction

1.1 Strawberry

Strawberry is a small fruit crop of great importance throughout the world and is appreciated for its unique flavor and nutritious quality. This fruit belongs to the genus Fragaria in the Rosaceae family and the Rosoideae subfamily. Fragaria consists of 23 species including diploids, tetraploids, hexaploids, and octoploids. Those four basic fertility groups are associated with their ploidy level or chromosome number (Folta and Davis, 2006).

The diploid woodland strawberry *Fragaria vesca* or fraise de bois ($2n = 2x = 14$) is the most common native species and is considered as a reference genomic system for the Rosaceae family. *F. vesca* has one of the smallest genomes among cultivated plants which was estimated at ~240 Mb (Shulaev et al., 2008; Shulaev et al., 2011). The woodland strawberry was widely grown until the late 1800s when it was replaced by *Fragaria* × *ananassa*.

The cultivated strawberry, *F.* × *ananassa*, is an accidental hybrid between *Fragaria chiloensis* and *Fragaria virginiana* that was first found in France in the 18th century (1766) by the French Botanist Antoine Nicholas Duchesne. The name *F.* × *ananassa* was accorded due to the perfume of the fruit that smelled like pineapple (*Ananas)* (Hancock et al., 2008). The genome composition of the octoploid ($2n = 8x = 56$) *F.* × *ananassa* is among the most complex of the crop species. Cultivated *F.* × *ananassa* is grown all over the world but natural populations of *F.* × *ananassa* are restricted to coastal California, Oregon and Washington whereas *F. vesca* inhabits North and South America, Europe, Asia and Hawaii (Hancock and Luby, 1993).

Complete genome sequencing of the woodland strawberry was carried out in 2010 placing the Rosaceae plant family at the cutting edge of the genomics revolution and greatly enhancing the efficiency of identifying gene networks (Peace and Norelli, 2009). Strawberry yields and fruits quality are greatly influenced by a number of factors including the effect of photoperiod and temperature, tolerance to different soil conditions such as alkalinity, salinity and winter hardiness.

1.2 Strawberry production and composition

1.2.1 Production

The cultivated strawberry *F.* × *ananassa* is an economically important crop plant because of its production of large berries. The strawberry world production in 2013 reached

approximately 6 million metric tons with a cultivable surface of around 214,000 ha. Global distributions of production of 49.7% in Asia, 25.2% in America, 19.2% in Europe, 5.4% in Africa and 0.5% in Oceania were recorded by FAOSTAT in 2013 (http://faostat3.fao.org). The top 5 producers of strawberries in 2013 were China (2.99 million metric tons), United States of America (1.36 million metric tons), Mexico (0.379 million metric tons), Turkey (0.372 million metric tons) and Spain (0.312 million metric tons). In addition to Spain which is the world's largest exporter in 2013, Italy, Poland and Germany are also belonging to the most important producing countries in Europe.

After apples, strawberries were the second most important fruit grown in Germany. In 2013 strawberries were grown in 2,400 holdings and on an area of 19,434 ha. 142,780 metric tons of strawberries were harvested in the open and 6,900 metric tons in protective environments. The main regions for strawberry production were located in Lower Saxony (4,307 ha), North-Rhine Westphalia (3,573 ha), Baden-Wuerttemberg (3,872 ha) and Bavaria (2,766 ha). Statistics revealed that Germany imported vegetables and strawberry plants worth around 35.8 million euros whereas the exports amounted to 46.3 million euros in 2013 (Horticulture in Germany).

The hybrid octoploid *F.* × *ananassa* covers around 60% of the world production due to the organoleptic properties and health benefits of the fruit regarded as significant quality factors for both consumers and the food industry.

1.2.2 Composition

The most frequently analyzed compounds from strawberry are the phenolic compounds such as phenolic acids, flavonols (kaempferol and quercetin derivatives), anthocyanins (cyanidin and pelargonidin derivatives), proanthocyanidins, galloylglucoses and ellagitannins. Besides phenolic compounds**,** strawberry fruits contain nutritionally important chemical constituents such as vitamins and amino acids. Folate is one of the most promising nutrients in strawberry with 10 to 100 µg/ 100 g of fresh weight (Tulipani et al., 2008). Moreover, the strawberry is a source of several other vitamins such as thiamin, riboflavin, niacin, vitamin B6, vitamin A (retinol) and vitamin E. Strawberry has been also qualified as a good source of minerals such as potassium, magnesium, calcium, iron and phosphorus **(Table 2)**.

Strawberry is composed of 90.9% of water, 0.7% of protein, 0.3% of total lipid, 2% of fiber and 4.9% of sugar with a calorific value of 32 kcal/100 g of wet weight. Fiber and fructose contents contribute in regulating blood sugar levels by slowing digestion and controlling the calorie absorption. This fruit is also a source of healthy essential fatty acids because its seed

oil is rich in unsaturated fatty acids (around 72% polyunsaturated fatty acids) (Giampieri et al., 2012).

Table 2. Nutrient constitution (left) and polyphenol composition in strawberries (right) (adapted from Giampieri et al., 2012).

Type	Nutrient	Per 100 g
Proximates	Water (g)	90.95
	Energy (kcal)	32
	Protein (g)	0.67
	Ash (g)	0.40
	Total lipid (g)	0.30
	Carbohydrate (g)	7.68
	Dietary fiber (g)	2
	Sugars (g)	4.89
	Sucrose (g)	0.47
	Glucose (g)	1.99
	Fructose (g)	2.44
Minerals	Calcium (mg)	16
	Iron (mg)	0.41
	Magnesium (mg)	13
	Phosphorus (mg)	24
	Potassium (mg)	153
	Sodium (mg)	1
	Zinc (mg)	0.14
	Copper (mg)	0.048
	Maganese (mg)	0.386
	Selenium (µg)	0.4
Vitamins	Vitamin C (mg)	58.8
	Thiamin (mg)	0.024
	Riboflavin (mg)	0.022
	Niacin (mg)	0.386
	Pantothenic acid (mg)	0.125
	Vitamin B6 (mg)	0.047
	Folate (µg)	24
	Choline (mg)	5.7
	Betaine (mg)	0.2
	Vitamin B12 (µg)	0
	Vitamin A, RAE (µg)	1
	Lutein + zeaxanthin (µg)	26
	Vitamin E, αtocopherol (mg)	0.29
	β-tocopherol (mg)	0.01
	Ɣ-tocopherol (mg)	0.08
	δ- tocopherol (mg)	0.01
	Vitamin K, phylloquinone (µg)	2.2

Class	Group	Compound
Flavonoid	Anthocyanins	Cyanidin-3-glucoside
		Cyanidin-3-rutinoside
		Cyanidin-3-malonylglucoside
		Cyanidin-3-malonylglucosyl-5-glucoside
		Pelargonidin-3-galactoside
		Pelargonidin-3-glucoside
		Pelargonidin-3-rutinoside
		Pelargonidin-3-arabinoside
		Pelargonidin-3,5-diglucoside
		Pelargonidin-3-malylglucoside
		Pelargonidin-3-malonylglucoside
		Pelargonidin-3-acetylglucoside
		Pelargonidin-dissacharide (hexose þ pentose) acylated with acetic acid
		5-Pyranopelargonidin-3-glucoside
	Flavonols	Quercetin-3-glucuronide
		Quercetin-3-malonyglucoside
		Quercetin-rutinoside
		Quercetin-glucoside
		Quercetin-glucuronide
		Kaempferol-3-glucoside
		Kaempferol-3-malonyglucoside
		Kaempferol-coumaroyl-glucoside
		Kaempferol-glucunoride
	Flavanols	Proanthocyanidin B1 (EC-4,8-C)
		Proanthocyanidin trimer (EC-4,8-EC-4,8-C)
		Proanthocyanidin B3 (C-4,8-C)
		(+)-catechin
Phenolic acids	Hydroxycinnamic acids	p-coumaroyl hexose
Hydrolyzable tannins	Ellagitannins	Ellagitannin
		Bis-HHDP-glucose
		Galloyl-HHDP-glucose
		HHDP-galloyl-glucose
		Galloyl-bis-HHDP-glucose
		Dimer of galloyl-bis-HHDP
		Sanguiin H-6
		Methyl-EA-pentose conjugates
		Ellagic acid pentoside
		Ellagic acid

1.3 Benefits of strawberry

1.3.1 Antioxidant capacity

The antioxidant power of fruit is closely correlated to the presence of efficient oxygen radical scavengers. Wang and Lin (2000) reported that blackberries, raspberries and strawberries are good sources of natural antioxidants. Strawberries have a greater antioxidant capacity (2 to 11 fold) than apples, peaches, pears and kiwi fruit (Scalzo et al., 2005; Wang et al., 1996). Anthocyanins cyanidin-3-glucoside, pelargonidin-3-glucoside, and pelargonidin-3-rutinoside

were demonstrated to be the most potent antioxidants (Zhang et al., 2008) and vitamin C was found to be one of the most important components responsible for more than 30% of the total antioxidant capacity (TAC) of strawberry extracts whereas flavonols did not significantly contribute to this activity (Tulipani et al., 2008). Giampieri et al. (2014) proved that total antioxidant capacity of total phenols, flavonoids, anthocyanins, vitamin C, and folates were significantly different according to the different genotypes in strawberry.

Henning et al. (2010) observed that the consumption of strawberries was associated with a significant increase in antioxidant activity in serum, which stimulate the defense against chronic diseases. Furthermore, strawberry juice extracts exhibited high levels of antioxidant capacity by owning the highest value regarding to singlet oxygen ($^1O_2$) scavenging activity and the second best value after blackberries in the antioxidant capacity inhibition of superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH•) (Wang and Jiao, 2000). According to Ayala-Zavala et al. (2004), the storage temperature significantly affects strawberry antioxidant capacity, anthocyanin, phenolic compounds, aroma compounds and overall the fruit quality.

1.3.2 Anticarcinogen and Antiproliferative activities

Strawberry extracts and their purified phenolic compounds showed *in vitro* human tumor cell antiproliferative activity by inhibiting the growth of human colon (HT-29 and HCT-116), prostate (LNCaP and DU145) (Heber, 2008; Zhang et al., 2008), oral (KB and CAL-27) (Zhang et al., 2008) and breast (MCF-7) tumor cell lines (Olsson et al., 2006). Seeram et al. (2006) demonstrated that increasing concentration of these compounds was correlated with the increasing inhibition of cell proliferation in all tested tumor cell lines (colon, prostate, oral and breast).

Besides, strawberries were demonstrated to be highly effective as a chemopreventive agent that acts by targeting the downregulation of the activity of two proteins which are the activator protein-1 (AP-1) and the nuclear factor kappa B (NF-κB) and consequently strawberries were proved to be implicated in the suppressing of tumor cell proliferation (Wang et al., 2005).

Interest in ellagic acid, tannins and associated polyphenols in plant extracts has increased greatly during the last decade due to their effectiveness as an antimutagen and anticarcinogen, and its potential as an inhibitor of chemically induced cancer (Maas et al., 1991a,b). The inhibition of cancer by ellagic acid (Pinto et al., 2010) appears to occur through the inhibition of the metabolic activation of carcinogens (Mandal et al., 1987) and the carcinogen

detoxification by stimulation of enzyme (e.g., glutathione S-transferase) activity toward benzo(α)pyrene-4,5-oxide and l-chloro-2,4-dinitrobenzene substrates in mice (Das et al., 1985). Vattem and Shetty (2005) proposed also a mechanism of action of ellagic acid through membrane-associated pathways that activate redox mechanisms which would be dysfunctional in cancer cell lines inducing therefore cell death while Cerdá et al. (2005) suggested that the retention of ellagitannins and ellagic acid within the gastrointestinal tract may influence cancer cell proliferation in the epithelial cells, from esophagus to colon.

In vitro and *in vivo* evidence indicates in fact that phenolics may modulate cell signaling in the cancer cell by inhibiting proliferation of these cells through the demethylation of tumor suppressor genes inducing apoptosis (Wang et al., 2013; Seeram et al., 2006) and suppressing tumor angiogenesis (Atalay et al., 2003).

1.3.3 Human immunodeficiency virus inhibition

Ellagic acid and some ellagitannins have shown inhibitory properties against replication of human immunodeficiency viruses (HIV) (up to 75% of inhibition) (Asanaka et al., 1988; Take et al., 1989), avian myeloblastosis virus (AMV), reverse transcriptase, (up to 95% of inhibition) and cellular DNA polymerases α (up to 81% of inhibition) and ß (up to 99% of inhibition) (Take et al., 1989).

1.3.4 Anti-UV radiation activity

Strawberry have a photoprotective activity in human dermal fibroblasts subjected to UV-A radiation with an increase in cellular viability and a decrease in DNA damage (Giampieri et al., 2014). *In vitro* and *in vivo* studies performed by Yin et al. (2013) demonstrated that quercitrin blocked UV-B-induced oxidative stress and DNA damage.

1.3.5 Other effects

There is evidence that the addition of strawberries to the diet can positively affect risk factors for cardiovascular diseases by inhibiting inflammation (Basu et al., 2010; Das, 1994) and reducing aging-induced malfunctions (Hanhineva et al., 2011). Recent research results have demonstrated that, besides antioxidant activity, phenolics may engage with cellular signaling flow, controlling the action of transcription factors and subsequently affecting the expression of genes involved in cellular metabolism (Giampieri et al., 2014).

Ellagic acid which is widely used in blood research of factors affecting clotting or thrombosis, has been found to induce *in vivo* hypercoagulability in animals by activation of the Hageman

factor (Girolami et al., 1966) and to control hemorrhage in humans (Cliffton, 1967). It also may have some hypotensive and sedative effects according to research with rodents (Bhargava et al., 1968). Furthermore, ellagic acid was found to be an endogenous inhibitor of insect growth and a deterrent of insect feeding on certain plants (Klocke et al., 1986).

1.4 Structure and function of PR-10 proteins

1.4.1 Bet v 1 structure

The 159 amino acids long Bet v 1, is the major allergen from the white birch (*Betula verrucosa*) which was first cloned and sequenced by Breiteneder et al. (1989). On the basis of sequence similarity and phylogenetic relationships, protein families structurally related to Bet v 1, were classified into 11 families including Bet v 1, START, AHA 1, CoxG and *SMU440-related proteins.* With 15 structures, Bet v 1 was reported to be the family which contains the highest number of protein structures with non-identical sequences in Protein Data Bank (Radauer et al., 2008). The major birch pollen allergen is a member of the ubiquitous PR-10 family with a sequence identity of 55% (Breiteneder et al., 1989; Radauer et al., 2008).

Pathogenesis-related (PR) proteins, one of the most commonly expressed proteins during plant defense mechanism, are induced upon pathogen attack, wounding and environmental stresses such as salinity, cold and ultraviolet radiation. According to Radauer et al. (2008) and van Loon et al. (2006), PR-proteins have been classified into 17 (PR1-PR17) families based on sequence similarity and biochemical functions.

PR-10s are small proteins (154 to 163 amino acids) that are encoded by multigene families and are constitutively expressed in different organs. Those 17 kDa proteins, slightly acidic (Somssich et al., 1988) and resistant to proteases, are generally intracellular and cytosolic. Despite multitudinous researches and studies, the role of PR-10 is still not well understood and requires further investigations. For this purpose, the structure of numerous PR-10 proteins was determined such as for Bet v 1 and yellow lupine (LIPR-10 and SPE-16) **(Table 3)**.

The determination of the crystal structure of a Bet v 1a variant in 1996 and the identification of the hydrophobic cavity was a primordial step in understanding its function (Gajhede et al., 1996). Later, the affinity of Bet v 1 towards a range of ligands was described using a fluorescent assay (Mogensen et al., 2002) and the crystal structure of two PR-10 proteins from yellow Lupine was reported (Biesiadka et al., 2002). The crystal structures of Bet v 1 in complex with a set of ligands including ANS (1-anilino-8-naphthalene sulfonate), deoxycholate (Marković-Housley et al., 2003), kinetin and naringenin revealed that these

ligands occupy different regions within the hydrophobic pocket and that differences in ligand binding exist between the different isoforms (Kofler et al., 2012).

Table 3. PR-10 protein structures in the PDB (Protein Data Bank) (adapted from Fernandes et al., 2013).

Subclass	Source	Name	Method (resolution in Å or number of NMR models)	PDB code (chain ID)	Cavity volume (Å^3)	Ligands
Major pollen and food allergens	White birch	Bet v 1*a*	X-ray (2.0)	1bv1 (A)	2963	
		Bet v 1*a*	NMR (20)	1btv	2780	
		Bet v 1*a* (M139L)	NMR (23)	1b6f	2927	
		Bet v 1a/Fab	X-ray (2.9)	1fsk (A, D, G, J)	2528	
		Bet v 1*l*/ deoxycholate	X-ray (1.9)	1fm4 (A)	3133	Deoxycholate
		Bet v 1*a* (E45S)	X-ray (3.1)	1llt (A)	3102	
		Bet v 1*a* (108G/N28T/ K32Q/E45S)	X-ray (2.15)	1qmr (A)	2913	
		Bet v 1*a*	X-ray (1.51)	4a88 (A)	2337	
		Bet v 1*j*	X-ray (1.16)	4a8u (A)	2445	
		Bet v 1*a*/ANS	X-ray (1.96)	4a80 (A)	2341	ANS
		Bet v 1*j*/ANS	X-ray (1.23)	4a8v (A)	2244	ANS
		Bet v 1*a*/ANS+ deoxycholate	X-ray (2.05)	4a81 (A)	2151	ANS, Deoxycholate, Na^+
		Bet v 1*a*/ deoxycholate	X-ray (1.54)	4a83 (A)	3017	Deoxycholate
		Bet v 1*a* (F30V)/ deoxycholate	X-ray (1.50)	4a84 (A)	3043	Deoxycholate
		Bet v 1*a*/naringenin	X-ray (1.24)	4a87 (A)	2434	Naringenin, Na^+
		Bet v 1*a*/kinetin	X-ray (1.40)	4a85 (A)	2900	Kinetin
		Bet v 1*a*/ANS+kinetin	X-ray (1.58)	4a86 (A)	2217	ANS, kinetin
		Bet v 1*a*/NDSB-256	X-ray (2.10)	4a8g (A)	2167	NDSB
	Cherry	Pru av 1	NMR (22)	1e09	2932	
		Pru av 1(E45W)	NMR (24)	1h2o	2550	
	Celery	Api g 1	X-ray (2.9)	2bk0 (A, B)	2846	
	Carrot	Dau c 1	X-ray (2.7)	2wql (A, B, C, D)	2483	
	Soybean	Gly m 4	NMR (20)	2k7h	2970	
	Strawberry	Fra a 1e	NMR (20)	2lpx	3649	

Afterwards, Seutter von Loetzen et al. (2014) identified the natural ligand of Bet v 1, namely Q3OS (quercetin-3 *O*-sophoroside). This binding of ligands may play a role during the inflammation response and Bet v 1 recognition by IgE.

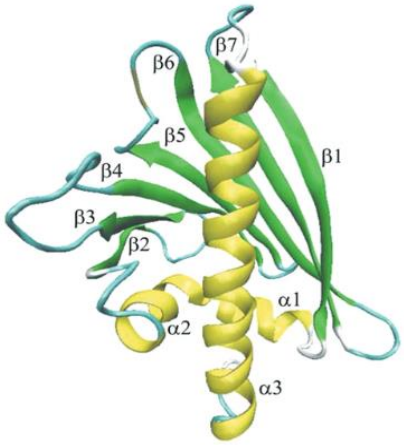


Figure 1. Structure of the pollen allergen, Bet v 1 from Betula pendula consisting of a seven-stranded antiparallel β sheet and three α helices (Chakrabortya and Dutta, 2011).

The crystal and NMR structures of the most abundant Bet v 1 isoform, Bet v 1a (Bet v 1.0101) consists of a seven-stranded antiparallel β-sheet (β1–β7), two short α-helices (α1 and α2) connecting β1 and β2, a long C-terminal α-helix (α3) and a hydrophobic cavity between β2 and β3. This cavity is a glycine-rich motif which is highly conserved throughout the PR-10 family **(Figure 1)** (Kofler et al., 2012; Seutter von Loetzen et al., 2014; Marković-Housley et al., 2003). Kofler et al. (2012) proved that the hydrophobic cavity of Bet v 1 is accessible to solvent and small molecules at three distinct entry sites. The first opening (entry ε1) is located at the flexible loop connecting β7 with α3. This entry is flanked by eight residues (Phe_{62}, Pro_{63}, Phe_{64}, Pro_{90}, Gln_{132}, Ala_{135}, Ser_{136}, and Met_{139}), creating a relatively nonpolar access route to the cavity. A second small opening (entry ε2) with a diameter of ∼6 Å is surrounded by residues Tyr_5, Thr_7, Val_{133}, and Lys_{137}. The third entry (entry ε3) is located at the opposite side of the protein. It is partly formed by the glycine-rich loop and framed by the residues Ile_{23}, Leu_{24}, Asp_{25}, Asp_{27}, Thr_{52}, Lys_{54}, Tyr_{81}, and Ile_{102}.

The connection sequences between α-helix or/and β-strand are, in general, short loop structures (from Leu_1 to Leu_9). The conserved P-loop motif is localized in the Leu_4 region between β2 and β3 and is strikingly the most rigid structural element in the PR-10 fold (Biesiadka et al., 2002; Jain and Kumar, 2015). Saraste et al. (1990) suggested that this motif is presumed to be involved in ATP or GTP binding and was shown to play a considerable role in the RNase activity for some PR-10 proteins (SPE-16) (Wu and al., 2003; Liu and Ekramoddoullah, 2006). However, according to other studies, PR-10 proteins were demonstrated to lack any affinity for ATP (Koistinen et al., 2005) and the glycine-rich loop is conformationally different from the P-loop (GxGGxGxxK[TS]) (Saraste et al., 1990; Biesiadka et al., 2002).

All those outcomes suggested that the physiological role of Bet v 1 is most likely the binding, transport or storage of small lipidic plant mediators such as steroids, cytokinins or flavonoids.

Although Bet v 1 is capable of binding several types of physiologically relevant ligands in a centrally placed cavity in the protein structure and that it is well characterized at a biochemical and immunological level, the natural and molecular function of this 17 kDa protein remains elusive (Mogensen et al., 2002; Mogensen et al., 2007).

1.4.2 Fra a 1 structure

The Fra a 1 allergen in strawberry *F.* × *ananassa* is homologous to the major birch pollen allergen Bet v 1. Several isoforms of the Fra a protein have been described in strawberry,

including Fra a 1E and Fra a 3 (Muñoz et al., 2010; Musidlowska-Persson et al., 2007; Casañal et al., 2013a).

It was postulated that Fra a proteins are required for the normal accumulation of flavonoids and the development of color in strawberry fruits (Karlsson et al., 2004; Muñoz et al., 2010). However, their function, as that of PR-10 proteins in general, is still not clearly understood. NMR structural model of Fra a 1E (Seutter von Loetzen et al., 2014) as well as crystallization and preliminary X-ray analysis of the Fra a 1E and Fra a 3 isoforms were successfully performed (Casañal et al., 2013a). Using the sitting-drop vapour-diffusion method, Fra a 1E crystals were detected in the shape of rods and trigonal prisms whereas Fra a 3 crystals were obtained only in the presence of (+)-catechin in the shape of hexagonal prisms **(Figure 2)**. Unfortunately, the Fra a 2 protein did not produce any crystals, neither alone nor in the presence of any flavonoids compounds ((+)-catechin, quercetin-3-*O*-glucuronide, myricetin and pelargonidin-3-*O*-glucoside).

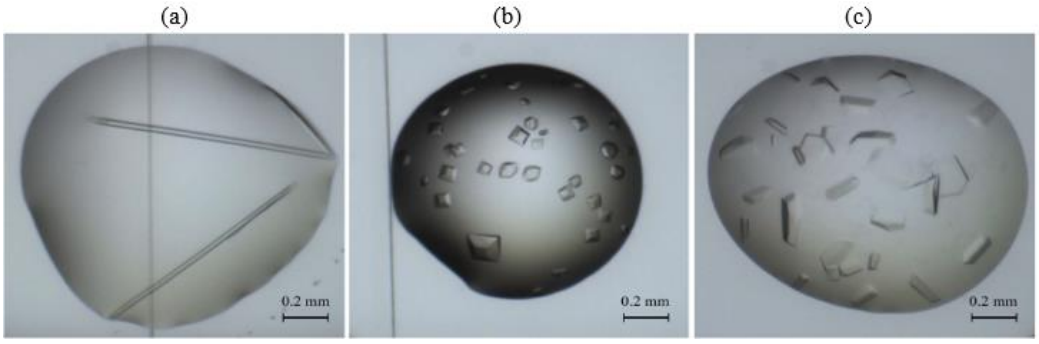


Figure 2. Fra a 1E and Fra a 3–catechin crystals. Two different crystallization conditions were identified for Fra a 1E; crystals were obtained in the shapes of rods (a) and trigonal prisms (b). Crystals of Fra a 3 with the shape of hexagonal prisms were only obtained in the presence of catechin (c) (Casañal et al., 2013a).

The structure of Fra a 1E **(Figure 3a)** consists of a seven-stranded antiparallel β-sheet (residues Tyr_6–Ser_{12}, Lys_{40}– Glu_{46}, Gly_{52}–Thr_{58}, Tyr_{67}–Asp_{76}, His_{80}–Glu_{88}, Glu_{97}–Ala_{108} and Gly_{111}–Thr_{123}) that is closed by a long C-terminal (residues Glu_{131}–Asp_{154}) and a short V-shaped α-helix (residues Ala_{16}– Ile_{34}) with a kink at Asp_{26}. The helices and the β-strands encompass a very large hydrophobic cavity, as for Bet v 1 homologous proteins. The binding cavity of the Fra a 3 protein in complex with catechin is stabilized both by polar and hydrophobic interactions **(Figure 3b, c)**.

Comparison of the structures of the apo forms of Fra a 1E and the Fra a 3-catechin complex point out that loops Leu_3, Leu_5 and Leu_7, surrounding the ligand-binding cavity, show significant flexibility in the apo forms and that ligand binding induces important conformational changes. Fra a 1E and Fra a 3 show a high structural similarity and a typical

START fold conformation. The major difference between the two Fra a proteins consists of the conformation of the loops surrounding the open end of the cavity. Fra a 1E backbone is also similar to those of PR-10 proteins, like Bet v 1 from *Betula berrucosa* (Gajhede et al., 1996; Spangfort et al., 1997) or LIPR-102.B from yellow lupin (Fernandes et al., 2009).

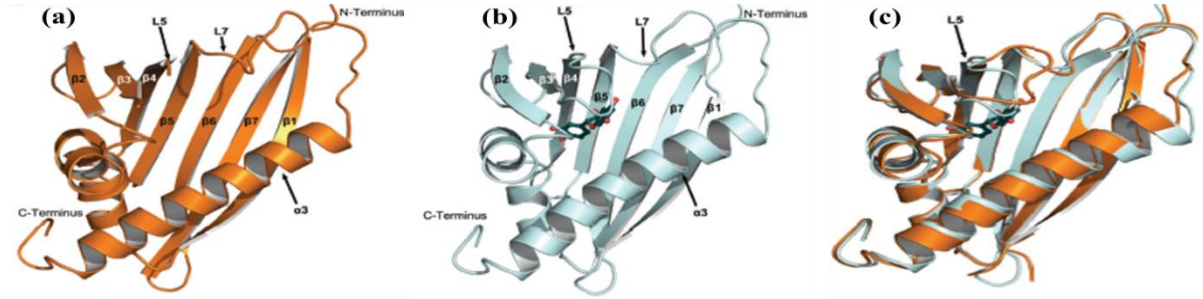


Figure 3. Structure of the Fra a 1E and Fra a 3-catechin complex. (a), Fra a 1E shown in ribbon representation. (b), Fra a 3-(+)-catechin complex, the protein and ligand are shown in ribbon and ball-and-stick representations, respectively. (c), superposition of Fra a 1E (orange) and Fra a 3-catechin (cyan) structures (adapted from Casañal et al., 2013b).

The variability between the three Fra a proteins of amino acids facing the cavity was observed at certain positions such as Leu_{59} and Arg_{139}, involved in Fra a 3 (+)-catechin interaction which are replaced by Phe and Lys in both Fra a 1E and Fra a 2.

The structural analysis reported by Casañal et al. (2013a,b) suggest that Fra a proteins control flavonoid biosynthesis through binding to metabolic intermediates and that those proteins could also be involved in the transport of flavonoids from the Endoplasmic Reticulum to other cellular membranes such as the tonoplast or the plasma membrane.

1.4.3 Function of PR-10

1.4.3.1 RNase activity

It has been reported that numerous functions could be assigned to the PR-10 proteins. Besides the ligand binding activity (Mogensen et al., 2002; Neudecker et al., 2001), PR-10 proteins were demonstrated to be involved in enzymatic processes such as ribonuclease activity and secondary metabolite biosynthesis.

A ribonuclease from a callus cell culture of *Panax ginseng* was isolated and Moiseyev et al. (1994) was therefore the first to detect such activity in PR-10 proteins. RNase activity was also detected for Bet v 1 (Bufe et al., 1996; Swoboda et al., 1996), white lupin roots LaPR-10 (Bantignies et al., 2000), yellow lupine LlPR-10.1B (Biesiadka et al., 2002), birch pollen BpPR-10c (Koistinen et al., 2002a), cotton GaPR-10 (Zhou et al., 2002), seeds of SPE16 (Wu et al., 2002; Wu et al., 2003), hot pepper CaPR-10 (Park et al., 2004), yellow-fruit nightshade

SsPR-10 (Liu et al., 2006), peanut AhPR-10 (Chadha and Das, 2006), pea PsPR-10.1, PsPR-10.4 (Srivastava et al., 2006; Srivastava et al., 2007; Krishnaswamy et al., 2011), *Astragalus mongholicus* AmPR-10 (Yan et al., 2008), peach Pru p 1.01, Pru p 1.06D (Zubini et al., 2009), cacao TcPR-10 (Pungartnik et al., 2009), maize ZmPR-10, ZmPR-10.1 (Chen et al., 2006; Xie et al., 2010), saffron CsPR-10 (Gomez-Gomez et al., 2011), ginseng PgPR-10s (Lee et al., 2011) and biofuel crop JcPR-10a (Agarwal et al., 2013).

The glycine-rich motif was believed to be the most probable nucleotide binding site of PR-10 and therefore was suggested to bind the phosphate group of RNA (Bantignies et al., 2000). The ribonuclease activity was demonstrated to be influenced or not under certain conditions while for some other PR-10 proteins the RNase activity was not detected at all. In this context, Park et al. (2004) proposed that the phosphorylation of the CaPR-10 may increase the RNase activity while Chadha and Das (2006) and Pungartnik et al. (2009) suggested that the antifungal activity of TcPR-10 might be correlated to its RNase activity.

1.4.3.2 Post-translational modifications

Post-translational modifications of the isoforms of Bet v 1 have been deeply studied. Koistinen et al. (2002a) was the first to demonstrate that PR-10c protein is post-translationally modified by glutathione in both, *in vitro* and *in vivo* plants. The S-glutathiolated cysteine residue of PR-10c is not conserved among Bet v 1 homologous proteins suggesting a unique role for S-glutathiolation of PR-10c compared to the other members of Bet v 1 family. However, the cys_{82} was found in hazelnut (*Corylus avellana*) allergen sharing 85% identity with PR-10c and also in *Castanea sativa* plants (Lüttkopf et al., 2002).

Some studies established that PR-10s are phosphorylated. Park et al. (2004) postulated that phosphorylation of CaPR-10 is a resistance response of plants to biotic stress stimulated by avirulent pathogen and that the ribonuclease function was increased consequently by the phosphorylated state. Some PR-10 proteins from *Arachis hypogaea* were shown to be phosphorylated but their role in RNase activity was not shown (Jain et al., 2006). In contrast, Pungartnik et al. (2009) published that the phosphorylation did not influence the RNase activity of TcPR-10 or its substrate specificity and both phosphorylated and non-phosphorylated cocoa PR-10 degraded plant RNA.

1.4.3.3 Plant defense

PR-10 proteins are induced under biotic and abiotic stress and are therefore involved in defense mechanism for plant protection. Phytohormones such as abscisic acid (ABA),

ethylene (ET), jasmonic acid (JA) and salicylic acid (SA) are major signaling molecules in plants during the stress response and their involvements during induction of PR-10 proteins has been investigated in various studies (Agarwal and Agarwal, 2014; Amil-Ruiz et al., 2011). Also cytokinins (Carpin et al., 1998) and auxin (Poupard et al., 2001) were demonstrated to be implicated in stress response. In general, SA is especially important for attack by biotrophic pathogens in so-called systemic acquired resistance (SAR) and the JA/ET signaling pathway is involved in responses to wounding and abiotic stresses such as drought and high salinity and also in the defense signaling against necrotrophic pathogens (Takeuchi et al., 2011; Yamaguchi-Shinozaki and Shinozaki, 2006). Also, ABA has a crucial role in response to plant growth and development as well as in a wide range of abiotic stresses including drought, salt and cold.

Moreover, some PR-10 proteins were shown to possess antifungal activity such as, AhPR-10 of *Arachis hypogaea* (Chadha and Das, 2006) and TcPR-10 of *Theobroma cacao* (Pungartnik et al., 2009) through internalization of fungal mycelium. Other PR-10 proteins that possess antifungal activity are SsPR-10 from *Solanum surattense* (Liu et al., 2006), maize PR-10 proteins (Chen et al., 2006; Xie et al., 2010), CsPR-10 from *Crocus sativus* (Gomez-Gomez et al., 2011), JcPR-10a from *Jatropha curca* (Agarwal et al., 2013) and CaPR-10 from pepper (Park et al., 2004).

In a recent study, Fan et al. (2015) isolated a new member of the PR-10 protein family, Gly m 4l from resistant soybean ‘Suinong 10’, and showed a significant resistance upon *Phytophthora sojae* inoculum. These results suggested that Gly m 4l protein played an important role in the defense of soybean.

Furthermore, some PR-10 proteins also show antibacterial and antiviral activity. Ocatin inhibits the growth of several phytopathogenic bacteria such as *Agrobacterium tumefaciens*, *Agrobacterium radiobacter*, *Serratia marcescens* and *Pseudomonas aureofaciens* and fungi such as *Phytophthora cinnamomi*, *Fusarium oxysporum*, *Rhizoctonia solani*, and *Nectria hematococcus* (Flores et al., 2002). The PR-10 proteins from maize, ZmPR-10 and ZmPR-10.1 showed a strong antibacterial activity against bacteria *Pseudomonas syringae* (Xie et al., 2010). Besides, antiviral activity of pepper CaPR-10 was demonstrated to degrade viral RNA of tobacco mosaic virus (Park et al., 2004). Antinematode activity was as well reported for PR-10 proteins. The CpPRI, a papain inhibitor from *Crotalaria pallid* roots, showed nematostatic and nematicide effects against root-knot nematode *Meloidogyne incognita* parasite in bioassays (Andrade et al., 2010).

1.4.3.4 Other possible functions of PR-10 proteins

It has been postulated that PR-10 proteins may act as a storage protein. A vegetative storage protein (VSP) from white clover (*Trifolium repens L.*) stolons was characterized to accumulate under autumn and winter conditions. The VSP protein may have an additional or alternative function to its role in nitrogen storage and may confer putative tolerance to chilling in white clover (Goulas et al., 2007). Comparably, the PR-10 homolog ocatin, a major tuber protein from the Andean crop oca could act as tuber storage protein (Flores et al., 2002).

Two further proteins with supposedly enzymatic role were classified as PR-10 proteins, namely Hyp-1 and NCS proteins. Bais et al. (2003) published that the phenolic oxidative coupling protein (Hyp-1) from *Hypericum perforatum* which catalyze the condensation of two emodine molecules to the bioactive naphthodianthrone hypericin, shows approximately 40% sequence identity with classic PR-10 proteins. NCS enzyme, (S)-norcoclaurine synthase, sharing 28% – 38% sequence identity with classic PR-10 proteins, is involved in benzyl iso-quinoline alkaloid biosynthesis (Lee and Facchini, 2010; Samanani et al., 2004).

Sequence identity between NCS and Hyp-1 (35%) suggested a role of PR-10 proteins in enzymatic condensation reaction but recently such reaction for Hyp-1 has been questioned (Kosuth et al., 2007; Michalska et al., 2010).

In order to determine proteins putatively interacting with Mal d 1 (*Malus domestica*, PR-10), the yeast-two-hybrid (Y2H) system was used. A novel protein binding to Mal d 1 isoforms was isolated (Pühringer et al., 2003). This Mal d 1-Associated Protein (MdAP) comprises 190 amino acids and shares 45% identity with a protein in *Arabidopsis thaliana* and 68% identity with an orthologous protein in *F. vesca* named Fra a 1-Associated Protein (FaAP) **(Figure 4)**. FaAP consists of 193 amino acids and the deduced protein of the full length FaAP has a predicted molecular mass of 21.46 kDa with a calculated pI of 4.49 whereas MdAP has a molecular weight of 21.17 kDa and a pI of 4.52. In accordance with MdAP, FaAP is encoded by a single gene in the genome of the woodland strawberry (Pühringer et al., 2003; Shulaev et al., 2011). The genomic DNA of FaAP consists of two exons that are separated by an intron. The intron starts after 204 bp and has a length of 1,282 bp. FaAP is presumably not secreted and is not a DNA binding protein although it could enter the nucleus due to its small size (Franz-Oberdorf et al., 2017a).

Figure 4. Sequence alignment of FaAP and MdAP proteins using Geneious software. Thick green line shows identity between the two protein sequences. In both compared sequences, large sequence logo letters denote the same nucleotide while small sequence logo letters represent different nucleotides.

Using a novel time resolved DNA-switching system (Dynamic Biosensors), the binding of the orthologous FaAP to Fra a 1.04-1.08 was investigated in this work while Franz-Oberdorf et al. (2017a) studied the interactions between FaAP and Fra a 1.01-03. The SwitchSENSE method uses double-stranded DNA oligonucleotides (48 bp), which are end-tethered to gold microelectrodes. An epifluorescence was used to measure the orientation of the DNA levers to the surface in real time. Due to a non-radiative energy transfer by near-field interactions with the metal, the fluorescence is gradually quenched when the DNA levers tilt toward the gold surface. Alternating potentials that are applied to the electrodes repel or attract the negatively charged DNA and switch therefore lever orientation between a standing and a lying state **(Figure 5a)**. The fixation of capture molecules to the DNAs' top end allow the binding of target proteins from solution and consequently the DNA lever motion slows down **(Figure 5b)**. Binding kinetics can be followed in real time, and the association and dissociation rates k_{on} and K_{off} and the dissociation constant K_D can be defined.

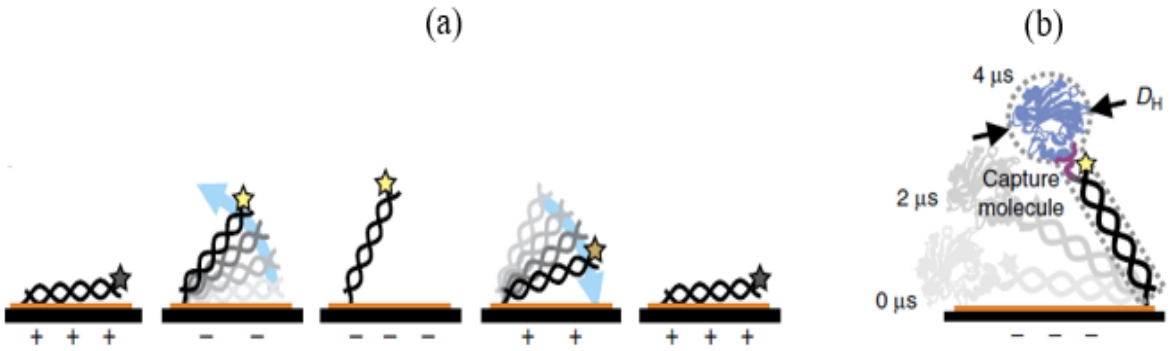


Figure 5. Schematic representation of the DNA lever switching. The fluorophore emission (star) is quenched in close proximity to the surface (a). Schematic of a DNA lever with a protein (effective protein diameter, DH) bound via a capture molecule (purple), which is covalently attached to one single strand (b) (adapted from Langer et al., 2013).

1.5 PR-10 related plant food allergy

Although fruit consumption is encouraged for health reasons, fruits may accommodate allergenic proteins causing serious problem which can considerably limit patient's life quality. Food allergy is an important public health problem for children and adults worldwide and is currently estimated to affect, according to the Food Allergy & Anaphylaxis Public Declaration (EAACI), more than 17 million Europeans, including 3.5 million under the age of 25. This adverse reaction is triggered when the immune system mistakenly recognizes a particular food or group of foods as aggressors to the organism (Canonica et al., 2013).

On the basis of their structural and functional properties, plant food allergens can be classified into families and superfamilies (Breiteneder and Radauer, 2004). The most widespread groups of plant proteins that contain allergens are the cupin (vicilins and legumins) and prolamin (2S albumins, nsLTPs, the cereal α-amylase and protease inhibitors and cereal prolamins) superfamilies and the protein families of the plant defense system (PR-proteins and proteases) (Breiteneder and Radauer, 2004; Breiteneder and Mills, 2005; Karlsson et al., 2004; Valério et al., 2015). Members of PR-proteins were shown to play a role in plant defense and allergenicity such as the PR-2 proteins (β-1,3-glucanases), the PR-3, the PR-4 and PR-8 proteins (chitinases), the PR 5 proteins (thaumatin-like proteins (TLP), the PR-10 (Bet v 1 homologous proteins) and the family of PR-14 proteins (nsLTPs) (Breiteneder and Ebner, 2000; Hoffmann-Sommergruber, 2002).

Bet v 1, one of the main causes of fruit allergies in pollen, shares common epitopes with major food allergens such as carrot (Dau c 1), celery (Api g 1), parsley (Pet c 1), soybean (Gly m 4), peanut (Ara h 8), melon (Cuc m 3) but also with fruits of the Rosaceae family like peach (Pru p 1), apple (Mal d 1), raspberry (Rub i 1) and strawberry (Fra a 1) (Marzban et al., 2005; Osterballe et al., 2005; Vieths et al., 2002; Karlsson et al., 2004).

Besides, Mal d 1, the major allergen from apple, was the first Bet v 1 homologous fruit allergen cloned and produced as a recombinant allergen by Vanek-Krebitz et al. (1995). This 18 kDa protein sharing 50% (Karlsson et al., 2004), 62% (Vieths et al., 1994) and 67.6% identity (Fahlbusch et al., 1995) to Bet v 1 was associated with Type 1 allergies in apple (Beuning et al., 2004). Both Bet v 1 and Mal d 1 are members of large families consisting of at least 20 (Swoboda et al., 1995a) and 15 (Atkinson et al., 1996) isoforms, respectively. The two proteins have been classified as belonging to the PR-10 class because of their high homology (Breiteneder et al., 1989; Matton and Brisson 1989; Swoboda et al., 1996; Pühringer et al., 2000; Liu and Ekramoddoullah, 2003).

In contrast to PR-14 (nsLTPs) proteins which are localized to the peel, PR-10 proteins were detected in the strawberry pulp (Marzban et al., 2012). The strawberry (*F.* × *ananassa*) fruit allergen Fra a 1, also a member of the Bet v 1, is considered as the main cause for allergic reactions after consumption of ripe strawberries in Northern Europe (Karlsson et al., 2004; Muñoz et al., 2010). Fra a 1 shows a sequence identity of 54 and 77%, respectively with corresponding allergens from birch and apple **(**Hjerno et al., 2006). Muñoz et al. (2010) reported that the total allergen content varied due to growth conditions. A naturally occurring white-fruited genotype contained less Fra a 1 protein than red-fruited varieties (seven-fold more abundant). This fact would explain why fruits of the white-fruited genotype were virtually free from the strawberry allergen and were tolerated by individuals affected by allergy (Hjerno et al., 2006).

Individuals with food allergies can manifest various types of immune responses. On this basis, food allergy can be divided into IgE- and non-IgE-mediated reactions. The percentage of the world's population suffering from Type I (IgE-mediated) allergic reactions has shown a dramatic increase in recent years (Knox and Suphioglu, 1996). In Europe and North America about 10% to 25% (Faber et al., 1996, Hoffmann-Sommergruber, 2002; Breiteneder et al., 1989) of the population suffer from IgE-mediated "atopic" diseases.

The major birch pollen allergen Bet v 1 is responsible for IgE binding in more than 95% of birch pollen allergic patient (Breiteneder et al., 1989) and is therefore the main cause of type I allergies observed in early spring (Faber et al., 1996). Cross-reactive IgE is the immunological basis for Bet v 1 food allergies (Ebner et al., 1995) that can cause symptoms ranging from the oral allergy syndrome (OAS) to anaphylactic shock (Amlot et al., 1987; Ebner et al., 1991; Vieths et al., 1995) but it is also possible that the presence of food allergen-specific IgE is not always accompanied by clinical symptoms (Bircher et al., 1994).

Contrarily to PR-10 proteins which are degraded during the passage through the gastrointestinal tract and therefore claimed to cause allergy only in patients previously sensitized by common epitopes of Bet v 1, the PR-14 proteins are very stable and responsible for true food allergies upon consumption of rosaceous fruits such as apple peach, cherry, apricot and strawberry, particularly in the Mediterranean area (Fernandez-Rivas et al., 2003; Marzban et al., 2005; Marzban et al., 2008; Pastorello et al., 1994). For this reason, it is important to investigate the presence of IgE antibodies to Bet v 1 and nsLTP in patients sensitized to Rosaceae food allergens or having clinical symptoms to Rosaceae foods to evaluate the risk for serious systemic reactions.

PR-10 proteins are very sensitive to proteases and acidic pH (Marzban et al., 2012). Also, heat treatment destroys the native three-dimensional molecular structure of PR-10 proteins, which is important for binding to the IgE molecule and the immediate reaction but does not affect linear peptides important for the late phase of cellular reaction. Therefore once cooked, this group of heat-sensitive and protease-labile PR-10 proteins is destroyed and could not cause sensitization directly (Valério et al., 2015).

Recent research performed in Munich revealed that the allergenicity of birch pollen was influenced by ozone as a prominent and crucial environmental factor leading to the increase of symptoms of pollen allergenicity among patients (Beck et al., 2013). This increase, observed mainly in industrialized countries and pollution which can cause nitration of allergens exogenously or endogenously, was attributed as primordial contributing factor (Ackaert et al., 2014; Ring et al., 2001; Saxon and Diaz-Sanchez, 2005).

1.6 *Verticillium* wilt disease

1.6.1 Overview

The genus *Verticillium* comprises a small group of plant pathogenic fungi that cause annually billions of dollars of damage to a variety of agricultural crops worldwide (Pegg and Brady, 2002). Among the ten species currently recognized in the genus *Verticillium sensu stricto*, (Inderbitzin et al., 2011; Zare et al., 2007), *Verticillium dahliae* is the most studied species (Hawksworth and Talboys, 1970).

The strawberry *F.* × *ananassa* belongs to the plant species susceptible to severe disease caused by the soil-borne pathogenic fungus *V. dahliae*. This type of *Verticillium* was described by Klebahn (1913) from Dahlia sp. cv. Geiselher in Germany. *V. dahliae* is not the oldest species of the genus, but it has the largest impact as a pathogen and thus has been conserved as the type of the genus (Gams et al., 2005). The mycelium of *Verticillium* is hyaline, simple or branched, septate and multinucleate. The conidia are ovoid to elongate and are produced on long phialides positioned in a spiral-like shape around the conidiophores (Fradin and Thomma, 2006).

Moreover, the control of *Verticillium* wilt is difficult and costly (Subbarao et al., 2007; Klosterman et al., 2009). In the absence of a suitable plant host, *Verticillium* species can remain dormant in the soil for years by means of small melanized resting structures and will only germinate in the proximity of a suitable host (Wilhelm, 1955).

1.6.2 The *Verticillium* life cycle

The life cycle of *Verticillium* spp. includes three vegetative phases: dormant, parasitic and saprophytic. In the dormant phase, the excess of carbon and nitrogen in root exudates stimulate the germination of long lasting multicellular structures called microsclerotia (Huisman, 1982; Mol, 1995; Olsson and Nordbring-Hertz, 1985).

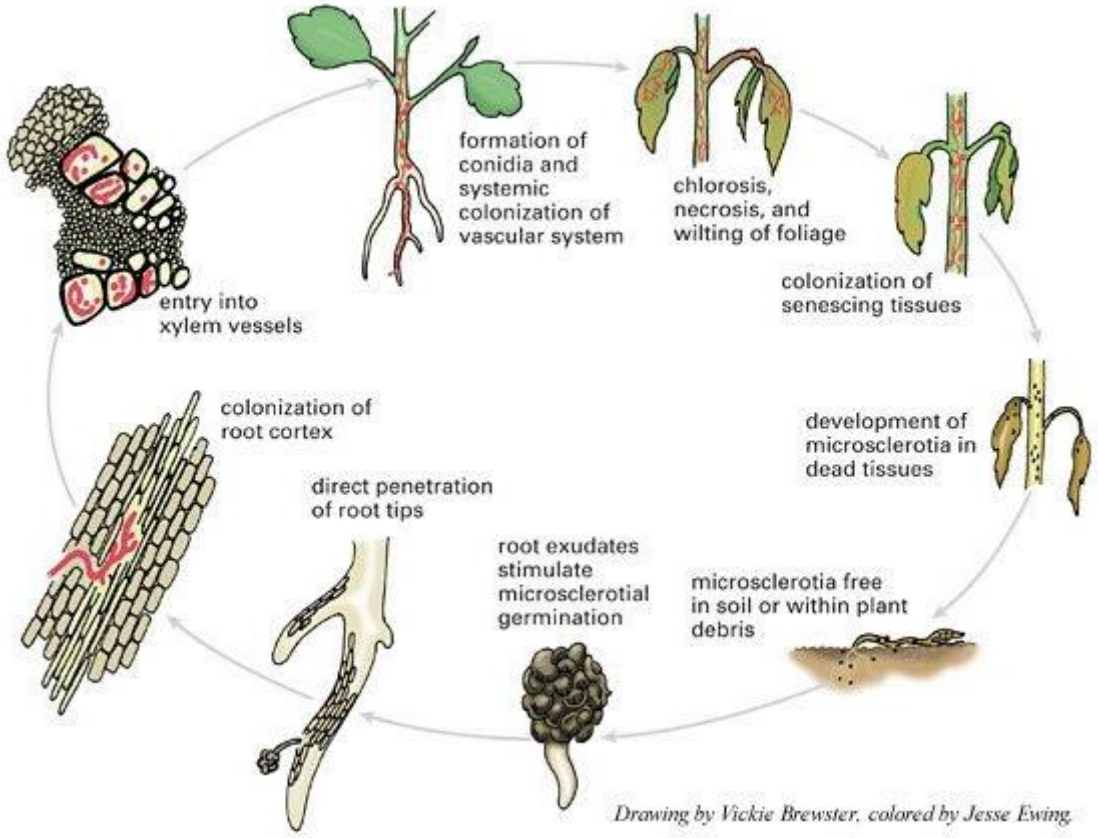


Figure 6. Life cycle of *Verticillium* spp. consists of three phases: dormant, parasitic and saprophytic. Adapted from a drawing of Vickie Brewster, colored by Jesse Ewing. (Source: http://www.apsnet.org/edcenter/intropp/lessons/fungi/ascomycetes/pages/verticilliumwilt.aspx).

Then, *Verticillium* spp. enters its parasitic stage by infecting susceptible plants either at the root tip or at the sites of lateral root formation (Bishop and Cooper, 1983). Once the vascular cylinder has been penetrated, *V. dahliae* begins to form conidia, which travel through the xylem system (Chen et al., 2004; Heinz et al., 1998). The plant becomes increasingly colonized, begins to wilt and develops typical symptoms of necrosis. During this stage, the pathogen enters the saprophytic phase of its life cycle, producing large numbers of microsclerotia from decaying tissue, which are released into the soil and can survive for 10-15 years (Wilhelm, 1955) **(Figure 6)**. *V. dahliae* is a monocyclic pathogen meaning that only a single round of disease and inoculum production occurrs in a growing season and that the

whole infection cycle need not be repeated in subsequent years (Fradin and Thomma, 2006; Pegg and Brady, 2002).

1.7 Objectives of the thesis

Muñoz et al. (2010) established that Fra a 1 proteins have an essential biological function in pigment formation in strawberry fruits. Recently, the work conducted by Franz-Oberdorf et al. (2016) in our laboratory aimed to identify the main strawberry fruit allergens by a basophil activation test (BAT).

The purpose of this study was to clone *Fra a 1* genes from *F.* × *ananassa,* according to the sequences provided by the *F. vesca* genome, to heterologously express the *Fra a 1* genes in *Escherichia coli* and to functionally characterize the corresponding recombinant proteins and define their gene expression levels in various tissues. The recombinant proteins were tested for RNase activity and used in phosphorylation assays and basophil activation test. Moreover, the binding of the Fra a 1-Associated protein (FaAP) to Fra a 1.04-1.08 by a novel time resolved DNA-switching system was also studied. Furthermore, this work aimed to investigate the molecular interplay between *in vitro* strawberry plants and the *Verticillium dahliae* pathogen in order to get molecular evidence about strawberry (*F.* × *ananassa*) defense response, to identify induced *Fra a 1* genes upon pathogen infection and to achieve insight into the hormonal response (ABA, SA, JA, GA and IAA) in strawberry.

Briefly, the purpose of the current study was to contribute to the understanding of the function of the Fra a 1 proteins by further investigating its functional aspects which would shed light on the molecular functions of the PR-10 proteins.

2 Materials and Methods

2.1 Materials

2.1.1 Plant material

Strawberry *Fragaria* × *ananassa* cv. Elsanta plants were provided from "Kraege International" company (Telgte, Germany) and were cultivated in a green house (Freising, Germany). Growing conditions were maintained at 25 °C (16 h /8 h, light/dark period). *In vitro F.* × *ananassa* plants were cultivated in a glass jar on 30 ml of modified MS medium under a light phase of 16 h. The average of the daytime temperature was 23.8 ± 1.4 °C whereas the temperature was around 22.0 ± 1.5 °C during the night.

2.1.2 Chemicals

The following chemicals were used in the current study:

Chemical	Manufacturer
Acetonitril	J.T. Baker, Griesheim
Acrylamide, 30%	Bio-Rad, München, Germany
Agar	Molekula, Taufkirchen, Germany
Agarose	Sigma-Aldrich, Steinheim
Ampicillin	Carl Roth, Karlsruhe
ATP	Carl Roth, Karlsruhe
Biochanin A	Sigma-Aldrich, Steinheim
Chloroform/Isoamyl alcohol (24/1)	Carl Roth, Karlsruhe
Citric acid	Carl Roth, Karlsruhe
Coomassie Brilliant Blue G250	Sigma-Aldrich, Steinheim
Diethyl pyrocarbonate (DEPC water)	Carl Roth, Karlsruhe
Dithiothreitol (DTT)	Carl Roth, Karlsruhe
Ethylenediaminetetraacetic acid (EDTA)	Carl Roth, Karlsruhe
Ethanol	J.T. Baker, Griesheim

Fast SYBR Green Master mix	Applied Biosystems, USA
Glycerol	Carl Roth, Karlsruhe
Glycine	Carl Roth, Karlsruhe
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Sigma-Aldrich, Steinheim
Jasmonic acid (JA)	Sigma-Aldrich, Steinheim
β-Mercaptoethanol	Carl Roth, Karlsruhe
Methanol	J.T. Baker, Griesheim
Nitrogen , liquid	Linde, München
Orange G	Fluka, Buchs
Potato dextrose agar (PDA)	Sigma-Aldrich
Potassium chloride (KCl)	Carl Roth, Karlsruhe
Phenol	Carl Roth, Karlsruhe
Polyvinylpolypyrrolidon (PVPP) for protein extraction	Sigma-Aldrich, Steinheim
Polyvinylpolypyrrolidon (PVP 40) for RNA extraction	Sigma-Aldrich, Steinheim
Sodium acetate (NaOAc)	Merck, Darmstadt
Sodium chloride (NaCl)	Carl Roth, Karlsruhe
Sodium sulfate	Fluka, Buchs
Tetramethylethylenediamine (TEMED)	Carl Roth, Karlsruhe
Torula yeast RNA	Sigma-Aldrich
Tris	Carl Roth, Karlsruhe
Tryptone	Carl Roth, Karlsruhe
Water for LC-MS	J.T. Baker, Griesheim
Western blotting substrate	Promega
Yeast extract	Carl Roth, Karlsruhe

2.1.3 Media, buffers and solutions

2.1.3.1 Media

LB-Medium	10 g/l Tryptone 5 g/l Yeast extract 10 g/l NaCl (pH 7)
LB-Agar	LB-Medium 15 g/l Agar
SOC-Medium	20 g/l Tryptone 5 g/l Yeast extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM Glucose (sterilize separately using 0.2 µm filter)

2.1.3.2 Buffers

Sodium phosphate buffer (0,1 M; pH 7.4)	77.4 ml 1 M Na_2HPO_4 22.6 ml 1 M NaH_2PO_4 Add 1 l of H_2O Adjust pH to 7.4
1x His-Tag wash/bind buffer	50 mM Sodium phosphate (pH 7.4) 300 mM NaCl 30 mM Imidazole
1x His-tag elution buffer	50 mM Sodium phosphate (pH 7.4) 300 mM NaCl 300 mM Imidazole
1x running buffer SDS (blotting)	25 mM Tris 192 mM Glycine 0.1% SDS

TE Buffer	1 mM EDTA (pH 8) 10 mM Tris-HCl (pH 8)
50x TAE Buffer	242 g/l Tris 57.1 ml/l Acetic acid 100 ml/l of 0.5 M EDTA (pH 8)
RNA extraction buffer	3% CTAB 3% PVP, K30 25 mM EDTA 2 M Sodium chloride 100 mM Tris/HCl (pH 8) Add 40 µl/ml ß-mercaptoethanol to the buffer before use and then heat to 65 °C for pre-warmed buffer
Semidry blotting Buffer	25 mM Tris 200 mM Glycine 20% Methanol
Blocking Buffer	5% BSA in washing buffer
Washing Buffer (TBST)	20 mM Tris 140 mM NaCl 0.1% tween 20
Detection Buffer	100 mM Tris (pH 9.5) 100 mM NaCl 5 mM $MgCl_2$
TFBI buffer for competent cells	30 mM $C_2H_3KO_2$ 10 mM $CaCl_2$ $2H_2O$ 50 mM $MnCl_2$ $4H_2O$ 100 mM RbCl 15% Glycerol

	Adjust pH to 5.8 and then filter
TFBII buffer for competent cells	100 mM MOPS 75 mM $CaCl_2$ 10 mM RbCl 15% Glycerol Adjust pH to 6.5 and then filter
PBS buffer	140 mM NaCl 8 mM Na_2HPO_4 2.7 mM KCl 1.76 mM KH_2PO_4

2.1.3.3 Other solutions

Bradford	100 mg/l Coomassie Brilliant Blue G250 50 ml/l Ethanol (96%) 100 ml/l Phosphoric acid (85%)
Staining solution	0.33g Coomassie Brilliant Blue G250 120 ml Methanol 24 ml Acetic acid 120 ml H_2O
Destaining solution	20% Ethanol 10% Acetic acid
Detection substrate NBT	5 mg in 100 µl of 70% DMF
Detection substrate BCIP	5 mg in 100 µl H_2O or in 100 µl DMF
Glycerol for cell stocks	65% Glycerol (v/v) 0.1 M $MgSO_4$ 25 mM Tris Adjust pH to 8 with HCl

10× DNA loading dye	0.21% Orange G 0.1 M EDTA (pH 8) 50% Glycerol
Colloidal coomassie staining	5% Aluminium sulfate- (14-18)-hydrate 20% Ethanol 0.1% Coomassie blue G250 2% ortho-Phosphoric acid
Internal standard LC-MS	50 mg Biochanin A 50 mg 4-Methylumbelliferyl-β-D-glucuronide 250 ml Ethanol
DEPC Water	0.1% of DEPC in Milli Q water Stirred for overnight and then autoclaved

2.1.4 Bacterial strains

E. coli JM 109	Promega, Mannheim, Germany
E. coli BL21 (DE3) pLysS	Novagen, Darmstadt, Germany

2.1.5 Vectors

pGEM® -T easy	Promega, USA
pQE70	Quiagen GmbH, Hilden, Germany

2.1.6 Enzymes

DNA polymerase	-DNA Polymerase (5 U/µl, New England Biolabs, Frankfurt, Germany) Ipswich, MA, USA -PhusionTM (2U/µl, Finnzymes, Espoo, Finland)
Reverse transcriptase	-M-MLV (Promega, Madison, WI, USA)

	-Superscript III (Invitrogen, Carlsbad, USA)
Restriction enzymes	BamHI, EcoRI, NheI, NotI, SmaI, SpeI, XbaI, XhoI, HindIII, Bgl (FastDigest, Fermentas, St. Leon-Rot)
Other enzymes	-DNase I and RNase-free (Fermentas) -RNaseA (New England Biolabs) -T4 DNA Ligase (Fermentas) -RNaseOUTTM Recombinant Ribonuclease Inhibitor (40U/µl, Invitrogen) -Calf Intestinal Alkaline Phosphatase (CIP) (New England Biolabs) -Lambda phosphatase (λ –PP) (BioLabs, New England)

2.1.7 Antibiotics

Antibiotics	**Stock solution**
Ampicillin	100 mg/ml in mQ water
Chloramphenicol	34 mg/ml in ethanol
Kanamycin	25 mg/ml in mQ water

2.1.8 Primers

Primers used for cloning were designed using Geneious software:

Primer name	**Primer sequence (*5′→3′*)**
*Fra a 1.04*_f	GATCGCATGCATGGGTGTCTTCACA
*Fra a 1.04*_r	GATCGGATCCTTAACAGTATTCATT
*Fra a 1.05*_f	GATCGCATGCATGGGTGTATTCACT
*Fra a 1.05*_r	GATCGCATGCTTAACAGTATTCATT
*Fra a 1.06*_f	GATCGCATGCATGGGTGTGTTCACT
*Fra a 1.06*_r	GATCGGATCCACAGTATTCATTAGGATT

*Fra a 1.07*_f	GATCGCATGCTGGGCGTGTTCACTTAT
*Fra a 1.07*_r	GATCGGATCCGTTGTATGCATCAGGGTT
*Fra a 1.08*_f	GATCGCATGCTGGGTGTGTTCACATAT
*Fra a 1.08*_r	GATCGGATCCGTTGTAGGCCTCAGGGTT

Primers of the 21 *Fra a 1* isoforms used for qPCR were designed using different software such as BioEdit, Clustal 2.1 and OligoAnalyzer:

Primer name	**Primer sequence (*5′→3′*)**
*Fra a 1.04*_f	AGCTCTTCTTTTCTTGTGTCACTT
*Fra a 1.04*_r	GGTTCCTACGCCTCCATCTC
*Fra a 1.05*_f	TCCTTCAACTTCTCAAACACCA
*Fra a 1.05*_r	GTTCCTACGCCTCCGTCTC
*Fra a 1.06*_f	TTTTATCATTTCCCAGTTTGTAAGC
*Fra a 1.06*_r	CCTTCAACGATTTCAGCACA
*Fra a 1.07*_f	TCAGTCTTCTCATCTTAAGCTTGC
*Fra a 1.07*_r	GTTCCAACACCTCCATCACC
*Fra a 1.08*_f	TTTCAATCTTCCCATTTTTAGCTC
*Fra a 1.08*_r	GTATTCGCTCCCTTCACCAA
*Fra a 1.01*_f	TCCTTATTCCCTGCTTGCTT
*Fra a 1.01*_r	AGGTGATCTTCTTGATGGTTCC
*Fra a 1.02*_f	CGTGGA GATCAAGGAAGAGC
*Fra a 1.02*_r	GGAACATCAGCGGAACAAAT
*Fra a 1.03*_f	GTTAAGGCCGGAAAGGAAAG
*Fra a 1.03*_r	GATCGAGCAGATTGACACCA
*04962*_f	CTTGTGTCATTTCCCAACTCA
*04962*_r	ATGGTTTCAGCACCCTTGAC
*07083*_f	TTCCCTTCAGCTCTTCTACATTG
*07083*_r	GAACGATTTCAGCGCTCTTC

*07077*_f	TCAGAAGCTAGCTGAGCCAAG
*07077*_r	TCACCTTCCAGGAATTCGAT
*32299*_f	CCGTTTACCTCATCAAATTATTAACTC
*32299*_r	TTCCCCGAGGTTGATCTTCT
*05185*_f	TGTCTTCAAGTACGAGGCTGAA
*05185*_r	CAATGGCATCAATTCTGTGC
*07063*_f	TCAACCTCATCAGTCACACCA
*07063*_r	CTTCGCCAAAGGTGATCTTC
*07076*_f	GCACAGAATCGATGAACTCG
*07076*_r	GCTTCTCCAATGGGGTTGTA
*07085*_f	TTCTCTGATCATTTCCCTTTTAGC
*07085*_r	CCAACTCCTCCATCACCTTC
*07081*_f	CGAATTGAGAATTTCGATCCTA
*07081*_r	TACTGCCTTCCCCAAAGGTA
*11094*_f	TCTGGTCAATTTCATCAGCAA
*11094*_r	TAATGGCCTGAGGCATGAGT
*07084*_f	TGGGTCTGTTCTTTGATCAATTT
*07084*_r	TCAGCATCGAGAACAAATGC
*07087*_f	TCACCTTATGGAGACCTAGCTATTG
*07087*_r	GCTGGGGTGCTTTCTTTGAC
*07078*_f	TTTGAGGTGATTAACACATAGATTACG
*07078*_r	ACCGCACTAGCCATGAATGT
Interspacer_f	ACCGTTGATTCGCACAATTGGTCATCG
Interspacer_r	TACTGCGGGTCGGCAATCGGACG

2.1.9 Commercial kits

Amine coupling kit	**Dynamic Biosensors, Germany**
Fast SYBR® Green Master Mix	Applied Biosystems, Foster City, CA, USA
Nucleo Spin® Extract II	Macherey-Nagel, Düren
pGEM®-T Vector System	Promega, Mannheim, Germany
Pro-Q Diamond gel stain	Invitrogen
QIAEX II Gel Extraction Kit	QIAGEN, Hilden, Germany
SensiMixTMSYBR Kit	Bioline GmbH
Wizard® Plus SV Minipreps DNA Purification System	Promega

2.1.10 Other materials

GeneRulerTM 1kb DNA Ladder	Fermentas
GeneRulerTM DNA Ladder Mix	Fermentas
Protein Marker Broad Range (14-66 kDa)	Sigma-Aldrich, Germany
96-*well* Microtiterplatte for qPCR	Applied Biosystems, Darmstadt
dNTPs	10 mM, Fermentas
dATP	100 mM, Fermentas

2.2 Equipment

2.2.1 Liquid chromatography ultraviolet electro-spray ionization mass spectrometry (LC-UV-ESI-MS)

HPLC	Agilent 1100 Series (Agilent Technologies Inc., Santa Clara, California, USA)
Pump	Agilent 1100 Quaternary Pump
Autosampler	Agilent Autosampler
Injection volume	5 µl
Separation column	Luna 3 µm C18 (2) 100 Å 150 x 2.0 mm

	(Phenomenex® Aschaffenburg, Germany) (Part Number: 00F-4251-B0)
Precolumn	Security Guard Cartridges C18 4 x 2 mm (Penomenex®)
Column temperature	25 °C
Solvents	A: Water plus 0.1% formic acid B: Methanol plus 0.1% formic acid
Flow Rate	0.2 ml/min
Gradient	0-30 min: 0-50% B 30-35 min: 50-100% B 35-50 min: 100% B 50-55 min: 100-0% B 55-65 min: 0% B Run-time: 65 min
UV-Detector	Agilent 1100 variable wavelength detector
Spray gas	Nitrogen (30.0 psi)
Spray gas	Nitrogen (30.0 psi)
Polarity	positive/negative
Scan range	*m/z* 100 to 800
Capillary voltage	± 4000 V
MS/MS	Auto-tandem MS^2
Collision gas	Helium 5.0 (4.21 x 10^6 mbar)
Collision voltage	1.0 V

2.2.2 Other equipments

Autoclave	Systec V95, Wettenberg, Germany
Centrifuge	Sigma 4K15C (Sigma, Osterode) Sigma 2K15 (Sigma, Osterode)

	Eppendorf 5415 R (Eppendorf, Hamburg) Eppendorf miniSpin (Eppendorf, Hamburg) Beckman Avanti J-25 (Beckman, Madrid)
Electrophoresis	Roth, Karlsruhe, Germany
NanoDrop ND-1000	peQLab Biotechnologie GmbH, Erlangen, Germany
pH meter	CG 820 (Schott Geräte, Mainz)
Scales	Scaltec SPB61 (Scaltec Instruments GmbH, Göttingen) Scout Pro SP U4001 (Ohaus, Pine Brook, New Jersey, USA) TP 214 (Denver Instrument, Bohemia, New York, USA)
Shaking incubator	GFL 3033 , Burgwedel, Germany
Spectrophotometer	Nicolet evolution 100 (Thermo, Cambridge, England)
StepOnePlus™ Real-time PCR system	Applied Biosystems, California, USA
Thermocycler	Primus 96 advanced (Peqlab Biotechnologie, Erlangen, Germany) SensoQuest labcycler (SonsoQuest, Göttingen)
Thermomixer	Eppendorf, Hamburg, Germany
Ultrasonic bath	RK103H (Bandelin Electronic, Berlin, Germany)
Ultrasonic Homogenizer	Bandelin Sonopuls UW2200 (Bandelin Electronic) Bandelin UW2070 (Bandelin Electronic, Berlin)
UV-Transilluminator	G:BoX (Syngene, Cambridge, UK)
Vortexer	VWR, Darmstadt, Germany
Water bath	Julabo, Seelbach, Germany

2.2.3 Software and internet resources

BioEdit	http://www.mbio.ncsu.edu/BioEdit/bioedit.html
BLAST	https://blast.ncbi.nlm.nih.gov/Blast.cgi
DataAnalysis and QuantAnalysis 6.2	Bruker Bremen, Germany
GeneiousTM Pro 5.6	https://www.geneious.com/
OligoAnalyzer 3.1	https://eu.idtdna.com/calc/analyzer
SAS	Statistic program (SAS Institute Inc., Cary, USA)
StepOnePlus™ 2.1	Applied Biosystems, California, USA
Genome Database for Rosaceae (GDR)	http://www.rosaceae.org/
National Center for Biotechnology Information (NCBI)	http://www.ncbi.nlm.nih.gov/

2.3 Methods

2.3.1 Polymerase Chain Reaction (PCR)

To amplify the cDNA, a polymerase chain reaction (PCR) was carried out using forward and reverse primers which were designed by Geneious software. Phusion polymerase and Taq polymerase were used for amplification and colony PCR Screening. PCR was performed in a reaction volume of 20 µl as mentioned below:

	Phusion polymerase	**Taq polymerase**
Buffer	4 µl of (5x HF buffer)	2 µl (10x ThermoPol buffer)
dNTP (2.5 mM)	1 µl	1 µl
Forward primer (10 µM)	1 µl	1 µl
Reverse primer (10 µM)	1 µl	1 µl
Template	1.5 µl	Bacterial cells
Polymerase	0.2 µl	0.3 µl
H_2O	up to 20 µl	up to 20 µl

PCR reactions were conducted under the following conditions:

	Temperature	Time (min, sec)
Initial denaturation	98 °C	2 min
Denaturation	98 °C	30 sec
Annealing	55 °C	30 sec
Extension	72 °C	1 min
Final Extension	72 °C	10 min

PCR program: 35 cycles

2.3.2 Agarose gel electrophoresis

After weighting out 0.8 g of agarose into an Erlenmeyer flask, 80 ml of TAE running buffer were added. The mixture was heated in a microwave for around 3 minutes. When the agarose was completely dissolved, 4 µl of ethidium bromide was added. The agarose was poured into a gel tray with the well comb in place. After sitting at room temperature for 20-30 minutes until agarose was completely solidified, the gel tray was placed in the casting apparatus and the loading was added to the nucleic acid samples to be separated with the appropriate marker. Electrophoresis was conducted for about 20 to 30 min and DNA or RNA fragments were later visualized by a UV box.

2.3.3 DNA purification from agarose gel

NucleoSpin® Extract II kit and QIAEX II Gel Extraction Kit were used for the purification of DNA fragments from TAE agarose gel and direct purification of PCR products. Samples were prepared according to the manufacturer's instructions.

2.3.4 A-Tailing

A-Tailing is an enzymatic method for adding a 3' terminal adenine overhang onto the PCR product amplified using a blunt-ended, proof-reading enzyme (Phusion). This procedure was done to facilitate the ligation of the PCR product into the pGEM T-easy vector. The A-Tailing was performed for 30 minutes at 72 °C as follows:

PCR Product	7 µl
dATP (2 mM)	1 µl
10x Taq polymerase Buffer	1 µl
Taq polymerase	1 µl

2.3.5 Ligation

The final step in the construction of a recombinant plasmid is the ligation of the insert into a compatible vector using the T4 DNA ligase enzyme which catalyzes the formation of covalent phosphodiester bonds. The reaction was set up overnight at 4 °C in the following way:

PCR product	6 µl
pQE70 vector	2 µl
10x Ligation buffer	1 µl
T4 DNA Ligase	1 µl

As control, 6 µl of water was added instead of the PCR product. Then, the transformation into bacterial cells were proceeded to rectify the correct ligation of the PCR products.

2.3.6 Transformation

A 5 µl aliquot of ligation solution was added to 100 µl of *E. coli* BL21 (DE3) pLysS competent cells. The mixture was incubated on ice for half an hour and a heat shock method was used for the transformation: 1 minute at 42 °C in water bath followed by a 2 minutes incubation on ice. Afterwards, 500 µl of SOC medium were supplemented to the Eppendorf tube and the reaction was carried out at 37 °C for one hour. Bacterial cells were plated out on LB plates containing the appropriate antibiotic for 18 h at 37 °C.

2.3.7 Restriction enzyme digestion

In order to generate compatible ends capable of being ligated together for the plasmid DNA and the vector, appropriate restriction enzymes which cleave at specific sites are determined. The restriction endonuclease approach was carried out for one hour at 37 °C as follows:

Plasmid DNA	1 µg
Restriction enzyme	1 µl (10 units/µl)
Buffer 10x	3 µl
H_2O	up to 30 µl

Then, an agarose gel was run to verify the correct size of the digested fragments.

2.3.8 Expression and purification of His-Tag Fra a 1 proteins

E. coli BL21 (DE3) pLysS cells were transformed with Fra a 1-pQE 70 construct. A unique colony was inoculated into 1 l of LB medium containing 100 µg/ml of ampicillin and 34

µg/ml of chloramphenicol. Cells were grown under shaking at 150 rpm overnight to an OD600 of 0.6-0.8. At this point, 1 mM IPTG was added to induce the expression of His-tag fusion protein at 18 °C, under shaking at 150 rpm for 16-20 h. Then, cells were harvested by centrifugation at 5,000 rpm for 10 min at 4 °C. After resuspension of the pellet in 10 ml of wash/bind buffer, cells disruption was carried out by sonication on ice, five times for 30 seconds. Following centrifugation at 10,000 rpm for 20 minutes at 4 °C, the supernatant was incubated with the resin in a gravity column for at least 1 hour at 4 °C under soft shaking. The column was washed twice with wash/bind buffer and then the bound proteins were eluted with an elution buffer. The correct size of the recombinant proteins was verified by SDS-PAGE.

2.3.9 Regeneration of His-Tag column (IMAC resin column)

Column regeneration was performed after 5 protein purifications using a nickel solution. The resin column was equilibrated with 5 ml of 50 mM of sodium acetate supplemented with 0.3 M of NaCl (pH 4). After an incubation step with 4 ml of 0.2 M of nickel chloride (pH 6) for 30 minutes, the column was washed again with 5 ml of NaOAc/NaCl solution and 10 ml of deionized water. Finally, the resin column was equilibrated with 5 ml of starting buffer and was therefore ready for a new purification.

2.3.10 Bradford assay

A 5 µl aliquot of the protein sample was mixed with water to a total volume of 100 µl and 1 ml of Bradford solution was added to each sample. After 5 minutes in the dark, the mixture color, initially brown, became blue by the formation of a complex between the dye and the proteins in solution. Then, the absorbance was determined at 595 nm using a spectrophotometer. A standard calibration curve using varying concentrations of BSA was used to define the concentration of each protein as follows:

Protein [µg/µl] = [OD 595 nm / concentration (BSA calibration curve)] / sample volume (µl)

2.3.11 SDS-PAGE

The SDS-PAGE gel consists of a stacking gel and a resolving gel. The 7% acrylamide gel was poured on the top of the separating gel after solidification and a gel comb was then inserted. After polymerization of the gel, 5 µg of heated proteins mixed with 4x protein loading dye were loaded into the wells. The electrophoresis was done in 1x SDS buffer for approximately

2 hours at 120 V constant. Following electrophoresis and in order to visualize the separated proteins, the gel was stained with Coomassie Brilliant Blue or processed further with Western blot.

	12% SDS gel (Resolving gel)	**7% SDS gel (Stacking gel)**
H_2O	2.45 ml	1.85 ml
30% Acrylamide	3	0.7 ml
1.5 M Tris (pH 8.8)	1.9	375 µl
10% SDS	75 µl	30 µl
10% APS	75 µl	30 µl
TEMED	3 µl	3 µl

2.3.12 Western blot

In order to detect specific proteins, a western blot was performed. After electrophoresis, proteins were transferred to a PVDF membrane which was activated in methanol and equilibrated in semidry blotting buffer for 5 minutes. Then, the polyacrylamide gel, the buffer soaked filter papers and the membrane were placed in the semidry blotter and an electric current of 10 V was applied. After 1 h, the PVDF membrane was incubated at room temperature for 60 minutes in blocking buffer under shaking and then washed 3 times in washing buffer for 5 minutes each. The incubation with the primary antibody was performed at 4 °C overnight. Afterwards, the membrane was rinsed again with washing buffer for 30 minutes and incubated with a secondary antibody for 1 h at RT. The PVDF membrane was washed later for 30 minutes in a washing buffer and for 10 minutes in a detection buffer. For detection, the membrane was incubated in 20 ml detection buffer which contains 50 µl of NBT and 25 µl of BCIP in the dark on the shaker, until bands appeared. Finally, the membrane was rinsed with water and scanned.

2.3.13 Preparation of chemically competent *E. coli* cells

In order to make chemically competent cells which are calcium chloride treated, one colony of fresh overnight culture of *E. coli* strain was added to 200 ml of LB medium containing the appropriate antibiotic. A shaking step (150 rpm) was performed at 37 °C until the absorbance at 600 nm was approximatively 0.5. Then, the culture was chilled on ice for 5 minutes and cells were poured into a 50 ml Falcon tube. Afterwards, the cell suspension was centrifuged for 5 minutes at 4,000 *g* and the pellet was gently resuspended in 30 ml of ice-cold TFBI

(transformation buffer I) buffer. Following an incubation step on ice for 90 minutes, a second centrifugation was achieved and the pellet was washed with 9 ml TFBII (transformation buffer II) buffer. The cell suspension was distributed in 100 µl aliquots, flash-frozen in liquid nitrogen and finally stored at -80 °C. At this temperature, the cells were competent for at least 6 months.

2.3.14 RNA extraction

Total RNA was isolated as described by Liao et al. (2004) using the CTAB method. A 20 ml aliquot of pre-heated extraction buffer was added to 1-2 g of lyophilized strawberry material. During the mixture´s incubation at 65 °C, Falcon tubes were inverted manually 3 times. Then, an equal volume of chloroform/isoamyl alcohol was added and the samples were again gently inverted by hand for 10 minutes. After centrifugation at 12,000 rpm (4 °C), the supernatant was transferred to a new Falcon tube and a 1/3 volume of 8 M LiCl was supplemented to the reaction. RNA precipitation was carried out overnight at 4 °C. After centrifugation at 12,000 rpm, for 30 minutes at 4 °C, the RNA pellet was gently dissolved in 500 µl 0.5% SDS. A second centrifugation step was following and the supernatant was then transferred to a new Eppendorf tube containing 2 volumes of 100% ethanol. The reaction was performed for 2 hours at -20 °C. After centrifugation of the precipitated RNA (30 minutes, 4 °C, 13,200 rpm), the pellet was washed with 70% and the 100% ethanol. Subsequently, the pellet was air-dried and then dissolved in 50 µl DEPC. The quality and quantity of RNA was determined using Nanodrop and 1% agarose gel.

2.3.15 Purification of RNA by DNase treatment

In order to remove any DNA contamination, extracted RNA was treated with DNase as follows:

RNA	5 µg
DNase buffer	3 µl
DNase	3 µl
DEPC water	up to 30 µl

After incubation at 37 °C for 1 h of the components above, 100 µl of DEPC water and 150 µl of phenol/chloroform/isoamyalcocol (25/24/1) was added to the Eppendorf tube. Samples were centrifuged (max speed, 4 °C) for 5 minutes and the supernatant was transferred to a new tube. Afterwards, a second centrifugation of the lower phase, mixed with 100 µl of

DEPC water, was performed. Then, both supernatants were combined and 0.1 volume of 3 M Na-acetate (pH 5.2; RNase free) and threefold volume of ethanol (100%, -20 °C) were added. The precipitation was conducted for 3 hours at -80 °C or alternatively overnight at -20 °C. Later, the precipitated RNA was centrifuged for 30 minutes and the pellet was washed successively with 500 µl of 70% and 100% ethanol. Finally, the dried pellet was resuspended in 20 µl of DEPC water and the RNA concentration was determined with NanoDrop.

2.3.16 First-strand cDNA synthesis

One µg of DNase-treated RNA was reverse transcribed to cDNA using 1 µl of random primers (10 µM) and 1 µl of 10 mM dNTP in 20 µl total volume. After a five minutes incubation time at 65 °C, the reaction mix was chilled on ice for 1 minute. Next, the following components were added to a nuclease-free-micro centrifuge tube as follows:

5x First strand buffer	4 µl
0.1 M DTT	2 µl
RNase OUT recombinant RNase inhibitor	1 µl

One µl of M-MLV RT was added after an incubation of 2 minutes at 37 °C. An incubation step at 25 °C for 10 minutes is recommended before performing the cDNA synthesis at 50 °C for one hour. Then, the temperature was raised to 70 °C to inactivate the reverse transciptase and the cDNA was stored at -20 °C.

2.3.17 Quantitative real time PCR (qPCR)

In order to test the expression of *Fra a 1* genes, different quantitative real time PCR experiments using the StepOne Software were performed. Primers were designed with different bioinformatics tools (BioEdit/ Clustal 2.1/ Primer3/ OligoAnalyzer 3.1). The amplification was carried out as follows in 20 µl total volume:

SYBR® Green Mix	10 µl
forward Primer (10 µM)	0.6 µl
reverse Primer (10 µM)	0.6 µl
template	2 µl
H_2O	6.8 µl

The PCR amplification program was optimized for 40 cycles. The amplification was carried out in 96-well plate and all samples were amplified in at least three technical replicates. A cDNA dilution of 1/20 for the target gene and 1/8,000 for the housekeeping gene were found enough sensitive for the gene quantification studies.

The Ct values (threshold cycle value) of each *Fra a 1* gene were normalized using the Ct value of the strawberry *FaRIB413* gene, corresponding to an internal RNA interspacer (16S-23S). The fold change of gene expression was determined using the following expressions:

ΔCt = Ct (target gene) – Ct (internal control gene)

$\Delta\Delta Ct$ = ΔCt (target gene) – ΔCt (reference gene)

Relative gene expression = Fold change = $2^{-\Delta\Delta Ct}$

2.3.18 Preparation of native protein extract from strawberry

Five hundred g of frozen strawberry fruits were homogenized with an Ultra-turrax under constant addition of 100 g PVPP and 1 l of demineralized water. The pH of the suspension was adjusted to 7.7 with 1 N of sodium hydroxide. Then, the suspension was equally divided into 50 ml Falcon tubes and centrifuged for 25 min at 4 °C and 21,000 rpm. The supernatant was dialyzed overnight against 20 mM Tris HCl at pH 7.7. The suspension was then filtered through a glass wool and ammonium sulfate was stirred into the obtained extract for 30 minutes on ice until 20% saturation was achieved. For 20% saturation, 114 g of ammonium sulfate per liter of extract was used. The ammonium sulfate was previously ground in a mortar, to facilitate dissolution in the extract. The resulting solution was further stirred for 30 min on ice and then centrifuged for 25 min at 4 °C and 21,000 rpm. The gelatinous pectin residue was discarded and the supernatant was dialyzed overnight against 20 mM Tris HCl (pH 7.7). The contents of the dialysis membrane was uniformly divided into 50 ml Falcon tubes and lyophilized. The freeze-dried protein was resuspended in minimal amount of mQ water by pipetting up and down and the strawberry extract was finally stored at 4 °C.

2.3.19 RNase activity

The ribonuclease activity was determined using an in-solution and in-gel RNase activity:

*In-solution RNase activity assay was performed by incubating the five recombinant Fra a 1.04-1.08 proteins in the presence of total RNA (1 µg) at different pH values for 4 hours at 37 °C. Ten µl of 100 mM citrate buffer at pH 3 was used, MES buffer (2-(N-morpholino) ethanesulfonic acid) at pH 5, phosphate buffer at pH 7.4 and CAPS buffer (N-cyclohexyl-3-aminopropanesulfonic acid) at pH 9. Denatured proteins at 100 °C for 15 minutes (boiled),

empty vector and phosphate buffer alone served as negative controls. RNA was separated on 1% agarose gel stained with ethidium bromide. In-solution RNase activity was also tested after dephosphorylation of Fra a 1.04, Fra a 1.05, Fra a 1.07 and Fra a 1.08 with alkaline phosphatase calf intestinal (CIP) and lambda phosphatase for 1 hour at 37 °C and 30 °C, respectively. One µg of the 4 phosphoproteins was also incubated in the presence of total RNA of strawberry fruit at 37 °C for 4 hours (pH 7.4). Fra a 1.06 was used as positive control whereas phosphate buffer and empty pQE70 vector were used as negative control. Controls were also dephosphorylated with CIP for 1 hour at 37°C.

*In-gel RNase activity was tested as described by Yen and Green (1991). The resolving gel was cast with 2.4 mg/ml of yeast torula RNA and an equal volume of 2x sample loading buffer was added to 5 µg of proteins. Gels were run at a constant voltage of 140 V for 2 hours. After one dimensional electrophoresis, SDS gels were washed twice with 25% isopropanol in 0.01 M Tris-HCl and then twice with 0.01 M Tris-HCl to remove the isopropanol, each step 10 minutes at RT. Following the washing steps, gels were incubated in 0.1 M Tris-HCl for 50 minutes at 51 °C. Afterwards, gels were stained once with 0.2% toluidine blue in 0.01% Tris-HCl for 10 minutes and destained twice in 0.01% Tris-HCl. Finally, gels were rinsed, equilibrated in 10% glycerol and dried in cellophane. In-gel RNase activity generate a gel with blue background and proteins with RNase activity were located on the basis of bright region observed at the molecular weight corresponding to the protein of interest. The activity was tested in the presence and absence of reducing agents. In-gel RNase activity was also performed with 10 µg of the 4 dephosphorylated Fra a 1.04, Fra a 1.05, Fra a 1.07 and Fra a 1.08 proteins, produced by treatment with alkaline phosphatase calf intestinal (CIP) and lambda phosphatase for 1 hour at 37 °C and 30 °C, respectively.

2.3.20 LC-UV-ESI-MS

For this approach an internal standard, containing 50 mg of biochanin A and 50 mg 4-methylumbelliferyl-ß-D-glucuronide which were dissolved by sonication for 10 minutes in 250 ml methanol, was prepared. For the extraction procedure, 50 mg of lyophilized tissues (leaves, roots and stems) of infected and non-infected strawberry plants were mixed with 250 µl of internal standard and 250 µl of methanol. Samples were vortexed for 1 minute, sonicated for 10 minutes and centrifuged also for 10 minutes at 13,200 rpm. Supernatant was taken into a new Eppendorf tube and the pellet was extracted again with 500 µl of methanol. Then, both supernatants were unified and treated with Speed-Vac System for 2-3 hours to evaporate the methanol. The dried pellet was resuspended in 35 µl LC-MS quality water, vortexed,

sonicated and centrifuged twice at max speed. Finally, the clear supernatant was taken into an LC-MS vial with micro-insert and analyzed by LC-UV-ESI-MS. The accumulation of metabolites in leaves, stems and roots of infected and non-infected *in vitro* plants was tested referring to their retention time and fragmentation pattern by the means of QuantAnalysis software.

2.3.21 *Verticillium* infection assay of strawberry *in vitro* plants

2.3.21.1 Propagation of *in vitro* plants

In order to propagate the *F.* × *ananassa in vitro* plants, a revised Murashige and Skoog (MS) medium was used **(Table 4)**. This culture medium containing all the nutritive elements (micro and macronutrients, vitamins and iron sources) was prepared by adding 20 g/l of sucrose, which is the best source of carbon for *in vitro* culture, and 6.5 g/l agar.

Table 4. Composition of the revised MS medium (Murashige and Skoog, 1962).

Major elements	**mM**
NH_4NO_3	20.6
$CaCl_2$ x 2 H_2O	3.0
$MgSO_4$ x 7 H_2O	1.5
KNO_3	18.8
KH_2PO_4	1.3
Minor elements	**µM**
H_3BO_3	100
$CoCl_2$ x 6 H_2O	0.1
CuSO4 x 5 H_2O	0.1
$MnSO_4$ x H_2O	100
KI	5
Na_2MoO_4 x 2 H_2O	1
$ZnSO_4$ x 7 H_2O	30
NaFe-EDTA	100
Organic constituents	**mM**
myo-inositol	550

thiamin	0.3
Glycine	26.6
Pyridoxine	2.4
nicotic acid	4.1

After adjusting the pH to 5.8 ± 0.2 by HCl or KOH (1 M) and in order to prevent contamination of the culture media and decrease the spread of plant parasites, the medium was autoclaved at 121 °C for 20 minutes. Then, after cooling down the agar to 45-50 °C, the medium was supplemented with 50 mg/l of citric acid and ascorbic acid and finally dispensed in jars. Once the agar was solidified, the *in vitro* plants were transferred into the jars and the strawberry culture was maintained for around 6 weeks.

F. × *ananassa in vitro* plants were grown for several weeks in a dedicated room with a 16 h photoperiod. The average of the daytime temperature was 23.8 ± 1.4 °C while during the night the temperature was around 22.0 ± 1.5 °C. This procedure was repeated until reaching the desired amount of *in vitro* plants.

2.3.21.2 *In vitro* plants *Verticillium* infection

F. × *ananassa in vitro* plants were artificially inoculated by the isolate E650 of *Verticillium dahliae* (Müncheberg, 2002), kindly provided by the Institute for Breeding Research on Horticultural Crops, Dresden **(Table 5)**.

Table 5. Instructions of line E650 of *Verticillium dahliae.*

Isolate	Reference	Culture	Origin	Year	Damage for Elsanta
E650	Hr. Lentzsch	*Fragaria* × *ananassa* cv. Elsanta	Müncheberg	2002	Weak-medium

Verticillium dahliae was cultured on potato dextrose agar using two layers of gauzes. The petri dishes were stored in the darkness for 3-4 weeks at 22 °C **(Figure 7)**. After one month, the gauzes were immerged and swirled in 10 ml of sterile distilled water. The conidia concentration was adjusted to 10^6 spores/ml using a Thoma-Neu hemocytometer.

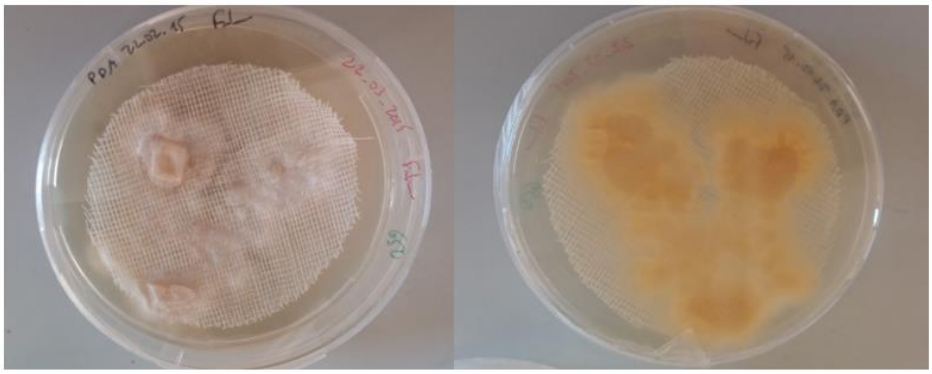


Figure 7. Culture of *Verticillium dahliae* in PDA medium. The bottom (right) and the top (left) of the Petri dish represent the evolution of the culture after 3-4 weeks.

This device contains two identical counting areas which were loaded independently by pipetting 10 µl of *Verticillium dahliae* suspension under the glass cover **(Figure 8).**

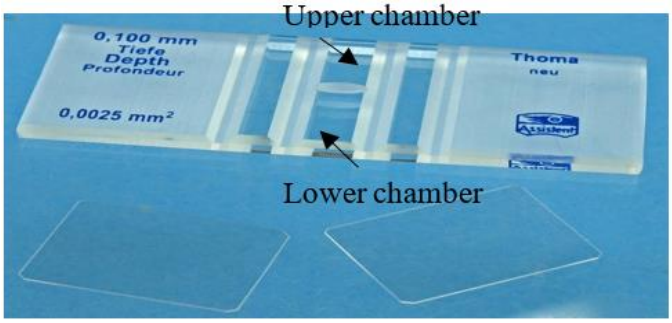


Figure 8. Thoma-Neu commercial chamber containing two identical counting areas. The frame of the counting chamber contains a large central square which is divided into 16 medium squares.With the 10x objective of the microscope the counting area was located while to count the cells the microscope was switched to 40x objective.

Thoma-Neu chamber's counting grid is 1.1 mm x 1.1 mm in size and 0.1 mm in depth. This grid is divided into 16 large squares of 0.2 mm edge length **(Figure 9)**. Each square has the same dimensions and contains 4 x 10^{-6} ml of suspension.

Volume = depth * area

= 0.1 mm * 0.2 mm * 0.2 mm

= 4 x 10^{-3} mm^3

=4 x 10^{-6} ml

Using a microscope, cells touching the upper and the left limits were counted for each square of the counting chamber. Then the cell density was calculated as follows:

$$\text{Cell density} = \frac{\text{number of live cells in square}}{\text{volume of square (ml)}}$$

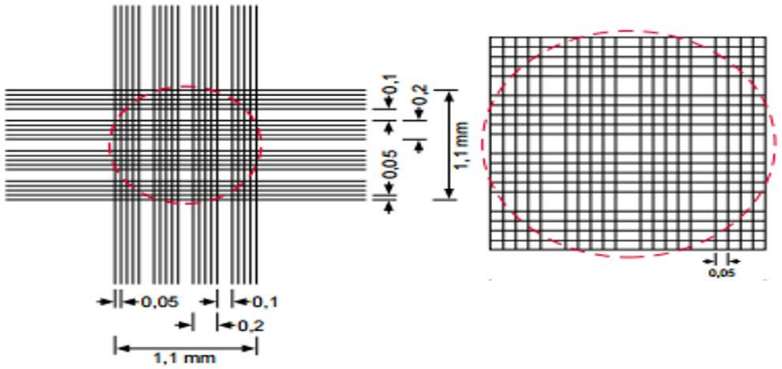


Figure 9. Thoma-Neu chamber´s counting grid details: 1.1 mm x 1.1 mm size and 0.1 mm depth. This grid is divided into 16 large squares. All the cells within each medium square and those touching the upper and the left sides of the square were counted.

In vitro plants were inoculated directly on the agar medium by piercing the pipette tip directly onto the roots at five different places (10^6 conidia/ml).

Control plants were similarly inoculated with sterile water. Plants were grown in a growing chamber and harvested at 1, 5, 10, 20 and 30 dpi. Five infected plants and five for the control were taken at each time point. Collected samples from leaves, roots and stems were immediately frozen in liquid nitrogen and stored at -80 °C until RNA isolation.

At all sampling time points, plants were visually assessed for the appearance of foliar symptoms using a 0-5 scale **(Figure 10)**.

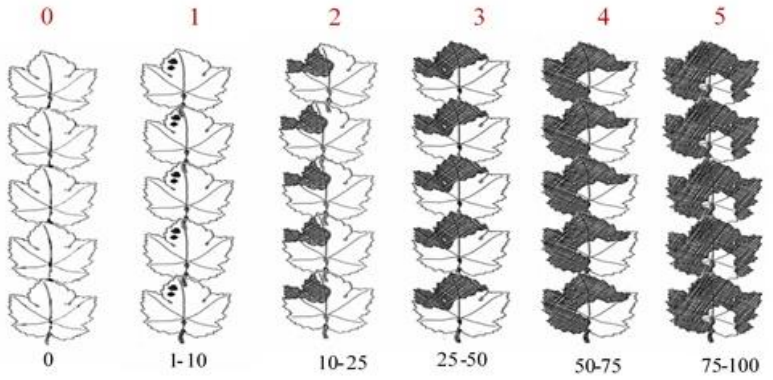


Figure 10. Schematic design of the infected leaves with *V. dahliae*. The scale employed for the disease visual assessment uses classes (0-5) based on a range of the percentage of symptomatic leaf area (0-100%).

2.3.22 Phosphorylation assay

2.3.22.1 Western blot

Equal amounts of the five Fra a 1.04-1.08 proteins (10 µg) were separated on one dimensional polyacrylamide gel electrophoresis (SDS-PAGE) and blotted to a PVDF membrane. For the

blocking buffer, a blot qualified BSA dissolved in a washing buffer was used. The membranes were probed with phosphoserine/ -threonine/ -tyrosine (SPM101) as primary antibody at a 1:1,000 dilution. After an overnight incubation at 4 °C, the membrane was washed and incubated at room temperature for 1 hour with an anti-mouse IgG conjugated to alkaline phosphatase as a secondary antibody at a 1:30,000 dilution. Detection step was carried out overnight under gentle agitation at room temperature. In this assay, pepsin was used as a positive control while lysozyme as a negative control.

2.3.22.2 Pro-Q Diamond phosphoprotein Gel Stain

The Fra a 1 phosphoproteins were stained with Pro-Q Diamond fluorescent gel according to the protocol of the manufacturer (Invitrogen). All steps of staining, destaining and washing were performed using gentle agitation on an orbital mixer (50 rpm). After polyacrylamide electrophoresis, the gel was fixed in 100 ml of a fix solution (50% methanol, 10% acetic acid) and incubated overnight at room temperature. Then, in order to remove residual methanol or acetic acid, the gel was washed 3 times in 100 ml of ultrapure water for 10 minutes. After a staining step with Pro-Q Diamond gel stain for 90 minutes in the dark, the gel was destained in 100 ml destain solution (20% acetonitrile, 50 mM sodium acetate, pH 4) for totally 90 minutes at room temperature. Finally, the gel was washed twice for 10 minutes each in ultrapure water. The proteins were visualized using a UV transilluminator box at an excitation wavelength of 300 nm.

2.3.22.3 Sypro Ruby Gel Stain

After staining with Pro-Q Diamond phosphoprotein Gel Stain, the gel was subsequently stained overnight in the dark with a total protein stain Sypro Ruby which is an organometallic ruthenium chelate stain designed for detection applications. Then, the gel was destained with a destain solution (10% methanol, 7% acetic acid) for 30 minutes and washed twice in ultrapure water. A 300 nm UV box was used for the detection.

2.3.22.4 Chemiluminescent Western blotting

Western blot analysis was performed by loading equal amounts of the five Fra a 1 proteins onto an SDS-PAGE gel. Proteins were transferred to a PVDF membrane and blocked with 5% BSA in washing buffer for 1 hour at RT. The membranes were probed with phosphoserine/ -threonine/ -tyrosine monoclonal antibody (SPM101) at 1:700 dilution. After an overnight incubation at 4 °C, the membrane was washed and incubated at room temperature for 1 hour

with a Goat anti-mouse IgG horseradish peroxidase conjugate (HRP) as a secondary antibody at 1:9,500 dilution. A substrate working solution was prepared by mixing equal parts of two chemiluminescent substrates: the Peroxide Solution and the Luminol Enhancer Solution. After an incubation time of 1 minute at room temperature, the membrane was removed from the solution, blotted with absorbent filter paper to absorb excess liquid and placed in a clear plastic wrap. The membrane was then developed in a dark room and the x-ray film was exposed on the top for around 30 minutes. Pepsin was used as positive control for this assay and lysozyme as negative control.

2.3.23 Protein interaction analysis: Dynamic Biosensors

2.3.23.1 Step conjugation of a biomolecule to a nanolever in a reaction tube

2.3.23.1.1 Nanolever modification

Samples were prepared according to the manufacturer's instructions: A complementary nanolevers (cNL) was added to phosphate buffer (50 mM Na2HPO4/NaH2PO4 pH 7.2, 150 mM NaCl) and vortexed until solids were completely dissolved. After suspending the cross-linker in 100 µl of sterile water, 10 µl of the freshly prepared linker solution were supplemented to one nanolever aliquot and incubated for 20 minutes at room temperature. In the meanwhile, purification spin columns were equilibrated by adding phosphate buffer (50 mM Na2HPO4/NaH2PO4 pH 8, 150 mM NaCl) on the top of the resin bed and samples were then loaded to the columns.

2.3.23.1.2 Ligand conjugation

A vivaspin centrifugal concentrator was used to reduce the volume of the protein fractions reaching an amout of 100 µg (ligand) which was added to the functionalized cNL and was incubated overnight at 4 °C.

2.3.23.1.3 Purification of ligand-cNL-conjugate

In order to perform a size analysis during the measurement, it has to be ensured that there is no free DNA on the chip and only 1:1 conjugates are present. For this purpose, a purification reaction was performed using the ÄKTA Start chromatography system which is equipped with an anion exchange column. The different fractions corresponding to the five Fra a 1 proteins were collected and spun down for 25 minutes using a centrifugal filter unit (3 kDa) **(Figure 11)**.

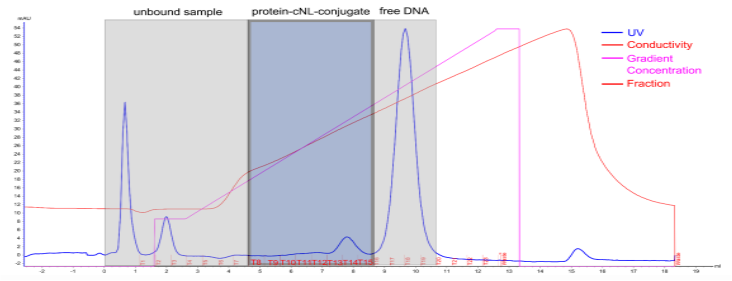


Figure 11. ÄKTA Start chromatogram of protein-cNL-conjugate purification.

A summary of the 3-step conjugation is shown in **Figure 12:**

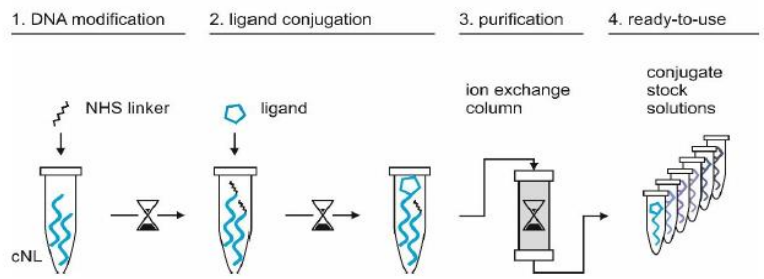


Figure 12. Overview of the 3-step conjugation workflow (*in vitro*).

2.3.23.2 Time-resolved DNA switching measurements

Using a phosphate buffer (10 mM Na_2HPO_4/NaH_2PO_4 at pH 7.4, 40 mM NaCl, 0.05% Tween), the concentration of FaAP-GFP (kindly provided by Katrin Franz-Oberdorf) was adjusted to 0.1 µM and 1 µM, and GFP (also kindly provided by Katrin Franz-Oberdorf) concentration was arranged to 1 µM. Protein interaction analysis of Fra a 1.04-1.08 was performed with the switchSENSE interaction analysis platform by Dynamic Biosensors using DRX 2400 auto-sampler. The switchSENSE biochip possesses 24 circular gold circular electrodes (d = 120 µm) which are addressable with different receptors for target molecules via double stranded DNA oligonucleotides (48 bp) **(Figure 13)**.

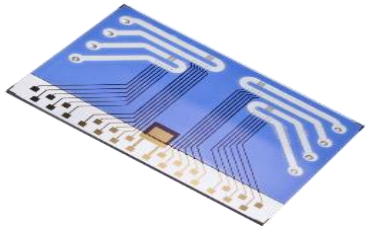


Figure 13. SwitchSENSE Biochip featuring 24 detection spots (gold circular electrodes with a diameter of 120 µm).

The fluorescence at repulsive and attractive potential was used as a parameter that indicates the free mobility of DNA strands on the surface. After a hybridization step of the ligand-cNL-conjugate and activation of the SwitchSENSE chip, the association-dissociation interaction was measured. Then, the ligand-cNL complex was washed away from the surface by DNA denaturation under basic pH conditions.

2.3.24 Statistical analysis

The statistical analysis was carried out using the statistical program SAS (SAS Institute Inc., Cary, USA). A one-way analysis of variance (ANOVA) was created for each inoculation day and then a comparison of mean values of the relative expression level for the 21 genes with the Tukey test was analyzed. The significance level was $p \leq 0.05$.

3 Results

3.1 Sequences and structural properties of Fra a 1 proteins

The genome sequence of the woodland strawberry (240 Mb) has been published on December 2010 (Shulaev et al., 2011). The availability of complete *F. vesca* genome sequences has made it possible to identify 21 isoforms of the *Fra a 1* allergens by means of *in silico* analysis. Orthologous *F. vesca* genes coding for *Fra a 1* isoforms have an open reading frame (ORF) from 399 to 639 bp. *Genes07080, 07086* and *07082* corresponding to *Fra a 1.01, 1.02, and 1.03*, respectively, with a length of either 483 or 480 nucleotides are encoding polypeptides of 159-160 amino acids and have been studied before (Muñoz et al., 2010). *Genes05122*, *07065*, *07088*, *05123*, and *07064* whose orthologues in *F.* × *ananassa* are *Fra a 1.04-1.08*, respectively are intronless and have an open reading frame of 483 bp. They are encoding polypeptides of 160 amino acids **(Table 6)** with the common feature of a P-loop motif (GxGGxGxxK [TS]) (Saraste et al., 1990). This motif is found in ATP and GTP-binding proteins but not present in all of them. Similarly, most PR-10 have an ORF from 456 to 489 bp and possess a single or no intron at the conserved position (Liu et al., 2006; Chen et al., 2008), indicating that the putative *Fra a 1* orthologous genes in *F. vesca* have a conserved gene structure, like the other pollen allergens. Most putative *Fra a 1* orthologous proteins, except for those encoded by *gene07078* (23.96 kDa), *gene07087* (21.73 kDa), *gene07084* (19.36 kDa), and *gene07063* (14.37 kDa) share a molecular weight ranging from 17.26 to 18.68 kDa. The orthologous Fra a 1.04-1.08 proteins showed 55.6% to 59.3% amino acid pairwise identity to Bet v 1 **(Table 6)**.

Table 6. Nomenclature of *Fra a 1.01-08* isoforms in *Fragaria* × *ananassa* and their orthologues in the *Fragaria vesca* genome.

Gene name in *F.* × *ananassa*	Gene name in *F. vesca*	GenBank accession no.	ORF (nt)	AA length	MW (kDa)	Pairwise identity related to Bet v 1 (%)
Fra a 1.01	*gene07080*	AM236319	483	160	17.76	53.1
Fra a 1.02	*gene07086*	GQ148818	483	160	17.49	58
Fra a 1.03	*gene07082*	GQ148819	480	159	17.44	58.4
Fra a 1.04	*gene05122*	KJ507735	483	160	17.51	55.6
Fra a 1.05	*gene07065*	KJ507736	483	160	17.48	57.4
Fra a 1.06	*gene07088*	KJ507737	483	160	17.47	56.2
Fra a 1.07	*gene05123*	KJ507738	483	160	17.58	59.3
Fra a 1.08	*gene07064*	KJ507739	483	160	17.52	58.6

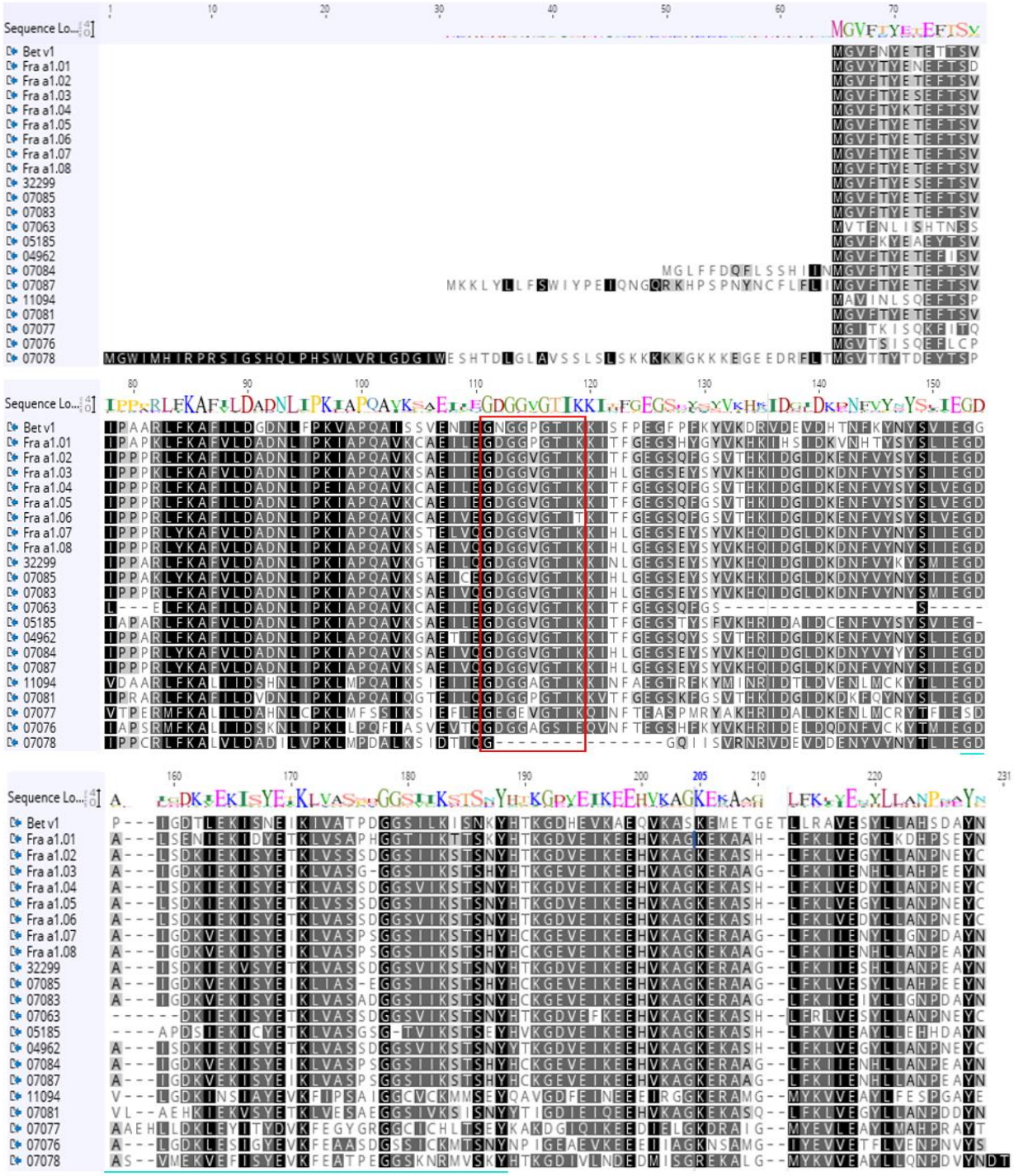


Figure 14. Multiple alignment of amino acid sequence of Bet v 1 and 21 similar sequences from *F.* × *ananassa* and *F. vesca*. The P-loop motif (GxGGxGxxK [TS]) is highlighted by a red box and blue underline indicate the Bet v 1 motif : G-D-A-[L/I]-[S/G]-D-K-[I/V]-E-K-I-S-Y-E-[T/I]-K-L-V-[S/A]-S-[S/P]-[D/S]-G-G-S-[V/I]-I-K-S-T-S-[N/H]-Y. Alignment was performed with Geneious software.

An alignment of the amino acid sequences of 21 orthologous *Fra a 1* genes extracted from the *F. vesca* genome and Bet v 1, identified the most similar and most divergent regions **(Figure 14)**. The glycine-rich loop motif is highly conserved throughout the Bet v 1 superfamily and is highlighted by a red box. This consensus sequence GxGGxGxxK [TS] (Saraste et al., 1990) was highly conserved in eighteen of the orthologous Fra a 1 proteins. For the protein encoded

by *gene07088* (orthologue of Fra a 1.06) the residue at position 119 which is conventionally a lysine (K_{119}) was replaced by a threonine (T_{119}). There was one AA exchange for 07076 (GxGGxGxx**E**) and 07077 proteins (GxG**E**xGxxK) while no P-loop motif was detected in the 07078 amino acid sequence.

Making a restricted comparison within the 21 isoforms, Fra a 1.04-1.08 proteins showed 115 identical sites of 160 AA in total (71.9%) and a pairwise identity of 84%. Furthermore, all those 5 sequences featured the Bet v 1 motif which is characteristic for the pathogenesis related proteins of the Bet v 1 family **(Figure 14, Figure 42)**.

3.2 qPCR Analysis

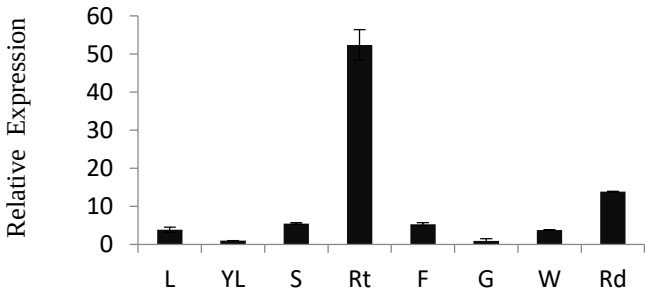
In order to evaluate the expression of the 21 *Fra a 1* isoforms in different tissues of the *F.* × *ananassa* strawberry plant (root, stem, leaf, young leaf, flower and fruit), quantitative real-time PCR analyses using the Fast SYBR Green master mix as a reagent were performed. Specific primers from the untranslated region (5′ UTR) of each of the 21 genes were designed. Five biological replicates were analyzed. The results were normalized with an inter-spacer 16-23S RNA housekeeping gene (*FaRIB413*).

A huge variation in transcript levels between the 21 isoforms was observed in all investigated *F.* × *ananassa* organs **(Figure 15)**. The expression levels varied from 182,670 fold of *gene05185* in roots to 0.17 fold for *gene07078* in red fruits. Thirteen isoforms were highly expressed in roots. *Fra a 1.04* (52 fold), *Fra a 1.06* (97 fold), *Fra a 1.07* (69 fold), *Fra a 1.08* (347 fold), *gene07063* (106 fold) and *gene07084* (50 fold) were strongly transcribed in roots with an intermediate expression in the late stage of fruit ripening. A comparable pattern was detected for *gene11094* (283 fold) and *gene07085* (275 fold) with a notable expression in leaves. *Genes07076* (37 fold) and *04962* (20 fold) were also significantly expressed in roots and showed an observable expression profile in leaves, flowers and red fruits. In contrast, *gene05185* was exclusively transcribed in roots with the highest expression value within those 21 isoforms. *Fra a 1.01* (287 fold) and *gene32299* (136 fold) were predominantly expressed in roots with an intermediate expression in leaves for both of them and in stems for *Fra a 1.01*.

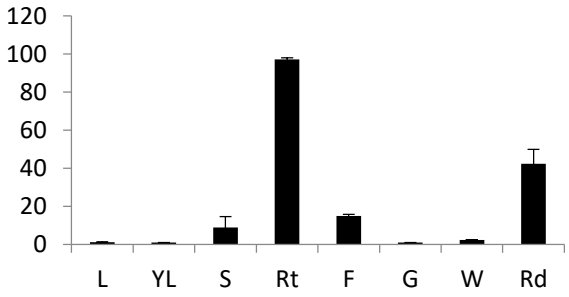
Interestingly, *Fra a 1.02* (231 fold) and *Fra a 1.05* (80 fold) were highly transcribed in the late stage of fruit ripening with a lower expression in roots, whereas *gene07077* (11 fold), *gene07087* (2 fold) and *gene07078* (1.6 fold) were expressed in all tissues with relatively higher transcript level in stems. *Gene07081* (76 fold) was a unique *Fra a 1* isoform, which was predominately expressed in flowers with a moderate transcription level in green fruits.

Fra a 1.03 (1,875 fold) was exclusively transcribed in leaves whereas *gene07083* (9 fold) was considerably expressed in leaves and also in roots and green fruits. Altogether, the qPCR analysis revealed that the 21 isoforms are distinctly and variably expressed within *F.* × *ananassa* tissues with a predominance of roots tissues and a significant expression level at the late stage of fruits ripening.

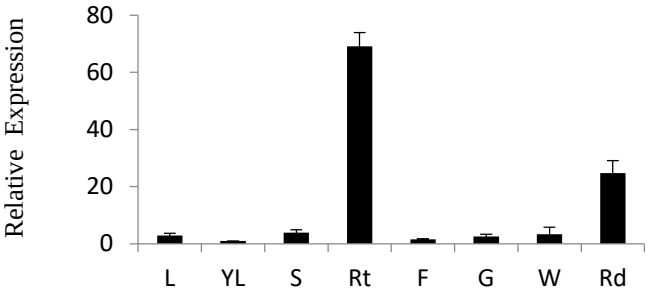
Fra a 1.04



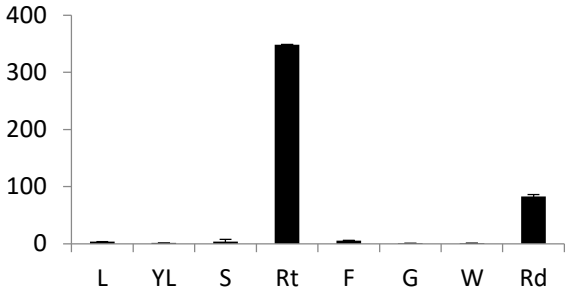
Fra a 1.06



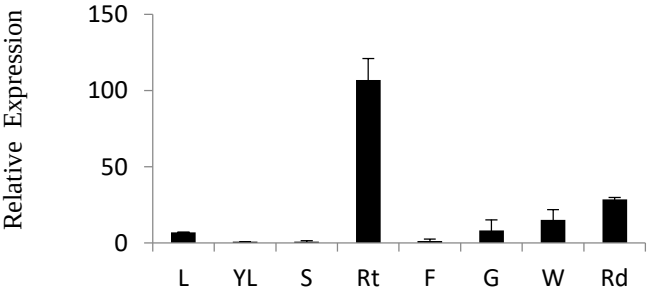
Fra a 1.07



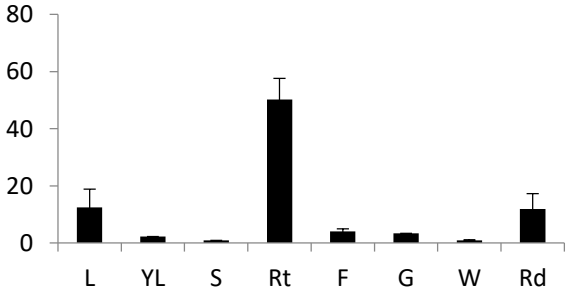
Fra a 1.08



gene07063



gene07084



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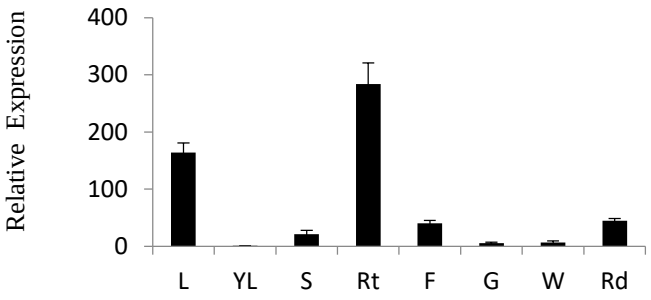
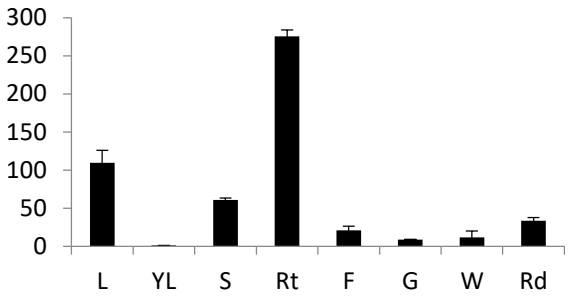
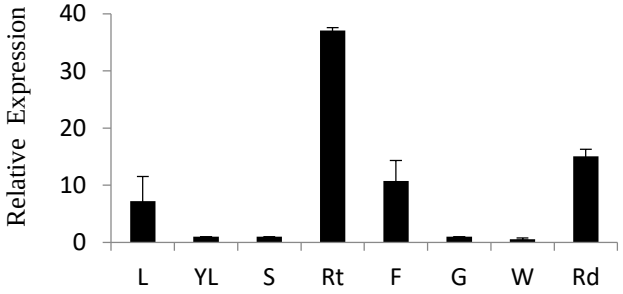
gene11094



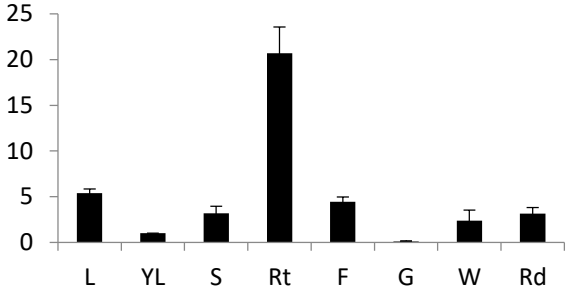
gene07085



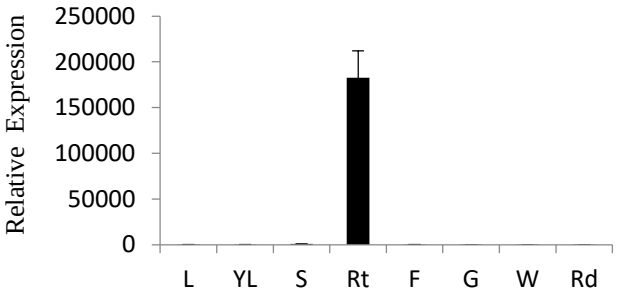
gene07076



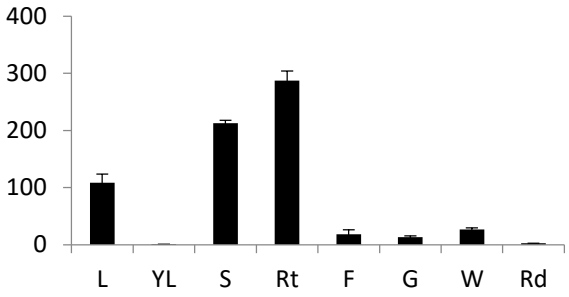
gene04962



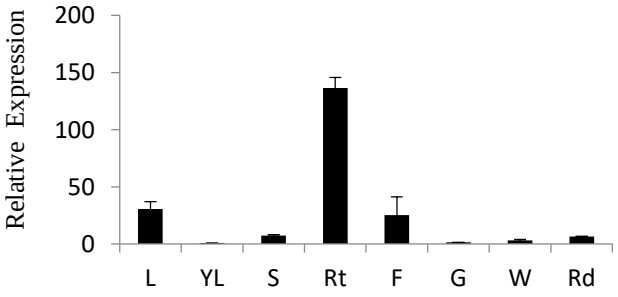
gene05185



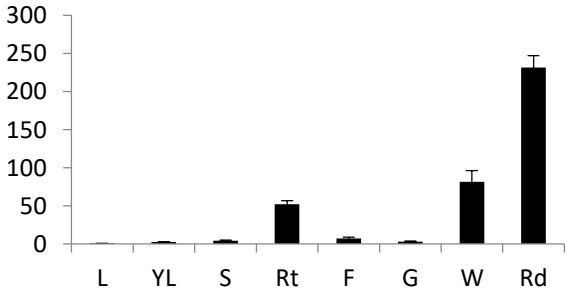
Fra a 1.01



gene32299



Fra a 1.02



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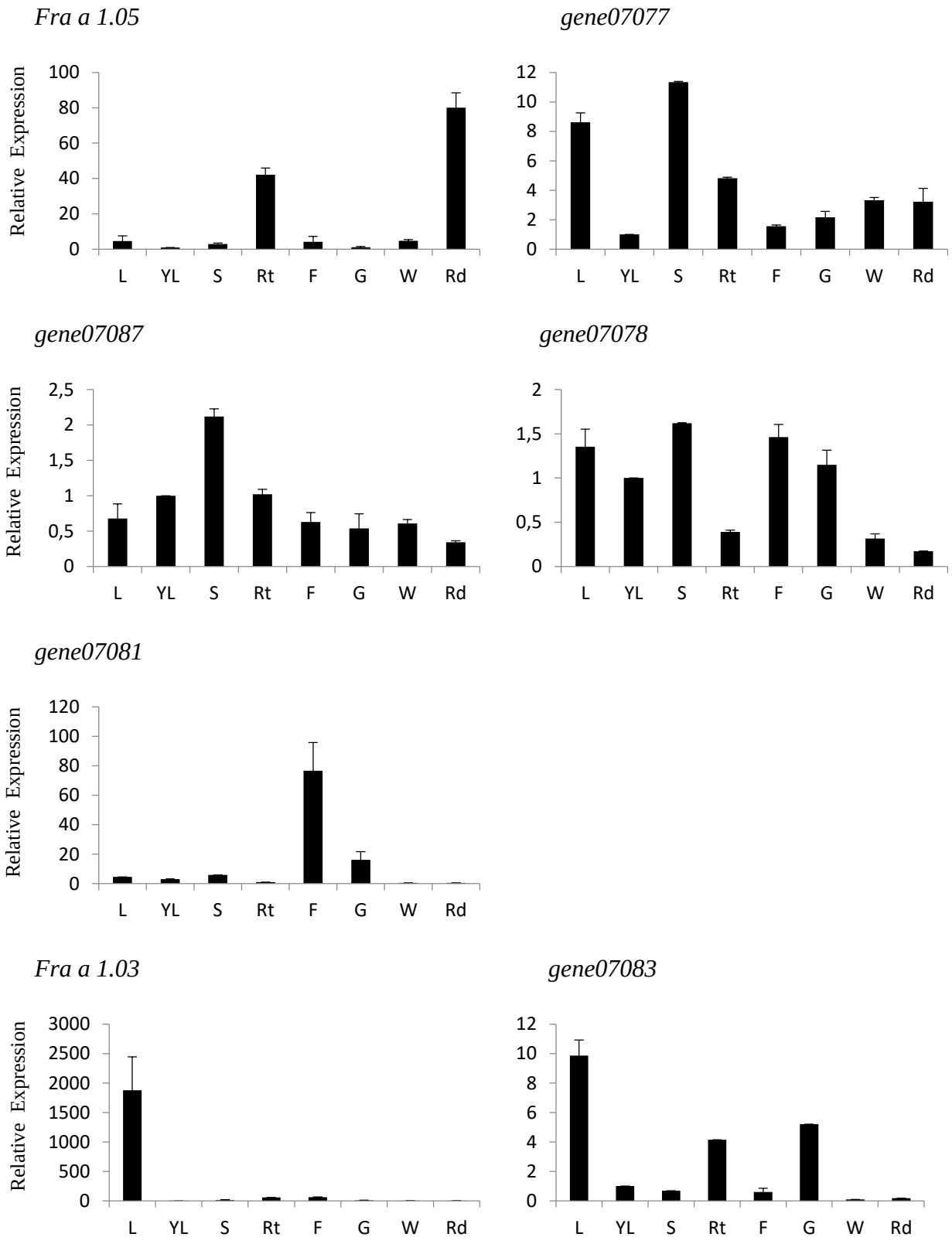


Figure 15. Relative gene expression of 21 *Fra a 1* genes in different tissues ((L) Leaf; (YL) Young leaf; (S) Stem; (Rt) Root and (F) Flower) of *F.* × *ananassa* cv. Elsanta and various stages of maturity of fruit ((G) Green; (W) White and (Rd) Red). The qPCR data were normalized to the interspacer gene *FaRIB413*. Young leaf was used as reference and values were set to 1. Values are mean of five biological replicates and are shown as relative changes. The error bars indicate the standard deviation of the means.

Taken together, **Figure 16** represents a summary of the qPCR analysis of the 21 *Fra a 1* isoforms. The qPCR analysis revealed significant expression levels of the 21 genes in root. In contrast to young leaf, white and green fruit, which showed low expression levels of almost all genes, both red fruit and root exhibit high transcript levels of the cluster comprising *Fra a 1.04, Fra a 1.06, gene07063, Fra a 1.02 and Fra a 1.05. Genes07077, 07087* and *07078* were highly expressed in stems, *gene07081* in flower while *gene07083* and *Fra a 1.03* were considerably expressed in leaf.

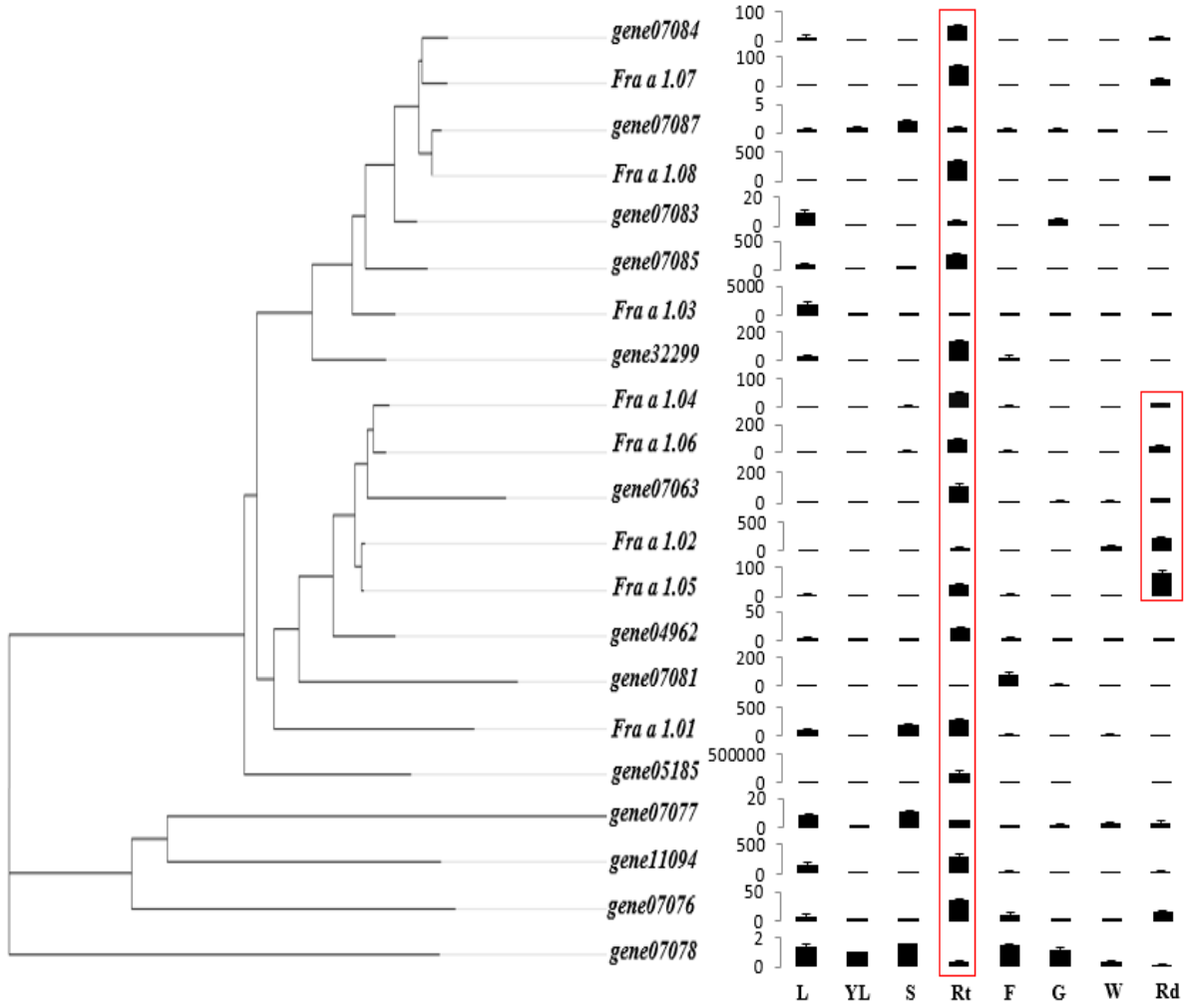


Figure 16. Phylogenetic tree of *Fra a 1* genes and summary of the qPCR analysis of the 21 *Fra a 1* isoforms in leaf (L), young leaf (YL), stem (S), root (Rt), flower (F), green (G), white (W) and red (Rd) fruit. Expression analysis was monitored by qPCR using specific primers for *Fra a 1* genes and the *FaRIB413* gene as reference gene.

3.3 Heterologous expression of Fra a 1 proteins

The full length coding sequences of the five isoforms *Fra a 1.04–1.08* were cloned into pQE70 vector which contains a 6x His-tag at the C-terminal end. Specific primers amplifying a nucleotide sequence of 483 bp length were designed. His-Fra a 1 proteins were successfully

expressed in *E. coli* BL21 (DE3) pLysS cells which are suitable for high level induction and expression of genes regulated by a T7 promoter.

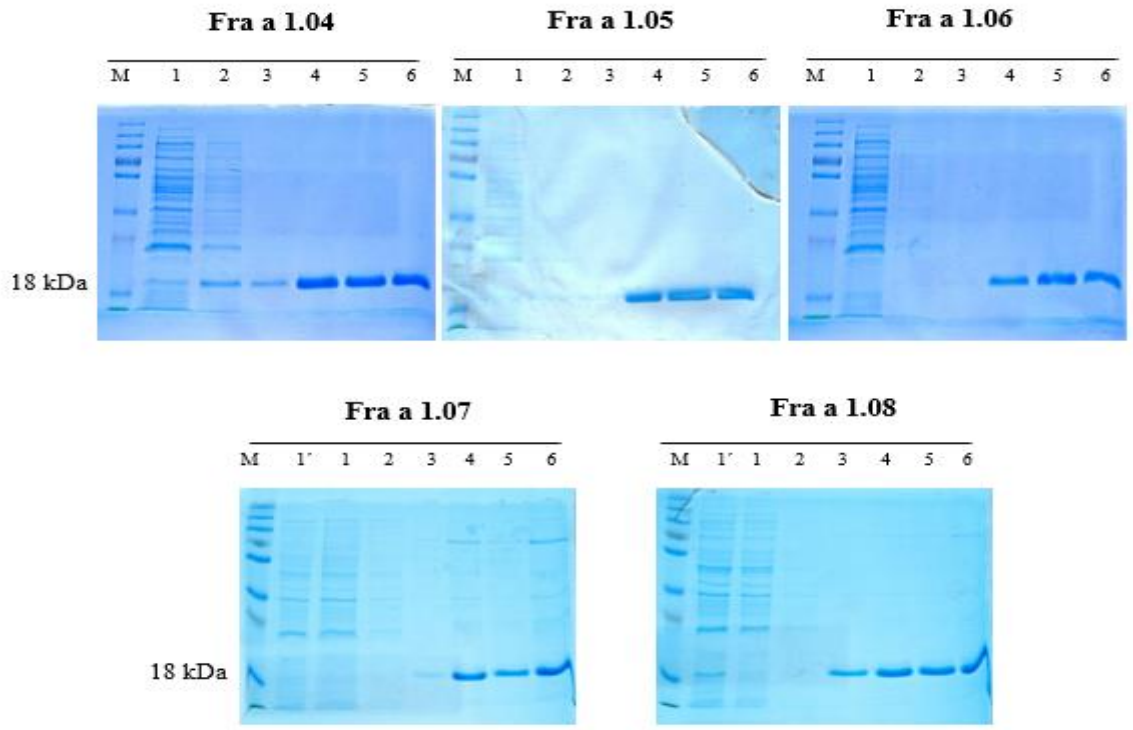


Figure 17. SDS-PAGE analysis of the five purified Fra a 1.04-1.08 proteins (10 µg); Lane M: molecular weight marker; Lane 1´: Crude extract, Lane 1: Flow through; Lane 2, 3: Washes; Lane 4, 5, 6: Elution fractions. Bands were visualized by Coomassie Brilliant Blue G250. A band of 18 kDa was revealed for the five Fra a 1 proteins.

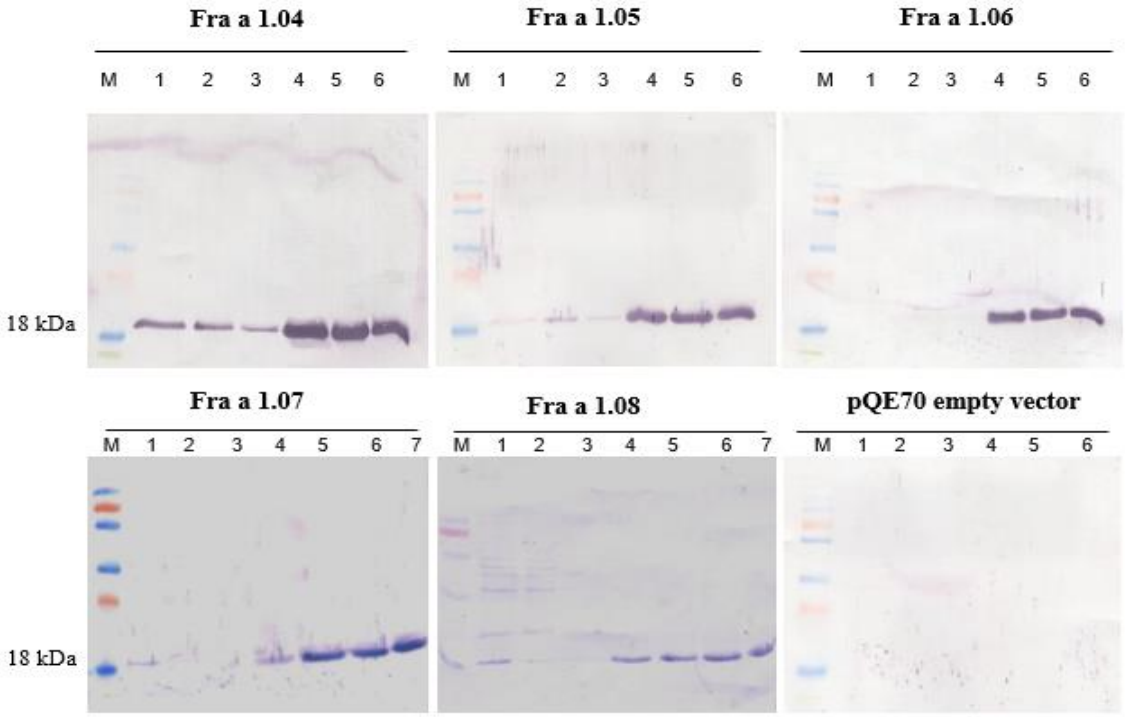


Figure 18. Western blot analysis of the five purified Fra a 1.04-1.08 proteins (10 µg); Lane M: molecular weight marker; Lane 1: Flow through; Lane 2, 3: Washes; Lane 4, 5, 6, 7: Elution fractions. Bands were visualized on polyvinylidene fluoride (PVDF) membrane with monoclonal anti-6× His AP antibody.

SDS-PAGE analysis of the samples purified by immobilized metal affinity chromatography (IMAC) **(Figure 17)** indicated bands at about 18 kDa (calculated values: 17.5 kDa for Fra a 1.04, 17.5 kDa for Fra a 1.05, 17.5 kDa for Fra a 1.06, 17.6 kDa for Fra a 1.07 and 17.5 kDa for Fra a 1.08). All the cited proteins revealed a high degree of purity of the eluted fractions. A Western blot analysis of the five Fra a 1 proteins was also performed using the monoclonal anti-6× His Alkaline Phosphatase (AP) antibody. The immunodetection confirmed the results obtained with the Coomassie blue staining of the five Fra a 1 proteins **(Figure 18)**.

3.4 Ribonuclease activity

3.4.1 In-solution RNase activity

It has been reported that the PR-10 proteins possess ribonuclease (RNase) activity. The RNase activity of Fra a 1 proteins was assayed by incubating the proteins in the presence of total RNA isolated from strawberry fruit at different pH for four hours. Ribonucleolytic activity was demonstrated on agarose gel by the migration of the degraded products. Under acetic conditions (pH 3), the RNA appeared to be completely hydrolyzed by Fra a 1.06 and partially degraded when incubated with the four remaining Fra a 1 proteins as well as with the buffer **(Figure 19)**.

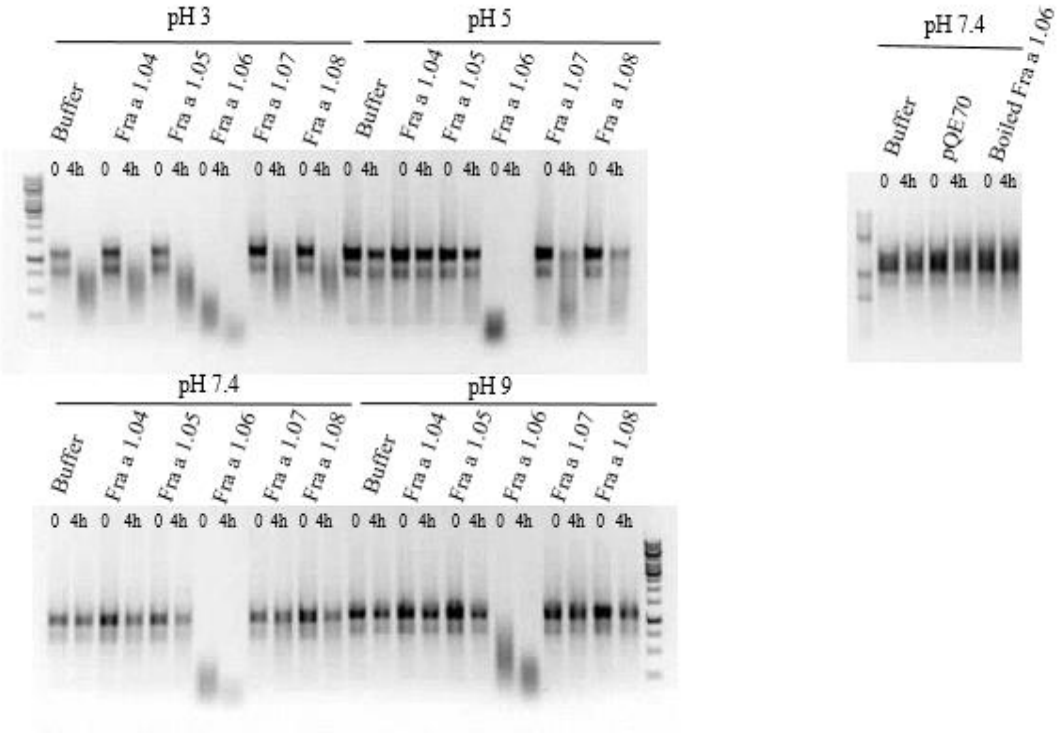


Figure 19. Ribonuclease activity of the recombinant Fra a 1.04-1.08 proteins (1 µg) tested against total RNA from strawberry fruit (1 µg) at different pH and different incubation times (0 and 4 h) at 37 °C. RNA hydrolysis products were separated using agarose gel. Negative controls were also evaluated.

Thus, RNA becomes chemically degraded after prolonged incubation at pH 3. At pH 5, total RNA degradation was detected for Fra a 1.06 after only a few minutes whereas Fra a 1.07 and Fra a 1.08 were unable of full hydrolysis even after 4 hours of incubation. No hydrolysis was detected for Fra a 1.04 and Fra a 1.05. Under basic conditions (pH 7.4 and pH 9), total RNA was completely hydrolyzed after a few minutes only for Fra a 1.06. For the rest of Fra a 1 proteins, no hydrolysis was observed **(Figure 19)**. No RNA degradation was detected when RNA was incubated with the buffer alone, with the lysate of *E.coli* transformed with pQE70 vector or with Fra a 1.06 thermally inactivated by incubation at 100 °C for 30 minutes.

3.4.2 In-gel RNase activity

The ribonuclease activity of Fra a 1.04-1.08 was also evaluated in polyacrylamide gel by incubating the five proteins with yeast torula RNA. A clear band of 18 kDa was observed for Fra a 1.06 whereas no detectable clear region at the corresponding molecular weight was visible for the rest of the four Fra a 1 proteins. Boiled Fra a 1.06 protein was used as a negative control **(Figure 20)**. Thus, only Fra a 1.06 possesses RNase activity confirming the result of in-solution RNase activity.

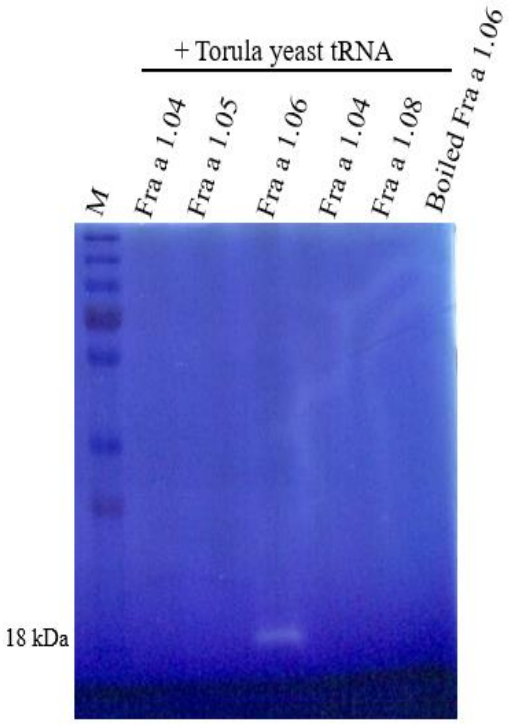


Figure 20. In-gel RNase activity of Fra a 1.04-1.08 proteins (5 µg). Polyacrylamide gel containing yeast torula RNA was used for electrophoresis. After staining with toluidine blue, a unique clear band was detected demonstrating that only Fra a 1.06 possesses the RNase activity. M: molecular weight marker.

3.5 Phosphorylation assay

3.5.1 Western blot of Fra a 1 proteins

It has long been reported that PR-10 proteins are post-translationally modified by phosphorylation (Ziadi et al., 2001) and that phosphorylation is required for defense response to microbial pathogen attack and stress (Park et al., 2004). A Western blot analysis of the five recombinant proteins was performed using a primary antibody raised against phosphoserine/ -threonine/ -tyrosine and an anti-mouse IgG conjugated to alkaline phosphatase as a secondary antibody **(Figure 21)**. Except for Fra a 1.06, which possesses ribonuclease activity, a band of about 18 kDa appeared for the recombinant proteins proving their phosphorylation. Treatment of Fra a 1.08 with alkaline phosphatase calf intestinal (CIP) clearly decreased the intensity of the signal indicating a loss of phosphates. Lysozyme and pepsin (tested at 2 different concentrations) were used respectively as negative and positive controls and were corroborating the results **(Figure 21)**.

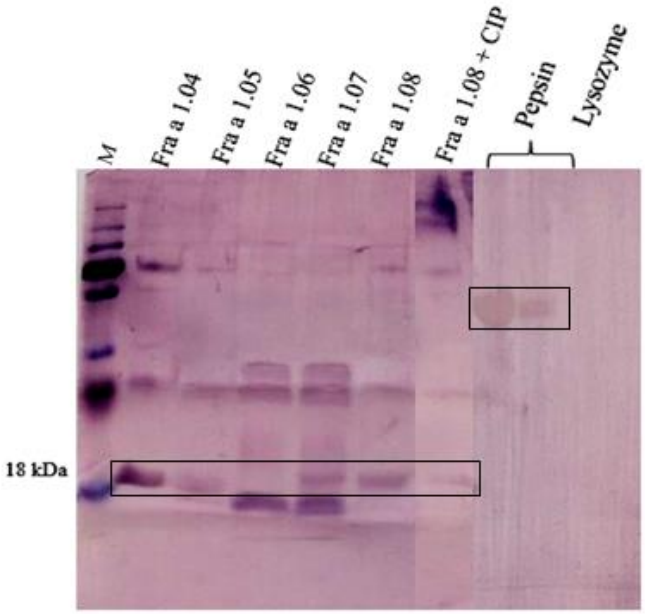


Figure 21. Western blot analysis using a phosphoserine/ -threonine/ -tyrosine antibody for the detection of phosphorylated Fra a 1.04-1.08 proteins. Positive (pepsin, 43 kDa) and negative (lysozyme, 14.3 kDa) controls were also used. Fra a 1.04, 1.05, 1.07 and 1.08 were shown to be phosphorylated due to the bands at 18 kDa. M: molecular weight marker.

3.5.2 Pro-Q Diamond and Sypro Ruby Gels Stain

In order to substantiate the Western blot outcome, a Pro-Q Diamond Phosphoprotein Gel Stain, which can detect phosphoproteins on SDS polyacrylamide gel by a mechanism that combines a chelating fluorophore and a transition metal ion, was performed. Fluorescent detection of phosphoserine, phosphothreonine and phosphotyrosine was possible due to

excitation at 555-585 nm. After one-dimensional electrophoresis the gel was stained with Pro-Q diamond in the dark and then destained with 20% acetonitrile and 50 mM sodium acetate. The dye selectively detects phosphoproteins. Except for Fra a 1.06, the four remaining Fra a 1 proteins bound the dye and produced clear signals confirming phosphorylation of the proteins Fra a 1.04, Fra a 1.05, Fra a 1.07 and Fra a 1.08 **(Figure 22**, left**)**. After staining with Pro-Q Diamond phosphoprotein Gel Stain, the gel was subsequently stained with a total protein stain to visualize total proteins and to check equal protein loading **(Figure 22**, right**)**.

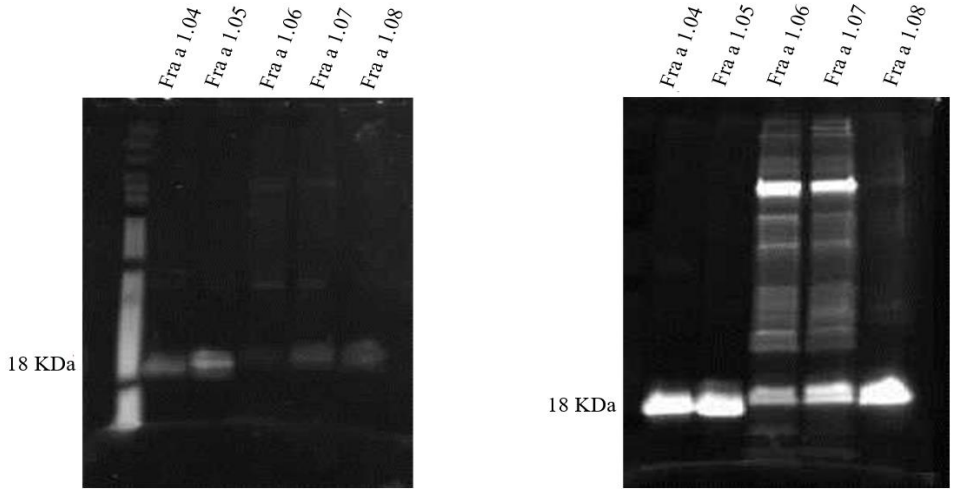


Figure 22. Phosphoprotein detection of Fra a 1.04-1.08 proteins using Pro-Q Diamond staining gel (left). Gel shown on the right was subsequently stained for total proteins detection with Sypro Ruby stain. Only Fra a 1.04, Fra a 1.05, Fra a 1.07 and Fra a 1.08 were demonstrated to be phosphorylated (left).

3.5.3 Chemiluminescent Western blotting

A chemiluminescent detection kit for the visualization of phosphoproteins, which yields the greatest sensitivity of any available detection method, was also performed.

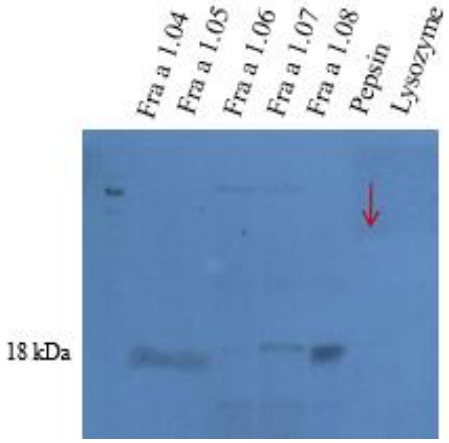


Figure 23. Chemiluminescent Western blotting of Fra a 1.04-1.08 (10 µg). Horseradish Peroxidase-conjugated antibody and an enhanced luminol-based chemiluminescent substrate were used for the chemiluminescent Western blotting detection. Exposure to X-ray film indicated that Fra a 1.04, Fra a 1.05, Fra a 1.07 and Fra a 1.08 are phosphorylated. Positive (pepsin, 43 kDa) and negative controls (lysozyme, 14.3 kDa) were also used.

The proteins were first probed with phosphoserine/ -threonine/ -tyrosine monoclonal antibody and then visualized with an anti-Mouse IgG antibody which is conjugated to the horseradish peroxidase and incubated with chemiluminescent substrates. Those substrates are two component-systems consisting of a stable peroxide solution and an enhanced luminol solution. After incubating the membrane with the substrates, a sheet of film was developed in a dark room. The exposure time was around 30 minutes. The bands at 18 kDa affirmed once again that Fra a 1.04, Fra a 1.05, Fra a 1.07 and Fra a 1.08 are phosphorylated **(Figure 23)**.

3.5.4 Protein dephosphorylation and Ribonuclease activity assay

3.5.4.1 In-solution RNase activity

Assuming that Fra a 1.04, Fra a 1.05, Fra a 1.07 and Fra a 1.08 proteins are phosphorylated and that only Fra a 1.06 possesses RNase activity, the question arose whether phosphorylation was influencing the RNase activity.

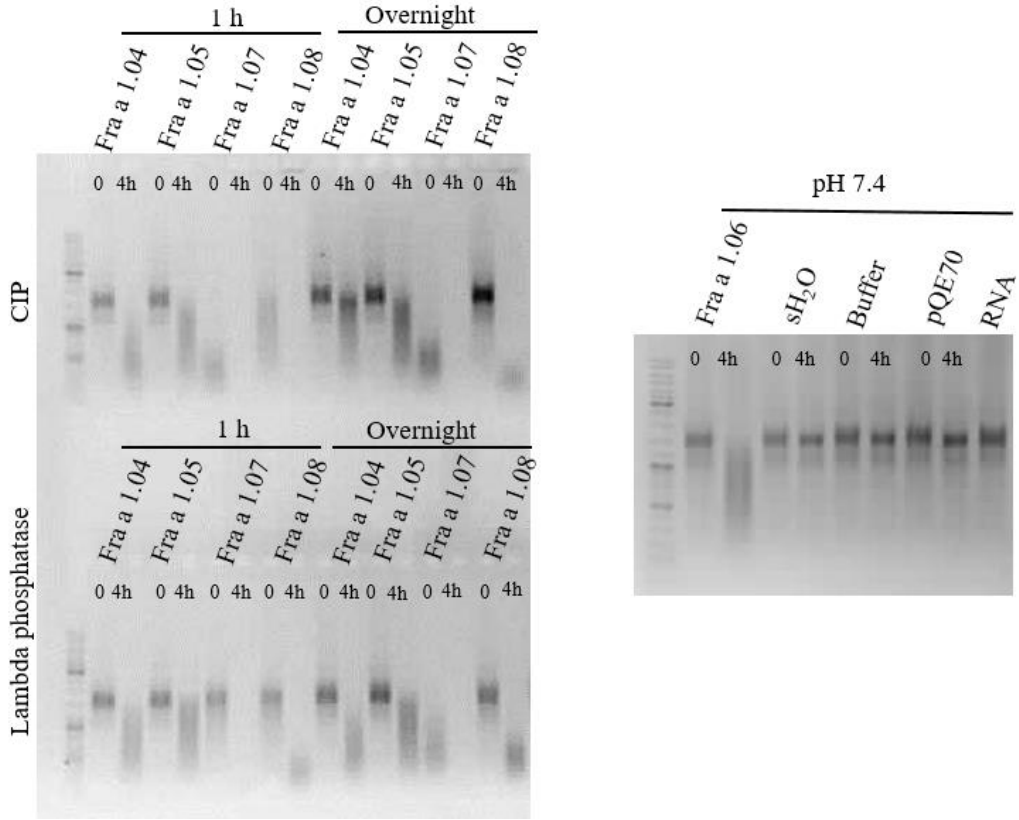


Figure 24. In-solution RNase activity of the dephosphorylated Fra a 1.04, Fra a 1.05, Fra a 1.07 and Fra a 1.08. Proteins were dephosphorylated for 1 h and overnight with calf intestinal alkaline phosphatase (CIP) and lambda phosphatase at 37 °C and 30 °C respectively. Negative (sH2O, buffer, empty vector pQE70, and only RNA) and positive controls (Fra a 1.06) were also used. After dephosphorylation, Fra a 1.04, Fra a 1.05, Fra a 1.07 and Fra a 1.08 were shown to possess RNase activity.

Thus, the recombinant Fra a 1 proteins were dephosphorylated by two phosphatases and the effect on their RNase activity was addressed. Alkaline phosphatase calf intestinal (CIP) treatment was performed at 37 °C, while lambda phosphatase hydrolysis was conducted at 30 °C, both of them for 1 hour and overnight. The in-solution RNase activity was tested by incubating the four recombinant proteins in the presence of total RNA isolated from strawberry at pH 7.4. Under these conditions, RNA was degraded by the four dephosphorylated proteins after 4 hours **(Figure 24**, left**)**. Concerning the dephosphorylation efficiency of CIP and lambda phosphatase, no significant difference was evaluated for both enzymes. Also, controls were treated with CIP and in-solution RNase activity was tested **(Figure 24**, right**)**. No degradation was detected when RNA was incubated alone, only with the empty vector (pQE70), with the buffer or with sH_2O. Fra a 1.06 which was used as a positive control was corroborating the efficiency of this assay.

3.5.4.2 In-gel RNase activity

To test the in-gel RNase activity, dephosphorylated Fra a 1 proteins were separated on 12% acrylamide gel containing 2.4 mg/ml total RNA of torula yeast. After electrophoresis, the gel was stained with toluidine blue and destained with Tris-HCl. The gel was incubated 50 minutes at 51 °C to allow the RNase to digest the RNA.

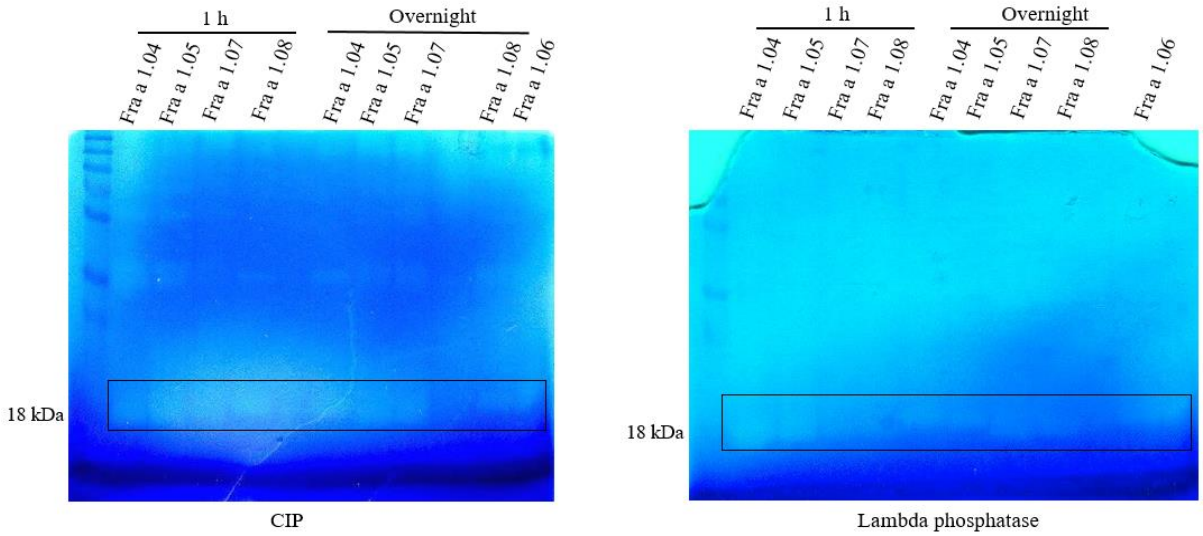


Figure 25. In-gel RNase activity of the dephosphorylated Fra a 1.04, Fra a 1.05, Fra a 1.07 and Fra a 1.08 proteins. Proteins were dephosphorylated with calf intestinal alkaline phosphatase (CIP) and lambda phosphatase at 37 °C and 30 °C, respectively for an incubation time of 1 h and overnight. The polyacrylamide gels showed a clear region at 18 kDa corresponding to proteins with RNase activity. Fra a 1.06 was used as positive control.

The obtained results **(Figure 25)** clearly proved that the four recombinant proteins dephosphorylated with CIP and lambda phosphatase exhibited ribonucleolytic activity against

total RNA, strengthening the results of the in-solution RNase activity. Using lambda phosphatase, the white bands at 18 kDa indicating RNase activity were weak for Fra a 1.07 and Fra a 1.08 after dephosphorylation for 1 hour but visible after overnight dephosphorylation whereas after CIP treatment RNase activity was observable after 1 hour and overnight treatments.

3.6 Protein interaction analysis: Fluorescence response and kinetic data

The determination of protein-protein interaction is a primordial step in understanding protein functions. Recently, it was shown that Fra a 1.01 – 1.03 proteins physically interact with FaAP (Fra a 1- Associated Protein) (Franz-Oberdorf et al., 2017a). In this work, supposed interactions of Fra a 1.04–1.08 proteins with FaAP fused to the green fluorescent protein (GFP) were monitored by the switchSENSE platform of Dynamic Biosensors **(Figure 5)**. This novel time resolved DNA switching system was applied and the molecular dynamics of proteins, which are set in motion by electrically actuated DNA levers, were investigated. Excited by green light, a dye attached to the levers emits fluorescence which is detected by a single photon counter. When the nanolever tilts to the surface, the dye emission becomes gradually quenched by the proximity of the electrode. Using this device, fluorescence response can be followed in real time **(Figure 26)** and the association and dissociation rates K_{on} and K_{off} as well as the dissociation constant K_D can be determined **(Table 7)**.

Figure 26 illustrates the time resolved normalized fluorescence signal of 48 bp labelled DNA before (gray line) and after (blue line) binding of the fusion protein FaAP-GFP. When Fra a 1.04-1.08 proteins, attached to the DNA´s top end bind to FaAP, the upward motion is slowed and lags behind the bare lever. Thus, protein-protein interaction between FaAP-GFP and the five tested Fra a 1 proteins was successfully detected. No binding was detected between Fra a 1.04-1.08 and GFP alone.

Kinetics data showed that FaAP bind to the five Fra a 1 proteins with different K_D values **(Table 7)**. In contrast to Fra a 1.05 which has the lowest affinity (2,181 nM) to FaAP-GFP, Fra a 1.04 was shown to have the strongest affinity (27.5 nM). Fra a 1.06, Fra a 1.07 and Fra a 1.08 proteins show intermediate affinity to FaAP-GFP with similar kinetic values ranging from 51.5 nM to 72.05 nM.

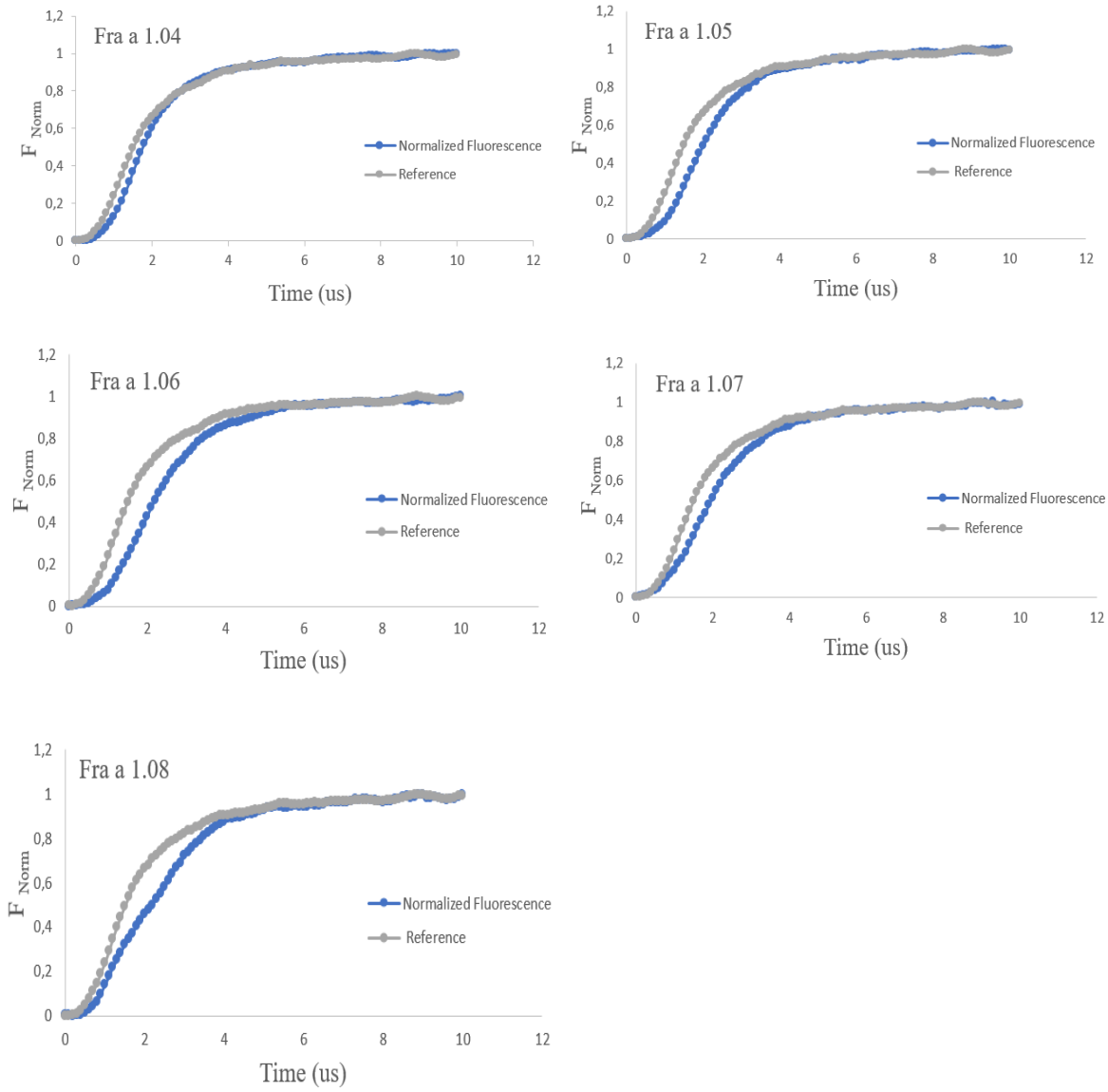


Figure 26. Time-resolved rising curves for DNA bound to Fra a 1.04–1.08 proteins (gray line) and after rinsing a solution of 0.1-1 µM FaAP-GFP (blue line). Interactions with FaAP-GFP were detected for the five Fra a 1.04-1.08 proteins. Y-axis: normalized fluorescence; X-axis: time in µs.

Table 7. Kinetic data obtained by the time-resolved DNA switching system for the interaction between FaAP-GFP and Fra a 1.04-1.08. Kinetic rate constants (K_{on}, K_{off}) and the dissociation constant K_D (K_{off} / K_{on}) were determined.

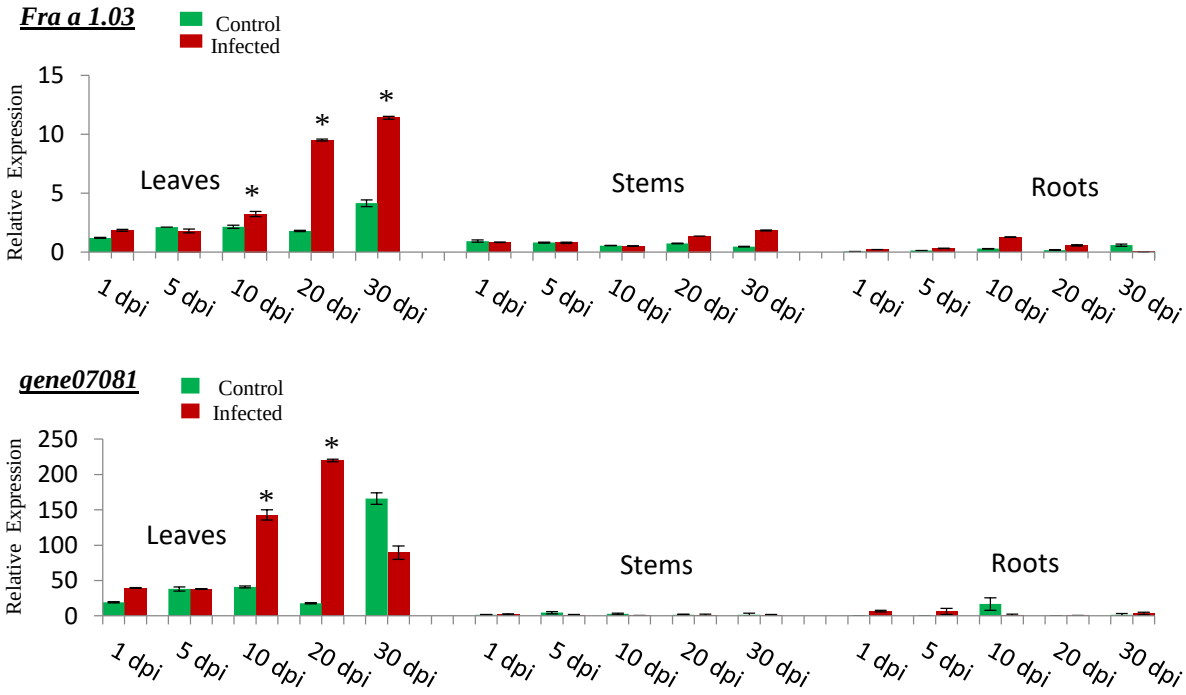
	Fra a 1.04	Fra a 1.05	Fra a 1.06	Fra a 1.07	Fra a 1.08
K_{on} [M^{-1} s^{-1}]	$2.29 *10^4$	$4.48 *10^2$	$2.54 *10^3$	$3.42 *10^3$	$3.75 *10^3$
K_{off} [s^{-1}]	$6.29 *10^{-4}$	$9.77 *10^{-4}$	$1.83 *10^{-4}$	$1.76 *10^{-4}$	$1.99 *10^{-4}$
K_D [nM]	27.5	2,181	72.05	51.5	53.1

3.7 Gene expression in response to *Verticillium* infection

3.7.1 Quantitative PCR analysis of 21 *Fra a 1* genes

In order to examine the effect of pathogen infection on the expression of *Fra a 1* genes, *in vitro* cultivated strawberry plants were infected with *Verticillium dahliae* and qPCR analysis was performed. Samples were taken at 1, 5, 10, 20 and 30 days post infection (dpi). Non-infected plants were also sampled and served as control. Quantitative real time PCR using the Fast SYBR Green master mix as a reagent, were accomplished and the method of $2^{-\Delta\Delta Ct}$ was exploited. Values were calculated as fold change of each sample relative to a reference gene (*FaRIB413*). The fold changes of each transcript (relative values) upon *Verticillium* infection were calculated and represented for each *Fra a 1* gene. The expression pattern of 21 *Fra a 1* isoforms in different tissues of the strawberry plants, after infection with *Verticillium dahliae* showed a wide range of response **(Figure 32)**.

Fra a 1.03 (11.4 fold relative expression) and *gene07081* (219.9 fold rel. exp.) were significantly upregulated in leaves after pathogen infection **(Figure 27).** This induction increased through the time and reached a maximum after 20 to 30 days of infection. For *gene07078* (2.7 fold rel. exp.), the highest induction level was detected in leaves already after 1 day of infection followed by a steady decrease until the 10th day and then the expression increased again. For *gene07083* (1.4 fold rel. exp.), the induction of expression was also significant in leaves at day 1 post inoculation (**Figure 27)**.



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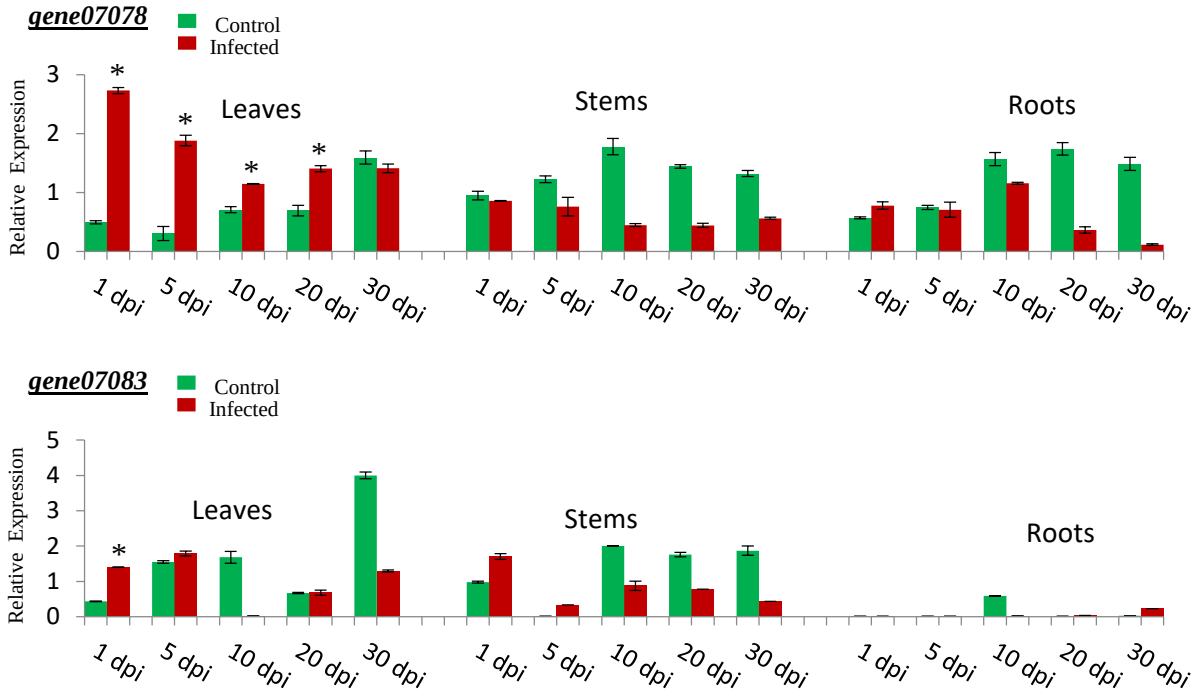
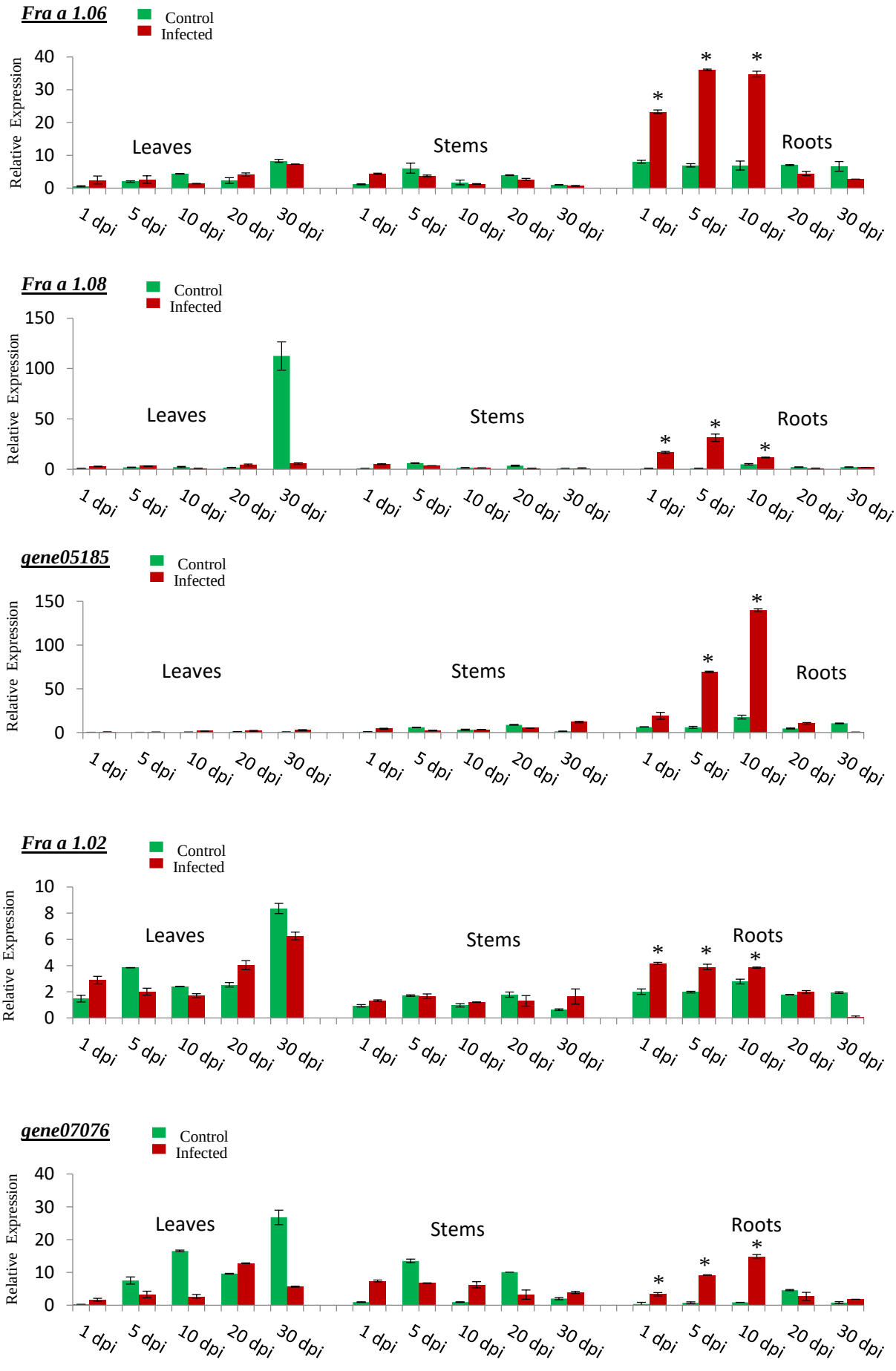


Figure 27. Relative transcript levels determined by qPCR of *Fra a 1.03*, *genes07081*, *07078* and *07083* which are highly induced in leaves. Strawberry plants were infected with *Verticillium dahliae* and the relative transcript levels were normalized to the control stem at 1 dpi (set to one). The expression level in the tissues was measured at 1, 5, 10, 20, and 30 dpi. Green bars represent the control while red bars represent infected tissues. The mean values (± SD) are obtained from five biological replicates and are shown as relative changes. Asterisks represent significant induction of a gene after statistical comparison with the transcript abundance of the control sample using the Tukey test. *FaRIB413* was used as a housekeeping gene for normalization.

Fra a 1.06, *Fra a 1.08* and *gene05185* were highly expressed in roots after pathogen infection **(Figure 28).** There was an up to 36.1 fold relative transcript level for *Fra a 1.06*, 31.3 fold rel. exp. for *Fra a 1.08*, and 140 fold rel. exp. for *gene05185*. The induction was more relevant at day 5 for *Fra a 1.06* and *Fra a 1.08* and at day 10 for *gene05185*. Interestingly, a quick and prominent upregulation was also observed in roots for *Fra a 1.02* (4.2 fold rel. exp.) at 1 dpi which declined slightly over time. Similarly, *gene07076* (14.8 fold rel. exp.) and *gene07063* (19.8 fold rel. exp.) were highly induced in roots but the maximum peak occurred at day 10. Moreover, the expression patterns of *gene04962* (9.0 fold rel. exp.) and *gene07077* (1.7 fold rel. exp.) were nearly similar in roots and increased from day 1 to day 5 followed by a constant diminution until day 30 **(Figure 28)**.


Fra a 1.06
Control
Infected
Relative Expression
40
30
20
10
0
Leaves
Stems
Roots
1 dpi
5 dpi
10 dpi
20 dpi
30 dpi
Fra a 1.08
150
100
50
0
gene05185
Fra a 1.02
8
6
4
2
gene07076

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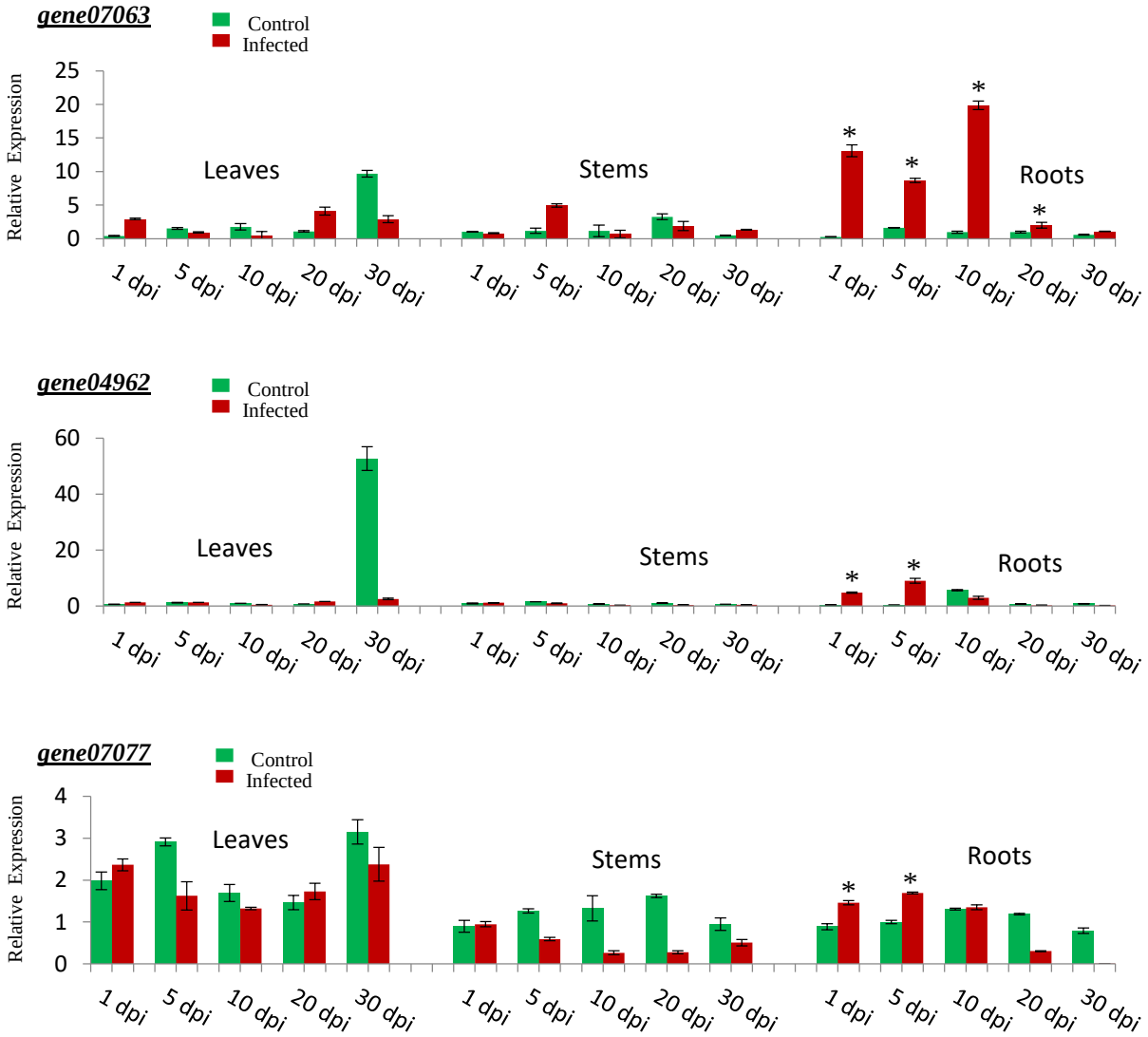
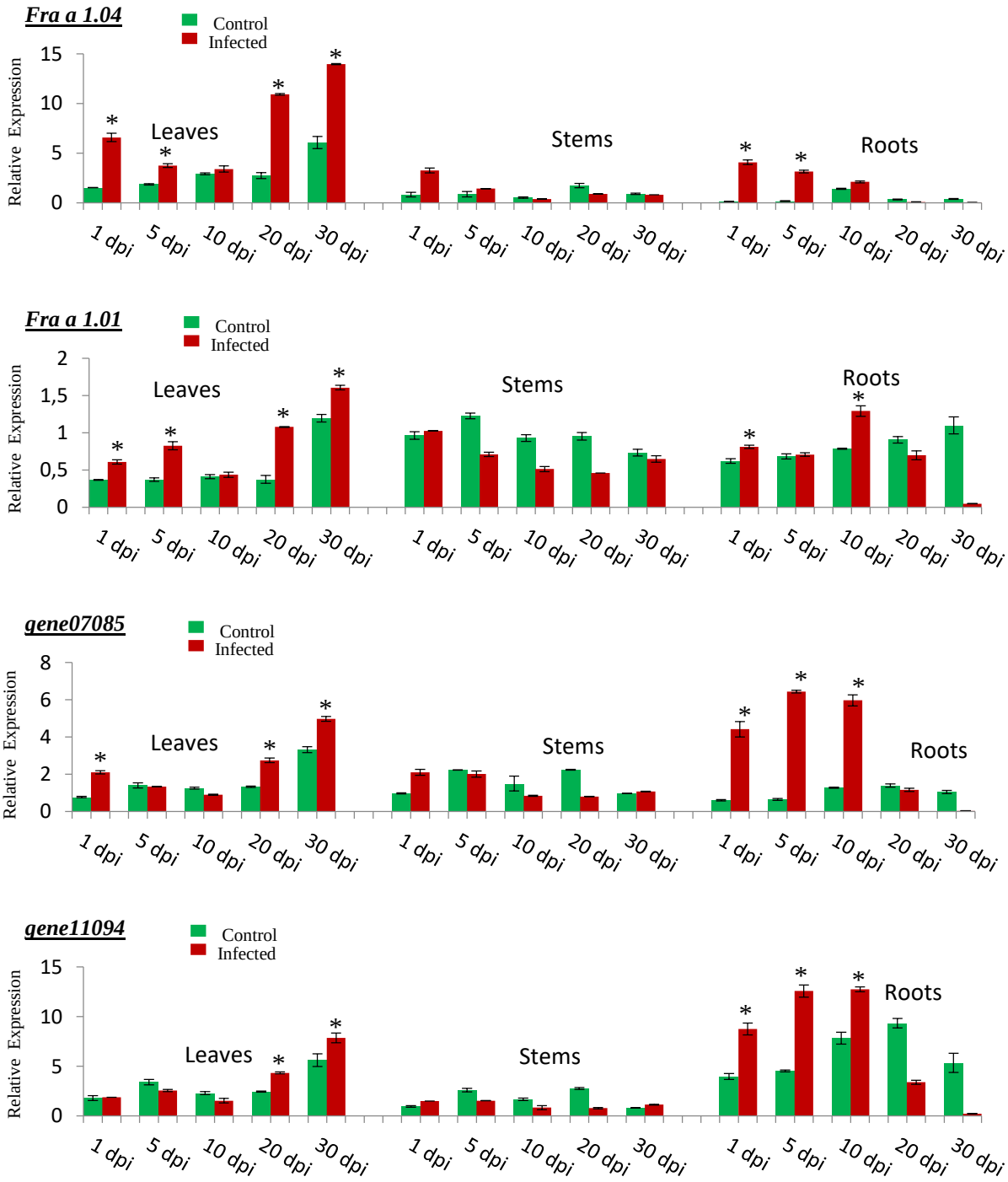


Figure 28. Relative transcript levels determined by qPCR of *Fra a 1.06*, *Fra a 1.08*, *gene05185*, *Fra a 1.02*, *gene07076* and *genes07063*, *04962*, *07077* which are highly induced in roots. Strawberry plants were infected with *Verticillium dahliae* and the relative transcript level was normalized to the control stem at 1 dpi (set to one). The expression level in the tissues was measured at 1, 5, 10, 20, and 30 dpi. Green bars represent the control while red bars represent infected tissues. The mean values (± SD) are obtained from five biological replicates and are shown as relative changes. Asterisks represent significant induction of a gene after statistical comparison with the transcript abundance of the control sample using the Tukey test. *FaRIB413* was used as a housekeeping gene for normalization.

Furthermore, *Fra a 1.04*, *Fra a 1.01*, *gene07085*, *11094 and 07087* were strongly induced in leaves and roots simultaneously **(Figure 29).** For the first three genes, the induction was more relevant in leaves after 20 dpi. There was an up to 14.0 fold rel. exp. for *Fra a 1.04*, 1.6 fold rel. exp. for *Fra a 1.01* and 5.0 fold rel. exp. for *gene07085* in leaves. In roots, an upregulation was detected at day 1 for *Fra a 1.04* (4.1 fold rel. exp.) followed by a constant decrease through the time, until day 30, whereas a strong induction of expression in infected roots was observed after 10 days for *Fra a 1.01* (1.3 fold rel. exp.) and 5 days for *gene07085* (6.4 fold rel. exp.). For *gene11094*, there was a high expression level in leaves namely at day

20 (4.3 fold rel. exp.) and day 30 (7.9 fold rel. exp.) post pathogen inoculation while in roots a progressive induction was detected until the 10th day (12.7 fold rel. exp.) followed directly by a severe decline from the 20th day (3.4 fold rel. exp.) of infection. *Gene07087* showed also a considerable upregulation in leaves at the first five days of infection (2.0 fold rel. exp.) but this increase gradually diminished in the course of time. In roots, this gene started to be induced from day 1 (0.9 fold rel. exp.) until day 10 (1.0 fold rel. exp.) followed by a drastic decrease at day 30 (0.1 fold rel. exp.) **(Figure 29)**.



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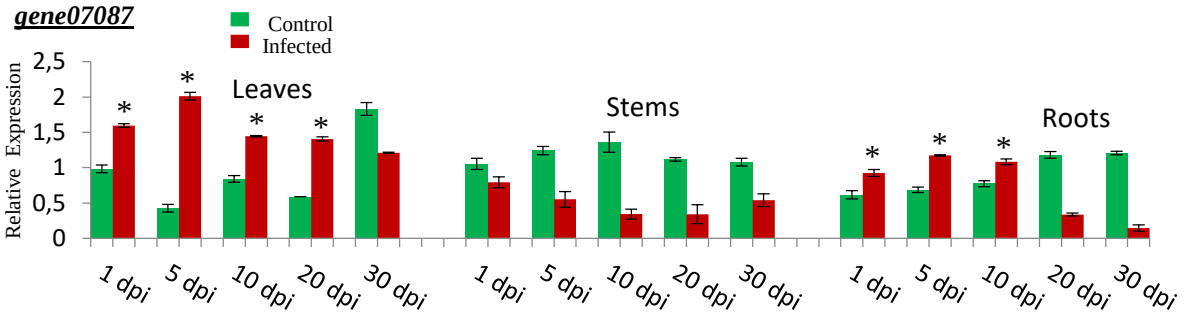

Figure 29. Relative transcript levels determined by qPCR of *Fra a 1.04*, *Fra a 1.01*, *genes07085*, *11094* and *07087* which are highly induced in leaves and roots. Strawberry plants were infected with *Verticillium dahliae* and the relative transcript level was normalized to the control stem at 1 dpi (set to one). The expression level in the tissues was measured at 1, 5, 10, 20, and 30 dpi. Green bars represent the control while red bars represent infected tissues. The mean values (± SD) are obtained from five biological replicates and are shown as relative changes. Asterisks represent significant induction of a gene after statistical comparison with the transcript abundance of the control sample using the Tukey test. *FaRIB413* was used as a housekeeping gene for normalization.

Interestingly, the expression of *genes32299* and *07084* was induced in all three tissues at the same time **(Figure 30).** In leaves, the expression pattern of both genes was very similar: it decreased progressively during the first 10 days post inoculation and augmented again until the 30th day (7.5 fold rel. exp. for *gene32299* and 18.4 fold rel. exp. for *gene07084*).

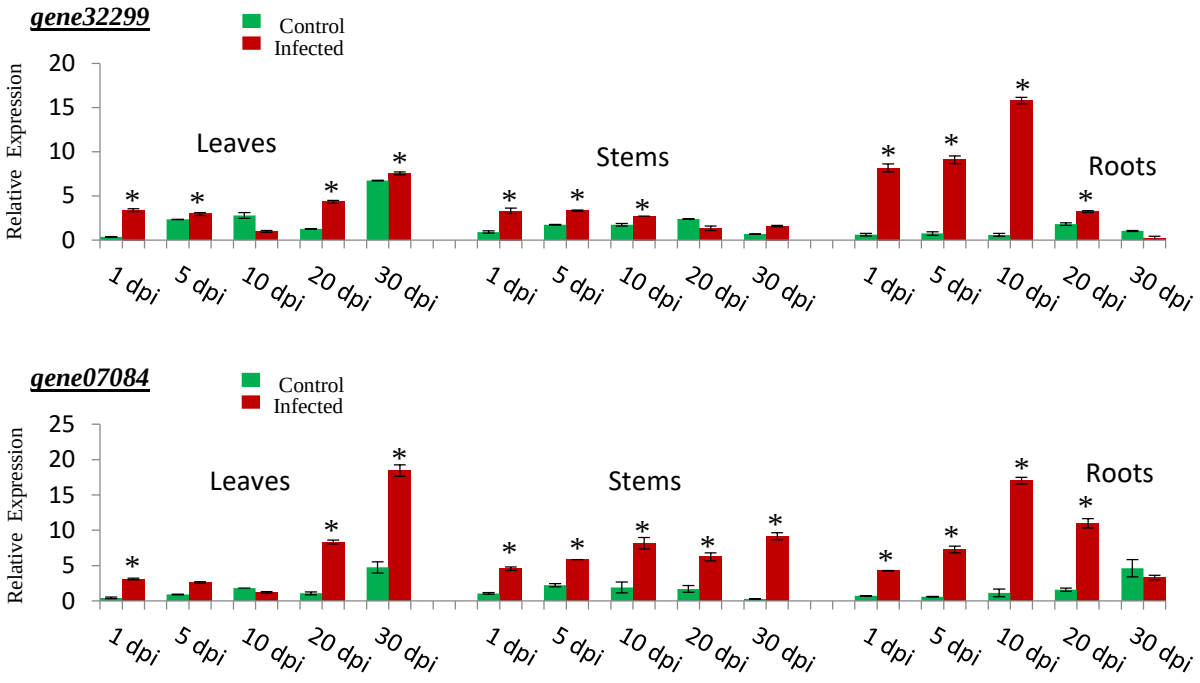

Figure 30. Relative transcript levels determined by qPCR of *genes32299* and *07084* which are highly induced in leaves, roots, and stems. Strawberry plants were infected with *Verticillium dahliae* and the relative transcript level was normalized to the control stem at 1 dpi (set to one). The expression level in the tissues was measured at 1, 5, 10, 20, and 30 dpi. Green bars represent the control while red bars represent infected tissues. The mean values (± SD) are obtained from five biological replicates and are shown as relative changes. Asterisks represent significant induction of a gene after statistical comparison with the transcript abundance of the control sample using the Tukey test. *FaRIB413* was used as a housekeeping gene for normalization.

In addition, those two genes were identically induced in roots. A gradual upregulation was observed until the 10th day of infection (15.8 fold rel. exp. for *gene32299* and 17 fold rel. exp. for *gene07084*) followed by a progressive diminution of expression until day 30 (0.2 fold rel. exp. for *gene32299* and 3.3 fold rel. exp. for *gene07084*). Moreover, *genes32299* and *07084* were the only two genes which were induced in stems. A steady decrease in the gene expression was detected for *gene32299* from the first day (3.3 fold rel. exp.) and until the 30th day while for *gene07084* there were two peaks of expression, one peak at the 10th day (8.2 fold rel. exp.) and the second peak at the 30th day (9.2 fold rel. exp.) **(Figure 30)**.

Interestingly, no induction of gene expression was detected for *Fra a 1.05* and *Fra a 1.07* **(Figure 31)**.

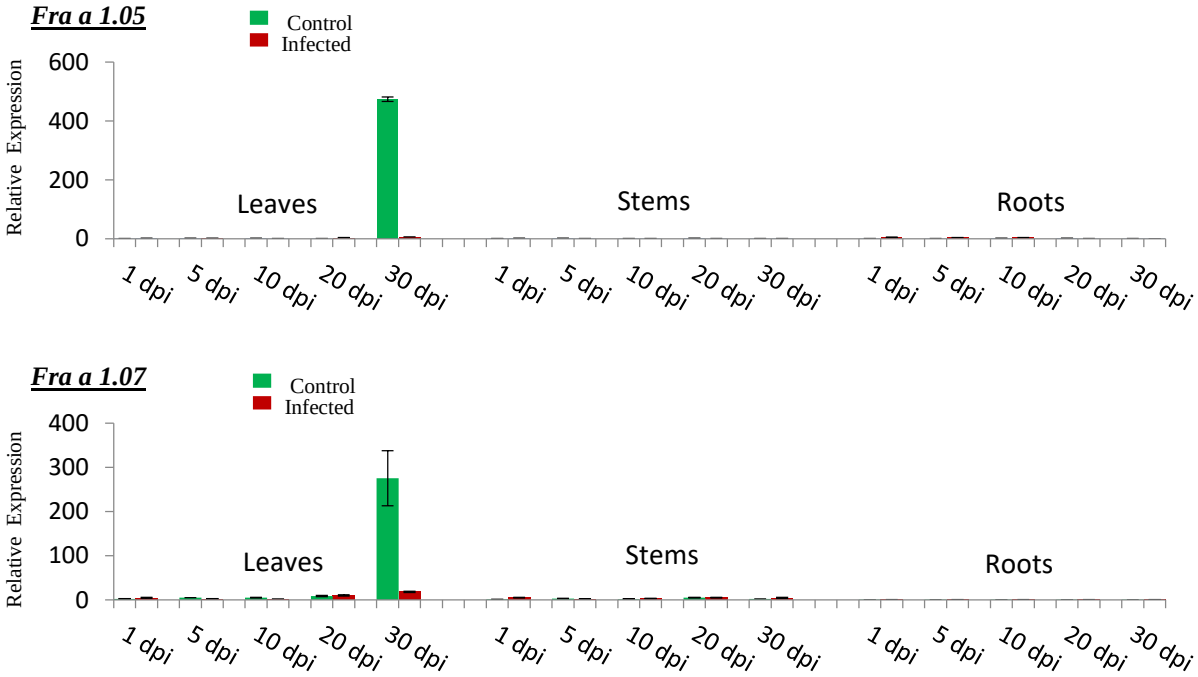

Figure 31. Relative transcript levels determined by qPCR of *Fra a 1.05* and *Fra a 1.07*. No induction was detected for both isoforms. Strawberry plants were infected with *Verticillium dahliae* and the relative transcript level was normalized to the control stem at 1 dpi (set to one). The expression level in the tissues was measured at 1, 5, 10, 20, and 30 dpi. Green bars represent the control while red bars represent infected tissues. The mean values (± SD) are obtained from five biological replicates and are shown as relative changes. *FaRIB413* was used as a housekeeping gene for normalization.

Altogether, a huge variation in transcript levels of the 21 isoforms was observed in the investigated *F.* × *ananassa* organs (leaves, stems and roots). The qPCR analysis indicated a clear upregulation of 19 isoforms after *Verticillium* infection and a different pattern of expression depending upon the analyzed strawberry tissue. Briefly, the highest levels of

induction were detected at later time point for leaves (20 to 30 days post pathogen inoculation) and early stage in roots (first 10 days post pathogen inoculation) **(Figure 32)**.

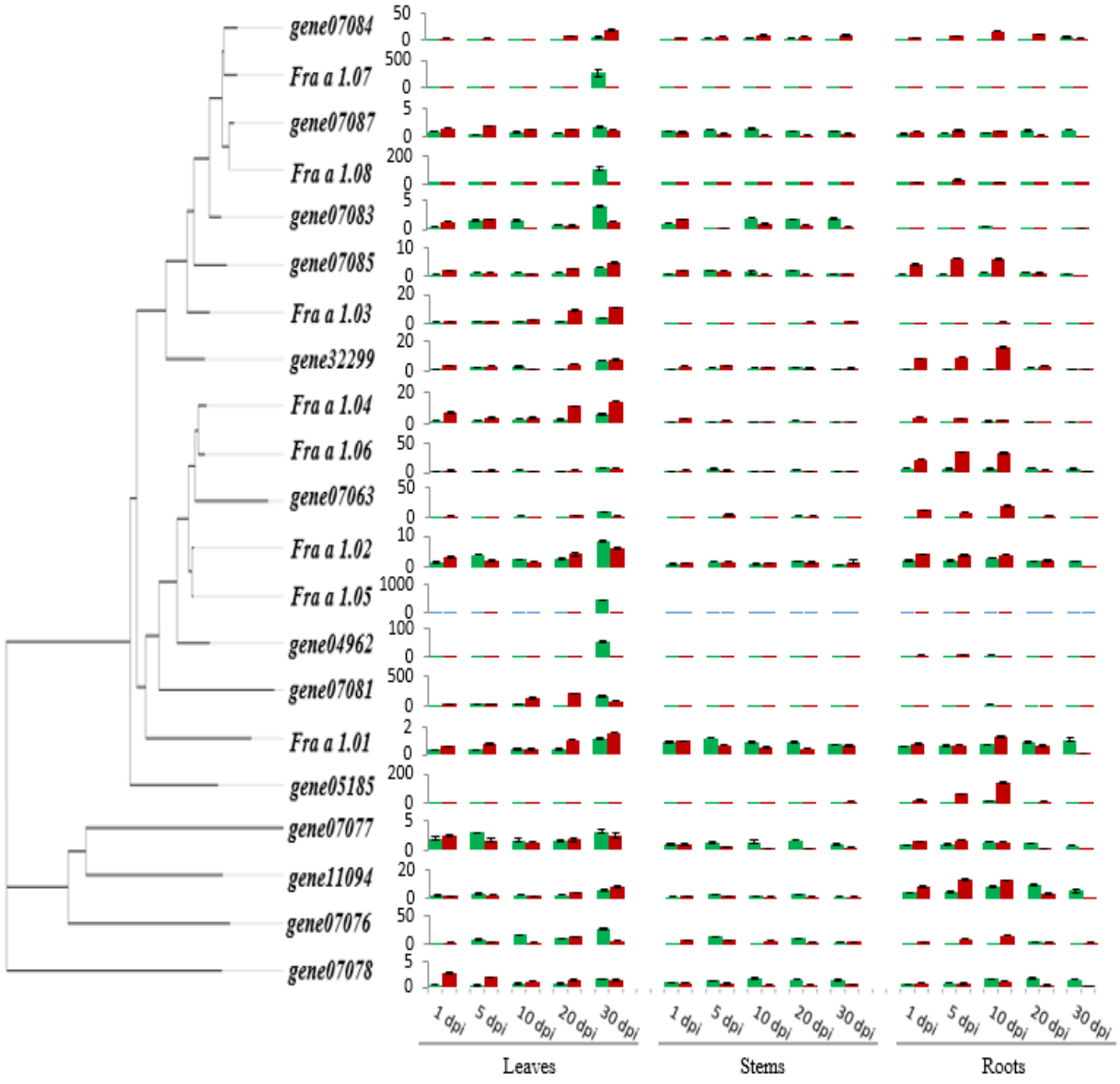

Figure 32. Phylogenetic tree of *Fra a 1* genes and summary of the expression profile of the 21 *Fra a 1* isoforms in untreated leaves, stems and roots of *in vitro* cultivated *F.* × *ananassa* plants (green bars) and infected tissues with *Verticillium dahliae* (red bars) at various time points post pathogen inoculation. qPCR using specific primers for *Fra a 1* genes and the reference gene was used for the expression analysis.

3.7.2 Visual evaluation of disease severity and fungal propagation

At all sampling time points, *F.* × *ananassa in vitro* plants were visually assessed for the appearance of foliar symptoms using a 0-5 scale **(Figure 33)**. Disease symptoms developed gradually and became visible 20 days post *Verticillium* inoculation. Scale 2 was attributed to plants at 20 dpi and scale 5 for plants at 30 dpi. For control and infected plants during the first 20 days, no symptoms were observed and therefore scale 0 was assigned **(Figure 33)**.

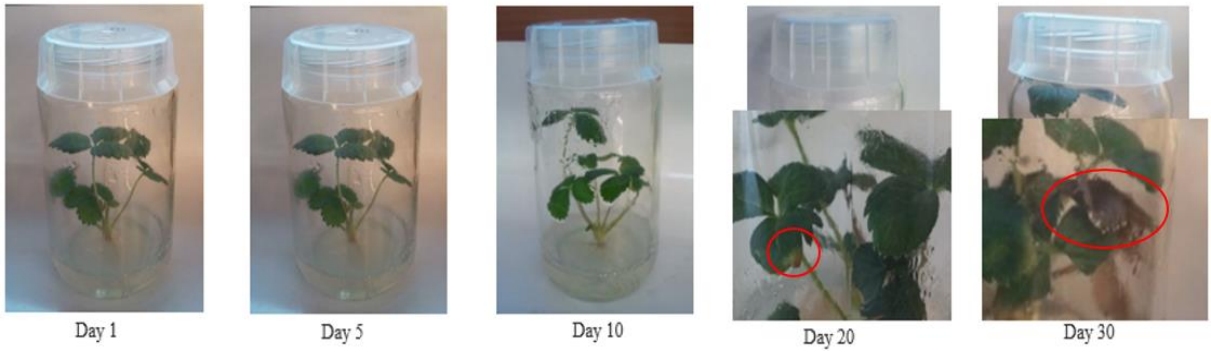

Figure 33. Development of disease symptoms in *in vitro* plants infected with *Verticillium dahliae* at different time points (1, 5, 10, 20, and 30 days).

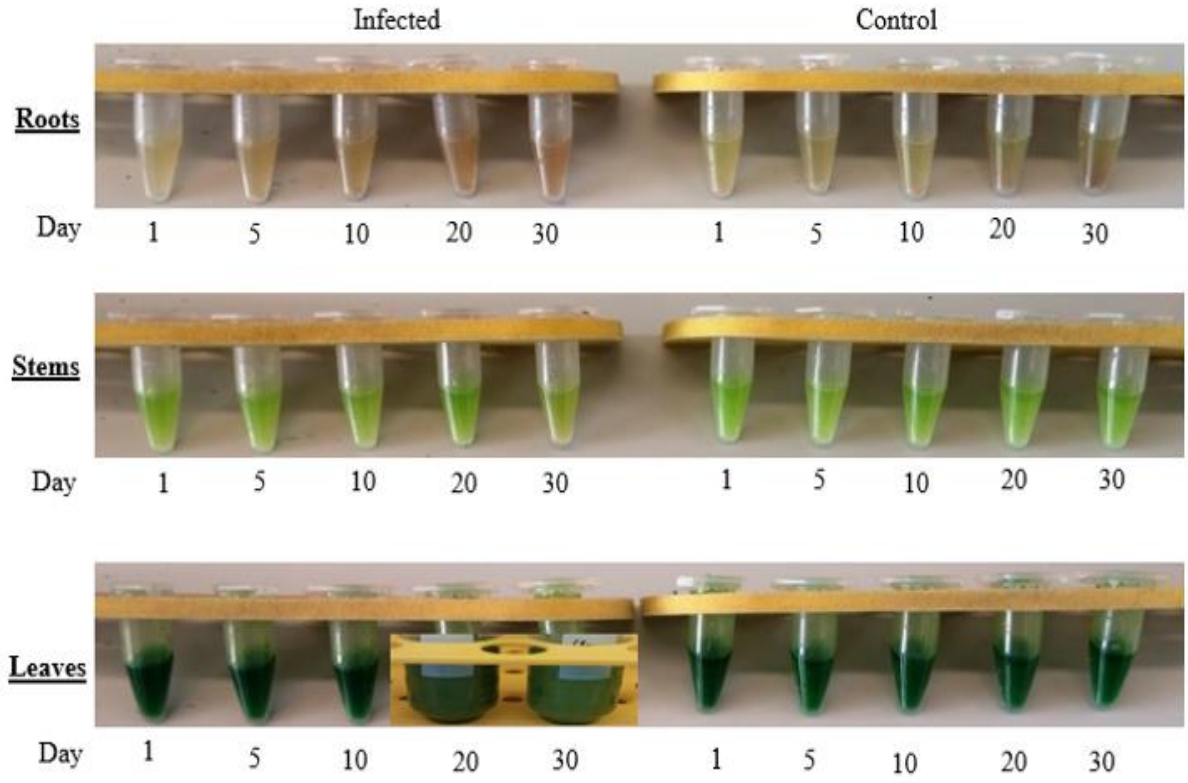

Figure 34. Comparison of methanol extracts of lyophilized tissues from control and infected organs at different time points post *Verticillium dahliae* infection (1, 5, 10, 20 and 30 days).

Samples were also evaluated on the basis of color modification after methanol extraction of lyophilized tissues **(Figure 34)**. Comparison of *Verticillium* infected roots and the control demonstrated that the color was yellowish at the first day and became progressively light brown at 30 dpi for the infected roots and yellowish brown for the control. Similarly, a color change was visible between 1 dpi (fluorescent green) and 30 dpi (fluorescent yellowish green) in infected stems but not for the control tissue whereas in infected leaves, the unique color

change occured at 20 dpi (dark green) and 30 dpi (light green) **(Figure 34)**. No color change was detected for the control leaves.

3.7.3 LC-UV-ESI-MS analysis

In order to test whether plant metabolites are involved in the defense response toward *Verticillium* infection in strawberry *in vitro* plants, tissues used for qPCR were also analyzed by LC-UV-ESI-MS **(Figure 35)**. The QuantAnalysis tool was used for quantification and results were normalized against the internal standard (biochanin A). The accumulation of the below mentioned metabolites is designed as the relative concentration (Rel. conc.) which is expressed in mg-equ./g dry weight of the internal standard biochanin A in freeze-dried strawberry tissues (leaves, stems and roots). Some metabolites such as catechin and epicatechin accumulated in stems and roots over time in infected tissues. Caffeic acid and citric acid were observed mainly in infected roots at 30 dpi. Significant accumulation of caffeic acid in control leaves and stems was also observed. Further compounds were also identified at early or late stage of infection. Tryptophan was observed in infected leaves and stems at 1 dpi as well as at late stage in infected leaves and roots (20-30 dpi). Significant accumulation of glutathione in its reduced form (GSH) was detected in control stems and roots over infection time with a peak of accumulation at 1 dpi in infected leaves. No significant accumulation was detected in control tissues for catechin, epicatechin, citric acid and tryptophan. Furthermore, the implication of some phytohormones on strawberry defense was also tested. Five plant hormones were identified by LC-UV-ESI-MS. Abscisic acid (ABA, 264.32 g/mol), salicylic acid (SA, 138.12 g/mol), jasmonic acid (JA, 210.27 g/mol), gibberellic acid (GA, 346.47 g/mol) and indole acetic acid (IAA, 175.19 g/mol) were eluted at 37.9 min, 35.5 min, 39.1 min, 30.8 min and 33.3 min, respectively. The retention times were confirmed by analyses of authentical reference materials. ABA, SA, JA and GA were accumulated in infected leaves at 5 dpi and at 10 dpi for IAA. The five phytohormones were as well detected in infected stems at various time points: during the first 5 dpi for SA, IAA and GA, and during the first 10 days post inoculation for JA. Likewise, ABA was identified during the first 10 days in infected stems with a peak at 30 dpi. Moreover, IAA and JA were the unique hormones to be discerned in infected roots at tardy stages (20 and 30 dpi). No relevant accumulation was observed in control tissues for the five phytohormones **(Figure 35)**.

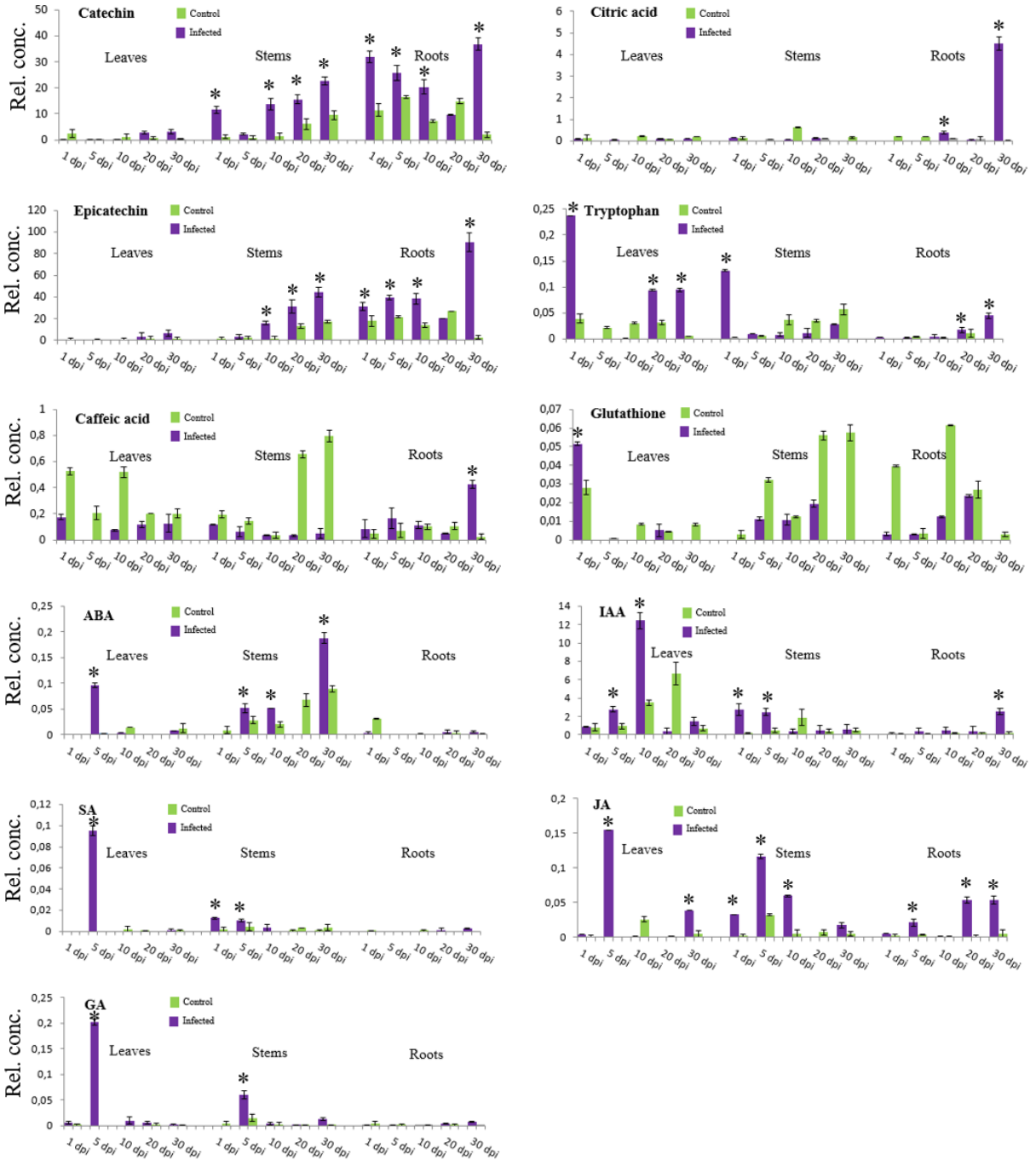

Figure 35. Relative concentration (± SD) of some metabolites and pytohormones in infected (with *Verticillium dahliae*) and uninfected Fragaria tissues at 1, 5, 10, 20, and 30 dpi. Relative concentrations (Rel. conc.) are expressed in mg-equ./g dry weight of the internal standard biochanin A in freeze-dried strawberry tissues (leaves, stems and roots). Asterisks (*) indicate significant differences for each metabolite after statistical comparison with its abundance in the control sample using the Tukey test.

3.8 Fra a 1 expression and RNA-seq

RNAsequencing (RNA-seq) is a recently developed approach to transcriptome profiling using deep-sequencing technologies and was performed on receptacle and achene tissues of three *F. vesca* botanical forms (Yellow Wonder "YW", Hawaii 4 "HW4" and Reine des Vallées "RdV") at three different ripening stages (green, white and ripe) (Härtl et al., 2017). The data

(kindly made available by Katja Härtl and Katrin Franz-Oberdorf) were used to determine the transcript levels of *Fra a 1* orthologues in *F. vesca* genotypes which were then summarized in the current work in a heat map **(Figure 36)**. Contrary to *genes07085* and *07077* which showed very prominent expression levels in achenes and receptacles, *Fra a 1.04*, *genes07063, 07081, 05185, 11094, 07076* and *07078* displayed low transcript levels in all tissues. Intermediate transcript levels of *Fra a 1.05, gene04962, Fra a 1.08, gene07083* and *gene32299* were observed in receptacle and achenes of the three *F. vesca* genotypes. While *genes07084* and *07087* displayed a meaningful expression level in ripe receptacle, *Fra a 1.01* was significantly expressed in all genotypes except ripe receptacle. Compared to *Fra a 1.02* and *Fra a 1.06* which were notably expressed at ripening stage in achenes and receptacles, *Fra a 1.07* was expressed mainly in ripe receptacle and *Fra a 1.03* was remarkably expressed in all achene genotypes. Altogether, the highest transcript level was detected in receptacles and achenes for *genes07085* and *07077* and in achenes for *Fra a 1.06, Fra a 1.02, Fra a 1.01* and *Fra a 1.03* **(Figure 36)**.

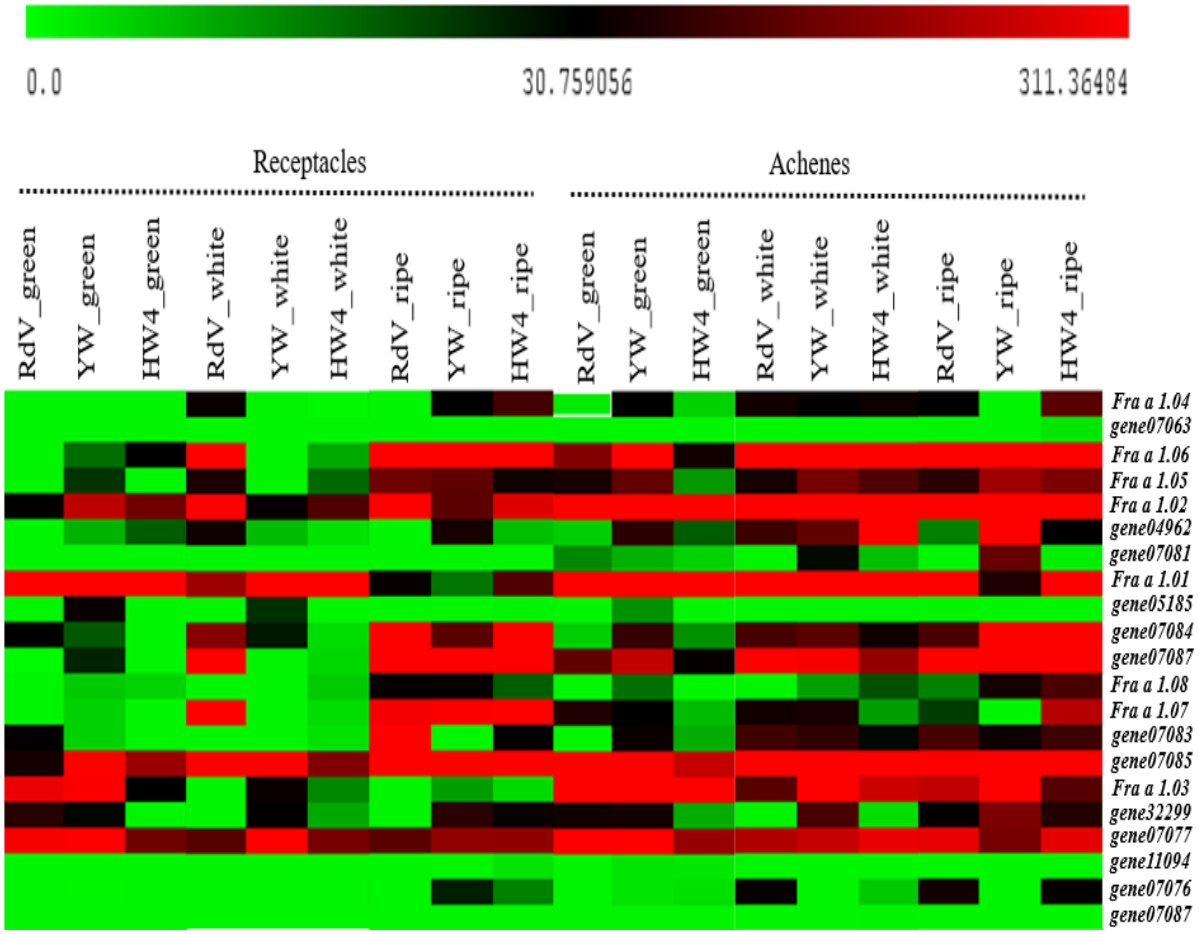

Figure 36. Heat map of 21 *Fra a 1* orthologues from three *Fragaria vesca* varieties (Yellow Wonder "YW", Hawaii 4 "HW4" and Reine des Vallées "RdV") at different developmental stages (ripe, white and green fruits) in receptacles and achenes. The expression signal of each gene at each stage is based on the normalization value in MEV (Multi experiment Viewer). Gene expression levels of the 21 *Fra a 1* genes are presented where the lowest values are shown in green and the highest values in red.

3.9 Basophil activation test

In order to identify the main strawberry fruit allergen, a basophil activation test (BAT) of Fra a 1.01-1.08 recombinant proteins was conducted by Franz-Oberdorf et al. (2016). In total, eight atopic patients (seven females and one male) with a type-I-allergy to birch pollen and two controls (females) were examined. The basophil activation test was based on the stimulation of whole blood cells measuring CD63 activation of basophils using CCR3 (C-C chemokine receptor type 3) as basophil marker. Activated basophils were counted by FACScan Flow cytometer as a cellular tool. The percentage of CD63+ positive cells represents the amount of activated basophils.

Recombinant Bet v 1, an empty pQE70 vector control, anti-FcεRI and fMLP (two positive controls) and the negative control of the kit Flow CAST were also subjected to BAT **(Figure 37)**. Bet v 1a, Fra a 1.06 and Fra a 1.07 exhibited a similar median of about 40% basophil activation. Fra a 1.01 and Fra a 1.08 showed a low allergenic potential with a median of 20% activated cells. Interestingly, Fra a 1.02 and Fra a 1.03 appeared to be the most active allergens in strawberry fruit with a median of more than 75% whereas Fra a 1.04 and Fra a 1.05 seemed to be hypoallergenic isoforms (median less than 15%). Considering the p-values of basophil activation, only Fra a 1.04, Fr a 1.05, pQE70 and the negative control indicated a significant low allergenic potential ($p \leq 0.05$) **(Figure 37)**.

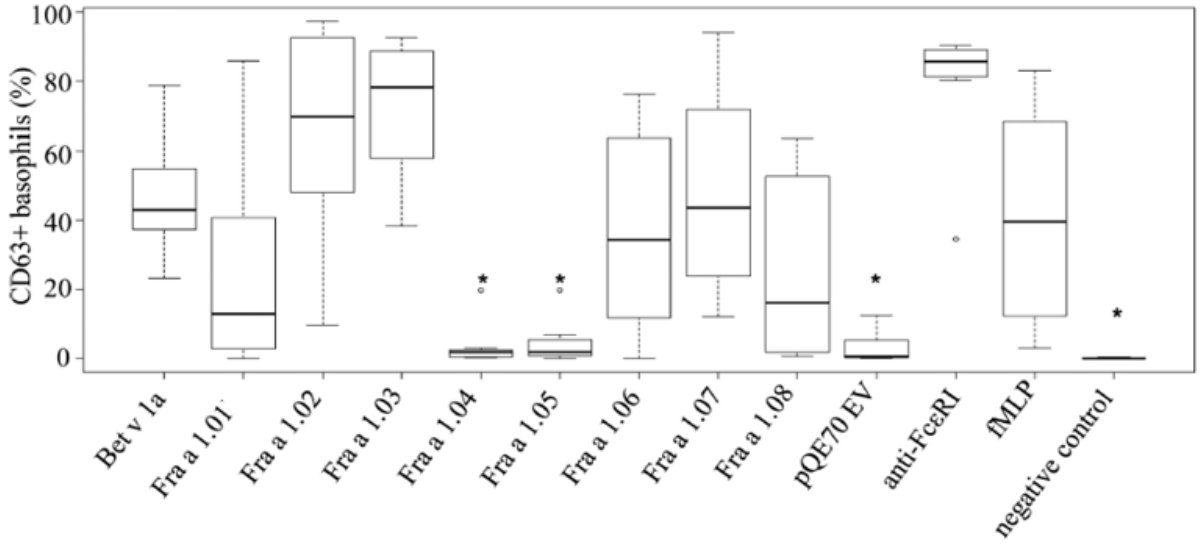

Figure 37. Activated basophils determined by flow cytometer after incubation of basophils with Fra a 1.01-1.08 recombinant proteins, Bet v 1a, pQE70 vector, two positive controls (anti-FcεRI and fMLP) and a negative control using a concentration of 50 ng/ml. p-value ≤ 0.05, indicated by an asterisk, was considered statistically significant (adapted from Franz-Oberdorf et al., 2016).

The activation of basophils was also analyzed for various concentrations of the allergens, ranging from 0.5 to 500 ng/ml. Basophil activation values were plotted against the concentration and the areas under the curves (AUC) were calculated **(Figure 38)**. The AUC of Bet v 1a was used as reference and set as 1. Fra a 1.02 and Fra a 1.03 were confirmed to be the main active allergens (AUC > 1), Fra a 1.01, Fra a 1.06, Fra a 1.07 and Fra a 1.08 were similar to Bet v 1a (AUC ~ 1) whereas Fra a 1.04 and Fra a 1.05 were hypoallergenic isoforms (AUC << 1).

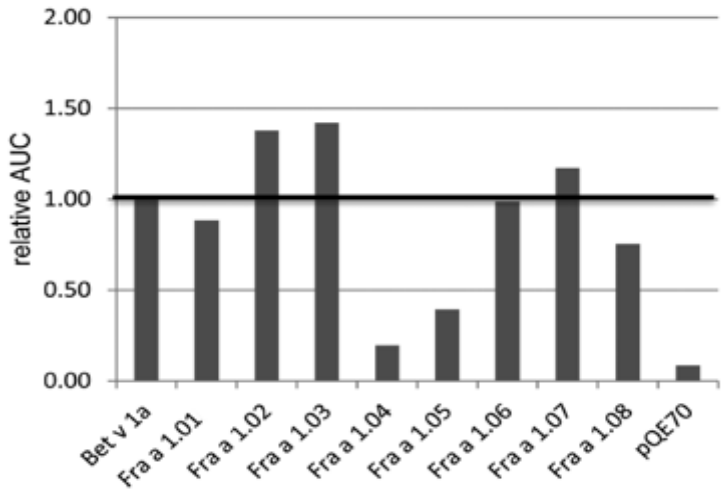

Figure 38. Relative area under the curve (AUC) calculated on the basis of basophil activation at different concentrations of the allergens, Bet v 1a and pQE70 vector. Bet v 1a was used as reference and set to 1 (adapted from Franz-Oberdorf et al., 2016).

4 Discussion

4.1 Structural properties of PR-10

As the octoploid *F.* × *ananassa* and the diploid *F. vesca* share a common ancestor, the availability of the genome sequence of the woodland strawberry (Shulaev et al., 2011) has made it possible to analyze the *Fra a 1* allergen family. Twenty-one isoforms of the *Fra a 1* allergen were identified in the *F. vesca* genome and five genes were cloned and their recombinant proteins were purified during this study. Likewise, at least eighteen *Mal d 1* genes of the multigene family of plant PR-10 proteins were identified in apple (Gao et al., 2005), eight PR-10 isoforms in yellow lupine (Handschuh et al., 2007), five PR-10 genes in pea (Tewari et al., 2003), and eight *Pru p 1* and *Pru d 1* isoforms in peach and almond, respectively (Chen et al., 2008).

Similarly to the intracellular PR-10 (IPR-10) proteins which have an ORF encoding a polypeptide of 151–162 amino acids with molecular masses of 15–18 kDa, Fra a 1.04-1.08 proteins are all 160 amino acids long. SDS-PAGE analyses of the recombinant proteins revealed a high degree of purity and protein bands were consistent with the expected molecular weights ranging from 17.5 to 17.6 kDa. Fra a 1.01 and Fra a 1.03, which were recently analyzed showed a molecular weight of 17.8 and 17.5 kDa, respectively (Casañal et al., 2013b). Interestingly, the proteins encoded by *genes07078* (23.96 kDa), *07087* (21.73 kDa), *07084* (19.36 kDa), and *07063* (14.37 kDa) exceeded this size. Likewise, (S)-norcoclaurine synthase PR-10 NCSs, which exhibit 28-38% identity and putative structural homology with the Bet v 1 protein family (Samanani et al., 2004), have an ORF of 630 bp encoding proteins with a molecular weight of 23.3 kDa.

The Fra a 1.04-1.08 proteins, analyzed in this study have sequence similarities varying from 55.6% to 59.3% amino acid pairwise identity to Bet v 1 **(Table 6)**. Apart from three exceptions, namely the proteins encoded by *genes07076, 07077* and *07078* **(Figure 14)**, the consensus sequence GxGGxGxxK[TS] was highly conserved in eighteen of the Fra a 1 proteins in which the Bet v 1 domain was also maintained. On this basis, the Fra a 1 isoforms were predicted to belong to the pollen allergen family.

Liu and Ekramoddoullah (2006) reported that four amino acids were revealed to be invariable across the PR-10 family which were also identified for Fra a 1.04-1.08: Gly_{47}, Gly_{52}, Asp_{76} and Leu_{152} **(Figure 42)**. Casañal et al. (2013b) established that the amino acids facing the cavity in Fra a 1E, Fra a 2 and Fra a 3 proteins are generally conserved but variability could be observed at certain positions explaining their different selectivity toward ligands. It was

shown that key amino acids Leu_{59} and Arg_{139}, involved in Fra a 3 (+)-catechin interaction were replaced by phenylalanine (Phe_{59}) and lysine (Lys_{139}) in both Fra a 1E and Fra a 2. The amino acid Phe_{59} was also conserved in Fra a 1.04-1.06 and changed to Leu_{59} in Fra a 1.07-1.08 while the arginine residue was detected only for Fra a 1.07 at position 140 and was replaced by Lys_{140} for the four rest of the Fra a 1 proteins. Further important residues detected by Casañal et al. (2013b) such as His_{70} and Tyr_{84} were also identified for Fra a 1.04-1.08. PR-10 genes are generally very similar and sequence divergence is therefore normally restricted. The sequences of the eight Fra a 1.01-1.08 proteins were compared using a pairwise sequence alignment and sequence similarities were calculated **(Table 8)**. The amino acid sequences showed at least 69.4% protein similarity (between Fra a 1.01 and Fra a 1.07) while a 99.4% of similarity was found between Fra a 1.02 and Fra a 1.05.

Table 8. Pairwise protein sequences similarity of the eight Fra a 1.01-1.08 proteins: Percentage of sequence similarities higher than 90% are highlighted in orange. Fra a 1.02 and Fra a 1.05 showed a 99.4% of similarity. Protein sequence similarities were performed using Geneious software.

	Fra a 1.01	**Fra a 1.02**	**Fra a 1.03**	**Fra a 1.04**	**Fra a 1.05**	**Fra a 1.06**	**Fra a 1.07**	**Fra a 1.08**
Fra a 1.01		79.4	75.6	76.3	78.8	76.3	69.4	70.6
Fra a 1.02			80.6	95.6	99.4	96.3	75.6	77.5
Fra a 1.03				78.1	80	78.8	88.1	90.6
Fra a 1.04					96.3	97.5	73.8	75.6
Fra a 1.05						96.9	75	76.9
Fra a 1.06							75	76.9
Fra a 1.07								96.3
Fra a 1.08								

The most striking feature of PR-10 genes was that those genes are generally very similar within species and more distant from gene copies of other plant species suggesting that the homogeneity of the PR-10 multigene family was maintained by concerted evolution (Wen et al., 1997). This phenomenon causes genes to evolve as a single unit with members exchanging genetic information through gene conversion and unequal crossing-over. As pointed out by Finkler et al. (2005), Schenk et al. (2009) and Lebel et al. (2010), such a high interspecific but low intraspecific variability has been observed in *Passiflora*, *Betula*, and *Vitis* genus.

4.2 qPCR analysis and *Fra a 1* gene expression

The expression pattern of the 21 *Fra a 1* isoforms was studied by quantitative real-time PCR in different plant tissues (root, stem, leaf and young leaf) and developmental stages (flower,

green, white and red fruits) of *F.* × *ananassa*. Because of the strong sequence similarity between the 21 isoforms, specific primers for qPCR were designed using the 5´ UTR region. Contrastingly, the design of specific primers for *Vitis vinifera PR-10 gene* transcripts was not possible even in the 5´ and 3´ UTRs as a consequence of the high similarity of *VvPR-10.1-a* and *VvPR-10.3-a* (Lebel et al., 2010).

As one of the best known and most frequently used reference genes in plants, *FaRIB413* was highly recommended for relative quantification of gene expression in strawberry (Amil-Ruiz et al., 2013). In fact, the *FaRIB413* RNA has been established to be a convenient internal control for strawberry expression studies across several tissues and experimental conditions using qPCR analyses (Benítez-Burraco et al., 2003). For all those reasons, *FaRIB413* was used as reference gene in this work.

Moreover, real time PCR data has been analyzed using the comparative C_t method also known as $2^{-\Delta\Delta Ct}$ method which offer the advantages of the ease of use and the ability to present data as "Fold change" in expression (Schmittgen and Zakrajsek, 2000; Livak and Schmittgen, 2001).

In the current work, the data revealed a wide variation of the expression levels of the 21 *Fra a 1* genes in the eight different tissues (leaves, young leaves, stems, roots, flowers, green, white and red fruits). Thirteen *Fra a 1* genes showed a strong induction in roots which is in conformity with a putative role in plant defense. Also, *Fra a 1E* was mainly expressed in roots while *Fra a 2*, encoding the major strawberry allergen in fruit showed an intermediate expression in the same tissue (Muñoz et al., 2010). In contrast to *gene05185* which was exclusively expressed in roots, ten genes within those thirteen isoforms were also transcribed in red fruits. Moreover, *Fra a 1.02* and *Fra a 1.05*, which exhibit 99.4% of protein sequence similarity **(Table 8)**, revealed a comparable expression pattern with a strong induction in the late stage of fruit ripening and an intermediate transcription level in roots. Muñoz et al. (2010) established that Fra a 2 was likewise highly transcribed in red fruit in coherence with the development of an allergic impact on individuals affected by allergy to red strawberries (Franz-Oberdorf et al., 2016).

Besides, at least one *Fra a 1* isoform was highly transcribed in flowers with an intermediate expression in green fruits (*gene07081*) and three *Fra a 1* isoforms were greatly expressed in stems with an observable expression in the seven remaining tissues (*genes07077*, *07087* and *07078*). Similarly, the *Mal d 1* genes were expressed in different tissues while some were expressed in the same tissue, namely in ripe fruit skins (*Mal d 1b, d–h* and *i*) or in leaves from disease-challenged seedlings (*Mal d 1b, d–f, i* and *j*) (Beuning et al., 2004).

Fra a 1-like PR-10 proteins are members of a small gene family of differentially expressed isoforms which are suggested to show increased gene expression in stress situations. Such expression of stress-induced proteins is triggered by pathogen infection (Swoboda et al., 1995b), plant hormones (Wang et al., 1999), wounding (Liu et al., 2005), salt stress (Jain et al., 2006) or cold (Yu et al., 2000). Like in the common morning glory (*Ipomoea purpurea*) of Convolvulaceae where the expressions of PR-10 genes were susceptible to both developmental and environmental changes (Lu et al., 2009), developmentally regulated isoforms that are expressed in high concentrations in pollen, seeds, fruits, or storage organs were identified as allergens cross-reactive with Bet v 1 (Vieths et al., 2002).

4.3 Ribonuclease activity

In this study, ribonuclease activity was tested for Fra a 1.04-1.08 by in-solution and in-gel RNase activity assays. Nucleolytic activity has been demonstrated in *in vitro* test for Bet v 1 (Bufe et al., 1996; Swoboda et al., 1996) as well as for PR-10 proteins from white and yellow lupine (Bantignies et al., 2000; Biesiadka et al., 2002). Most of the published reports on PR-10 were based on the amino acid sequence similarity between two ginseng ribonuclease proteins (PgPR-10**)** and PR-10 proteins suggesting that this family exhibits ribonuclease activity (Moiseyev et al., 1994). Among the five recombinant Fra a 1.04-1.08 proteins only Fra a 1.06 was proved to be able to catalyze RNA hydrolysis by both techniques. Similar to our results, Biesiadka et al. (2002) found that LlPR-10.1B from yellow lupine manifested RNase activity while its homologous LlPR-10.1A did not.

The RNase activity assays of Bantignies et al. (2000) requested six hours of incubation time for total hydrolysis of RNA, three hours for those published by Srivastava et al. (2006) while Zubini et al. (2009) reported thirty minutes to two hours for total hydrolysis. Results of this work showed that Fra a 1.06 protein was capable of full RNA degradation in a few minutes.

The P-loop structure has been considered to be the possible RNA phosphate binding site correlated with ribonucleolytic activity (Bantignies et al., 2000). The conserved RNase binding site P-loop structure in Bet v 1 protein was found in the Fra a 1 proteins. The P-loop motif of the five Fra a 1.04-1.08 proteins was identical from position 47 to 54 ($G_{47}xG_{49}G_{50}xG_{52}xxK_{55}$) notated also ($Gly_{47}xGly_{49}Gly_{50}xGly_{52}xxLys_{55}$) and differed only at position 55: Thr_{55} for Fra a 1.06 and Lys_{55} for the rest of the four Fra a 1 proteins (**Figure 42**).

In PR-10 proteins with RNase activity, tyrosine (Tyr/Y) and glutamic acid (Glu/E) residues were generally conserved. In Bet v 1, Glu_{96}, Glu_{148}, and Tyr_{150} were predicted to be implicated in its ribonuclease activity (Moiseyev et al., 1997). According to Agarwal et al.

(2013), the three amino acids Glu_{97}, Glu_{150} and Tyr_{152} together with the P-loop were implicated in the RNase activity of the JcPR-10a protein (biofuel crop) whereas Pungartnik et al. (2009) proved that in TcPR-10 (cacao), Glu_{97}, Glu_{149} and Tyr_{151} also with the P-loop were involved in this catalytic activity. Contrarily, the P-loop domain was not concerned in GbPR-10 and only Gly_{51}, Lys_{55}, and Glu_{96} were involved in this activity (Zhou et al., 2002). Tyr_{150} and Glu_{148} which play an important role in catalyzing RNA degradation in GaPR-10 (cotton) (Zhou et al., 2002), were replaced in AhPR-10 with Phe_{148} and $His_{150,}$ respectively (Chadha and Das, 2006). Also in AhPR-10, the Lys_{54} appeared to play a critical role in the RNase activity of the peanut protein while a site-directed mutagenesis of Lys_{55} on GaPR-10 decreased its RNase activity 50 to 60%. In Fra a 1.06 protein, the P-loop domain (AA 47-55) along with Glu_{97}, Glu_{149} and Tyr_{151} **(Figure 42)** residues could be suggested to play a role in its RNase activity.

Since PR-10 proteins are induced by biotic as well as abiotic stresses, their ribonuclease activity suggested a possible role in defense against pathogenic infections in order to protect plants during programmed cell death (PCD) at and around the plant infection sites or to act directly on the pathogen (Liu and Ekramoddoullah, 2006). The correlation of *in vitro* RNase activity with antimicrobial activity of PR-10 proteins was reported in many reviews. Besides the ability to inhibit growth of the oomycete pathogen *Phytophthora capsici*, CaPR-10 protein also exhibited ribonuclease activity against *Tobacco mosaic virus* RNA (Park et al., 2004). It was also demonstrated that the ribonuclease activity of SsPR-10 (yellow-fruit nightshade) and the peanut AhPR-10 played a crucial role during viral attack (Chadha and Das, 2006; Liu et al., 2006). Also, the maize ZmPR-10.1 protein has a high specific RNase activity in correlation with strong antimicrobial activity (Xie et al., 2010). Contrarily, for Bet v 1 (Bufe et al., 1996; Swoboda et al., 1996), LaPR-10 (Bantignies et al., 2000), and GaPR-10 (Zhou et al., 2002), only ribonuclease activity was revealed. Studies demonstrating the relevance of the RNase activity on antifungal activity of PR-10 proteins are also numerous. Indeed, experiments of Pungartnik et al. (2009) suggested that the RNase activity of TcPR-10 was in accordance with its antifungal activity against the basidiomycete cacao pathogen *M. perniciosa*. Similarly AhPR-10 appeared also to exhibit an antifungal activity through its RNase function (Chadha and Das, 2006). Likewise, Lam and Ng (2001) predicted that the *Panax notoginseng* RNase shows antifungal activity. In tobacco, Galiana et al. (1997) reported the involvement of plant RNase activity in defense against pathogenic fungus invasion. However, for ZmPR-10, the relationship between its RNase activity and its strong

resistance to *Aspergillus flavus* is not understood and still needs more clarification (Chen et al., 2006).

Other factors can influence the RNase activity. In the presence of Zeatin, the RNA hydrolysis of the peach major allergen Pru p 1.01 was shown to be considerably affected while no effect was detected for its homologous Pru p 1.06D. Also, post-translational modifications could affect this catalytic reaction. According to Park et al. (2004), the phosphorylation of CaPR-10 increased its ribonuclease function since the 19 kDa phosphorylated form of CaPR-10 has 12.4 fold higher RNase activity compared to the 18 kDa unphosphorylated form in degradation assays using yeast total RNA as substrate. Contrary, as reported by Pungartnik et al. (2009), phosphorylation did not influence the RNase activity of the cocoa TcPR-10 protein or its substrate specificity and both phosphorylated and non-phosphorylated TcPR-10 degraded plant RNA.

4.4 Phosphorylation assay

It has been established that PR-10 proteins are phosphorylated and phosphorylation site were found in hot pepper CaPR-10 (Park et al., 2004), cacao TcPR-10 (Pungartnik et al., 2009), potato PR-10a (Despres et al., 1995), peanut AhPR-10 (Parthibane et al., 2012), white lupin LaPR-10 (Bantignies et al., 2000) and Jatropha JcPR-10a (Agarwal et al., 2013).

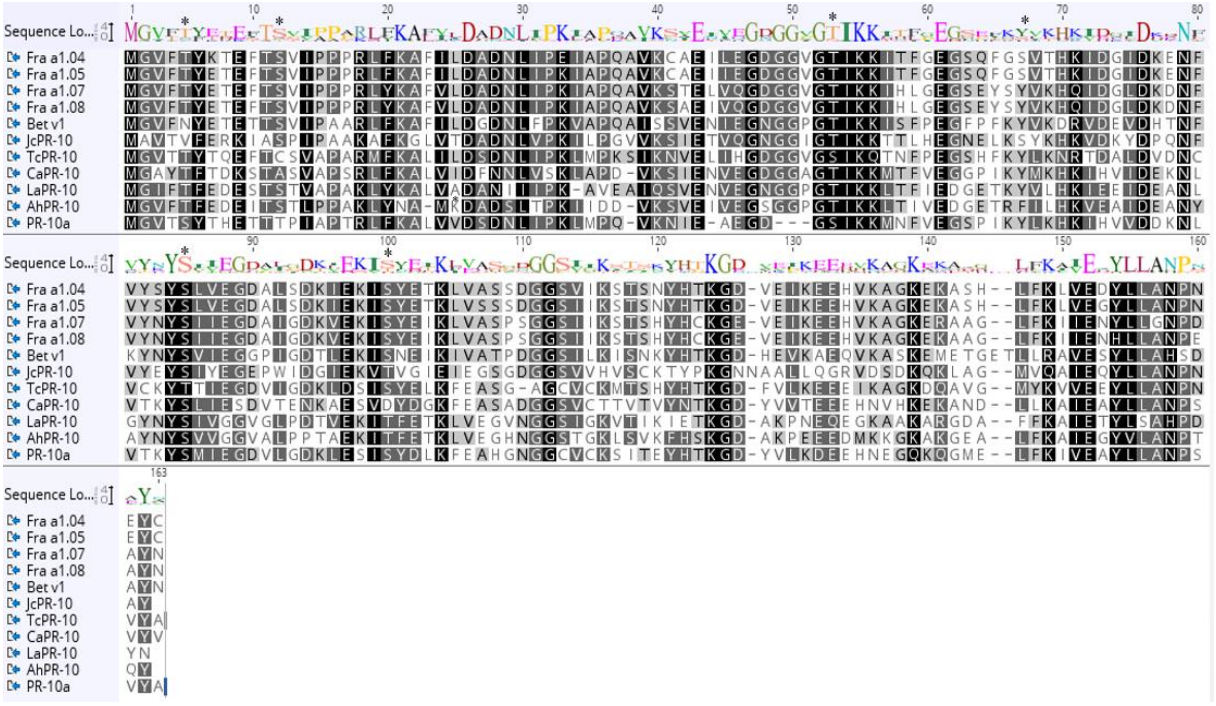

Figure 39. Protein sequences comparison of Fra a 1.04, Fra a 1.05, Fra a 1.07 and Fra a 1.08 with those of Bet v 1, JcPR-10 (biofuel crop), TcPR-10 (cacao), CaPR-10 (hot pepper), LaPR-10 (white lupin), AhPR-10 (peanut) and PR-10a (potato). Amino acids Thr_5, Ser_{12}, Thr_{53}, Tyr_{67}, Ser_{85} and Ser_{100} are predicted to be phosphorylated for Fra a 1 proteins. Phosphorylation sites are marked with an asterisk.

In this study it was shown that Fra a 1.04, Fra a 1.05, Fra a 1.07 and Fra a 1.08 are phosphorylated. Alignment of amino acid sequences of Fra a 1.04, Fra a 1.05, Fra a 1.07 and Fra a 1.08 with Bet v 1 (Basharat, 2016) and the six above-cited PR-10 proteins from plants was performed **(Figure 39)**. Phosphorylation profile study of the four Fra a 1 proteins using NetPhos 3.1 server (http://www.cbs.dtu.dk/services/NetPhos/) followed by a comparison of the eleven protein sequences revealed six putative phosphorylation sites for the Fra a 1 proteins: three serine residues (Ser_{12}, Ser_{85}, and Ser_{100}), two threonine residues (Thr_5 and Thr_{53}) and one tyrosine residue (Tyr_{67}) **(Figure 39)**.

Such post-translational modifications were demonstrated to play regulatory role in cellular function and gene expression of PR-10. Phosphorylation was revealed to be implicated or not in the RNase activity. Park et al. (2004) pointed out that in the hot pepper CaPR-10 protein, phosphorylation increases its ribonucleolytic activity to cleave invading viral RNAs and that phosphorylation of PR-10 may influence substrate specificity. While working on pepper PR-10, Choi et al. (2012) suggested that increased amount of LRR1 (Leucine-rich repeat protein), which recognize pathogen attack and activate defense genes, enhanced both phosphorylation and RNase activity of the pepper PR-10 proteins. Besides, Agarwal et al. (2013) indicated the presence of nine putative phosphorylation sites in JcPR-10a suggesting that it might regulate its activity. In the cocoa, Pungartnik et al. (2009) established that TcPR-10 phosphorylation has no influence on the RNase activity and that both phosphorylated and non-phosphorylated TcPR-10 degraded plant RNA while in *Arachis hypogaea*, PR-10 proteins were established to be phosphorylated but their role in RNase activity was not shown (Jain et al., 2006). In this work, after *Verticillium* infection, Fra a 1.06 showed a higher induction in roots than for Fra a 1.04, Fra a 1.05, Fra a 1.07 and Fra a 1.08 which suggested that the non-phosphorylated form of Fra a 1.06 increased the ribonuclease activity in roots in order to cleave invading fungal RNA of *Verticillium.*

In the current study, the phosphorylation of the recombinant Fra a 1 proteins was tested using different methods such as Western blot (Goodman et al., 2004; Sun et al., 2007), Pro-Q Diamond gel stain (Choi et al., 2012; Goodman et al., 2004; Jain et al., 2006) and a chemiluminescence assay (Park et al., 2004; Sun et al., 2007). It was shown that Fra a 1.04, Fra a 1.05, Fra a 1.07 and Fra a 1.08 are phosphorylated, except Fra a 1.06. Furthermore, the 4 phosphorylated Fra a 1.04, Fra a 1. 05, Fra a 1.07 and Fra a 1.08 proteins displayed no RNase activity whereas the unique Fra a 1 which was able to degrade RNA was the non-phosphorylated Fra a 1.06. After dephosphorylation of Fra a 1.04, Fra a 1.05, Fra a 1.07 and Fra a 1.08 the four recombinant proteins acquired RNase activity. Park et al. (2004) findings

proved that non-phosphorylated CaPR-10 also possessed RNase activity but this activity was much lower compared to the phosphorylated form.

Furthermore, the optimization of the conditions of phosphatase treatment is important when aiming to produce a completely dephosphorylated protein which was a fundamental process to detect RNase activity. Dephosphorylation depends on the phosphatase and its concentration as well as on sample preparation including buffer composition. Husberg et al. (2012) conducted *in vitro* dephosphorylation of the cardiac muscle proteome using different phosphatases (alkaline phosphatase (AP), lambda phosphatase (λ –PP) and protein phosphatases (PP1 and PP2A)) and found that, compared to PP1 and PP2A, AP and λ –PP displayed reduced substrate specificity. The two phosphatases AP and λ –PP were used in this study to dephosphorylate Fra a 1.04, Fra a 1.05, Fra a 1.07 and Fra a 1.08 and showed a very satisfying results.

As the recombinant proteins Fra a 1.04, Fra a 1.05, Fra a 1.07 and Fra a 1.08, which were produced in *E. coli*, were shown to be phosphorylated even in the absence of the kinase source, it seemed that bacteria such as *E.coli* have exploited the potential of protein phosphorylation. It was supposed for a long time that serine, threonine and tyrosine phosphorylation occurs only in Eukaryota (Bakal and Davies, 2000) whereas bacteria have histidine and aspartate kinases (Stock et al., 1990). In general, in a typical two-component system in bacteria, the autophosphorylated histidine kinase transfers the acquired phosphate to an aspartate residue which is the response regulator to a variety of environmental stimuli (Hoch, 2000; Podgornaia and Laub, 2013).

Recently, the capacity of bacterial serine/threonine and tyrosine kinases to phosphorylate multiple substrates has been deeply studied. Kinase phosphorylation sites were detected on many bacterial proteins such as those from *Bacillus subtilis* (Pietack et al., 2010; Ravikumar et al., 2014), *Mycobacterium tuberculosis* (Grundner et al., 2005; Baer et al., 2014) and *E. coli* (Grangeasse et al., 2003; Macek et al., 2008). Using high accuracy mass spectrometry in combination with phosphopeptide enrichment, Macek et al. (2008) reported 81 phosphorylation sites on 79 *E. coli* K12 proteins, with distribution of Ser/Thr/Tyr phosphorylation sites of 67.9%/ 23.5% /8.6%, respectively.

The issue of how and where Fra a 1 proteins are phosphorylated is challenging and remains ambiguous. Since this report is the first work proving the phosphorylation of Fra a 1 proteins, the determination of the 3D structure of the Fra a 1 proteins could help to get insight into such intriguing questions.

4.5 *Verticillium* infection of strawberry *in vitro* plants

4.5.1 *Fra a 1* gene expression

In vitro plants of *F.* × *ananassa* were infected with *Verticillium* and *Fra a 1* gene expression was examined using qPCR. In the present study, *Fra a 1* genes were demonstrated to be clearly induced by *Verticillium* infection. The inoculated leaves, stems and roots at 1, 5, 10, 20 and 30 dpi revealed strong expression levels with a wide range of response. *Fra a 1.03, genes07081, 07078* and *07083* were significantly induced in leaves. The peak of expression in leaves was attained during the last 10 days of infection (20-30 dpi) for *Fra a 1.03* and *gene07081,* and towards the first five days (1-5 dpi) for *genes07078* and *07083*. Similarly, PR-2, PR-3 and PR-4 expression was also induced in leaf apoplast of oilseed rape infected with *V. longisporum* (Floerl et al., 2008). In lettuce infected with *V. dahlaie*, qPCR indicated that putative cysteine protease (LsCP2) was expressed in senescent leaves at significantly higher levels in symptomatic leaves while using suppression subtractive hybridization, PR-3 and PR-5 genes were identified and expressed only in symptomatic leaves harvested 3 weeks after infection (Klosterman et al., 2011).

Transcription of *Fra a 1.06, Fra a 1.08* and *gene05185* was exclusively induced in roots. The same expression pattern was observed for *gene05185* which was exclusively transcribed in roots with the highest expression value within the 21 *Fra a 1* isoforms. Likewise, a significant upregulation was detected also in roots for *Fra a 1.02, genes 07076, 07063, 04962* and *07077*. The upregulation was mainly observed during the first 10 days post inoculation. Although plant roots are in close contact with a vast variety of surrounding microorganisms, these interactions are still poorly analyzed (Okubara and Paulitz, 2005; Millet et al., 2010). For a better understanding of the interaction between roots and vascular pathogens, *Arabidopsis* has been established as a host model for *V. dahliae* (Tjamos et al., 2005; Pantelides et al., 2010) and *V. longisporum* (Johansson et al., 2006). In *V. longisporum*-infected *A. thaliana* roots, detailed time course experiments revealed strongest induction of the selected genes at 8 dpi (Iven et al., 2012). In this study, a peak of expression in roots was reached around 10 dpi suggesting that the fungus is progressing through the time and then a decline of expression towards 30 dpi was revealed. This decrease is probably due to plant resistant responses followed by fungus elimination around 30 dpi.

It has been suggested that pectinaceous gels, formed in xylem vessels of plants infected with *Fusarium oxysporum*, are a substantial defense system, blocking pathogen colonization of the upper parts of stem and leaves and favoring only roots infection (Vander Molen et al., 1977).

Thus, according to this study and all the cited results, the high level of PR-10 proteins in roots could be proposed to be part of a constitutive defense mechanism in the plant organ that is most exposed to environmental stress.

Interestingly, *Fra a 1.04*, *Fra a 1.01*, *genes07085*, *11094* and *07087* isoforms were considerably expressed in both roots (early stage) and leaves (late stage) and hence correlates with the time course of infestation. This finding is also in accordance with the induction of *PR-10c* in leaves and roots of birch in response to oxidative stress (Koistinen et al., 2002b). Overall, the expression levels of the 17 above-cited *Fra a 1* genes revealed that leaves and roots differ considerably in executing their temporal defense responses.

A striking expression was observed for *genes32299* and *07084* which were induced in all three tissues (leaves, roots and stems) simultaneously. Several studies showed that *Verticillium* species enter the xylem vessels of the root and start sporulating after 2 to 5 dpi followed by a colonization of stem vessels few days later (Gold and Robb, 1995; Chen et al., 2004). In *Verticillium*-infected hop stems (Cregeen et al., 2015), the fungal biomass increased through the time in accordance with the transcript level of *gene07084* in stems while the expression of *gene32299* decreased steadily until 30 dpi. Also in stems of tomato (*Solanum lycopersicum*) infected with *V. dahliae*, the expression pattern of two *PR*-genes *Ve1* and *Ve2* revealed that both genes exhibit high transcript levels 10 - 12 dpi in the susceptible isolines (Fradin et al., 2009). *In Verticillium*-infected tomatoes, the host compatibility (susceptibility) or incompatibility (resistance) with *Verticillium* appeared to be determined in stems (Street et al., 1986; Robb et al., 1987). Moreover, during *Verticillium* colonization in this tissue, fungal elimination predominates because the resistant cultivar plant is able to activate defense responses faster (Robb et al., 1991; Gold and Robb, 1995) while in the susceptible cultivar the pathogen escapes from this elimination and spreads inside the plants vascular system (Chen et al., 2004; van Esse et al., 2009).

In strawberry, Shaw et al. (2010) suggested that the global performance of different genotypes in the presence of *V. dahliae* can be enhanced by both resistance and tolerance, and that tolerance may be less stable over the course of a season. Contrary to plant disease resistance which protect plants from pathogen attack, plant disease tolerance was proposed by Robb et al. (2007) as a specific phenomenon that includes down-regulation of genes associated with development of symptoms. Most of the 41 evaluated strawberry genotypes from the University of California breeding program were identified as highly susceptible to *V. dahliae* (Shaw et al., 1996) such as “Joliette” Strawberry (Chalavi and Tabaeizadeh, 2003). However, Shaw et al. (2010) demonstrated later that eleven strawberry (*F.* × *ananassa*) genotypes are

designated as resistant to *Verticillium* wilt. Strawberry resistance to a variety of pathogens has been bred into commercial varieties as a polygenic trait and has been reported to be quantitatively inherited (Żebrowska et al., 2006). The absence of symptoms in the presence of high levels of *V. dahliae* inoculum might result from a reduced incidence of systemic infection and from limited symptom expression despite pathogen entry into the xylem. The involvement of *PR*-genes in the *V. dahliae* resistant genotypes has not yet been well analyzed.

In the present work, after *Verticillium* inoculation, a visual assessment of the foliar symptoms of *F.* × *ananassa in vitro* plants was performed. The scale employed for the visual assessment of the disease was based on the percentage of symptomatic leaf area. Using a scale of 6-points ranking scale (0-5), the extension of the wilt symptoms on the leaf surface was recognized only towards 20 dpi corresponding to scale 2 while a value of 5 was attributed for plants at 30 dpi. The strawberry plants seemed to be "healthy" during the first 20 dpi (class 0) since no visible foliar wilting symptoms were observed. The diffusion of strawberry leaf symptoms began with class 2 equivalent to 10-25% of symptomatic leaf area at 20 dpi and only within 10 days the fungus was able to colonize almost the totality (75-100%) of the leaf surface producing necrotic leaves which were sometimes stunted in stature. This visual finding is in accordance with *Fra a 1* gene expression in leaves which revealed that the highest level of induction was detected at late stages (20 - 30 dpi). The disease severity was spectacular beyond 36 dpi as the *in vitro* plants faded completely resulting in wilting and plant death which explained consequently why plant material for gene expression analysis was not more recoverable after this time point. This finding is in agreement with the results of Żebrowska (2011), who found out that the response to the infection caused by *Verticillium dahliae* in two micropropagated *F.* × *ananassa* cultivars become evident only after 15 dpi and, differently to our results, the highest percentage of totally chlorotic microplants was obtained at 75 dpi for both subclones 'Filon' and 'Teresa'. While these findings clearly demonstrated an initiation of transcriptional responses in leaves, roots and stems during invasion by the *Verticillium* fungus for nineteen of the *Fra a 1* genes, *Fra a 1.05* and *Fra a 1.07* seemed to escape from the fungus colonization since no induction was detected in any tissue for both genes.

Studying defense gene expression implies the use of the suitable host-pathogen model systems and the culture conditions. In strawberry, many researchers have applied an *in vitro* screening system to obtain plants resistant or tolerant to *Rhizoctonia fragariae* and *Botrytis cinerea* (Orlando et al., 1997), *Colletotrichum acutatum* (Damiano et al., 1997), *Fusarium oxysporum* (Toyoda et al., 1991), *Phytophthora cactorum* and *Phytophthora fragariae* (Maas et al., 1993) and *Verticillium dahliae* (Sowik et al., 2001). Jain (2001) underlined that *in vitro*

selection, the technique used in this work, is a useful tool in identifying plants resistant or tolerant to stresses produced by phytotoxins from pathogens, cold temperature and salt toxicity. Sowik et al. (2008) stated in a later work, that susceptibility to infection by *Verticillium dahliae* in strawberry cultivars and breeding lines under *in vitro* conditions was similar to their response to infection in the field conditions.

Besides, a comparison of both qPCR analyses with and without *Verticillium* infection revealed that the transcript levels of the PR-10 proteins could be quite different or similar for the *in vitro* and soil grown strawberry plants: fourteen *Fra a 1* genes showed the highest transcript level and induction in the same tissue for both qPCR and only seven *Fra a 1* genes showed a difference in the highest expressed tissue (*genes07081, 07078, 07077, 07087, Fra a 1.02, Fra a 1.05 and Fra a 1.07*).

4.5.2 Tissues analysis by LC-UV-ESI-MS

4.5.2.1 Flavan-3-ols

In the present work, flavan-3-ols such as catechin and epicatechin were shown to accumulate in infected stems and roots especially at late stages after infection by the fungus *Verticillium dahliae*. This result suggested that the increased production of catechin and epicatechin during *Verticillium* inoculation imply a protective role in order to suppress fungus infection. Yamamoto et al. (2000) reported that (+)-catechin, which already pre-exsist in strawberry leaves, inhibited *Alternaria alternata*, and that the induction of the resistance response in the strawberry leaf to this fungus provoke the accumulation of (+)-catechin. Moreover, Puhl and Treutter (2008) stated that the accumulation of catechin-derived procyanidins was fundamental to inhibit the growth of *Botrytis cinerea* in immature strawberry fruits. Moreover, in *Centaurea maculosa* or spotted knapweed, (±)-catechin has been reported to be released from its roots (Blair et al., 2005) showing an inhibitory effect on soil microbial activity (Inderjit et al., 2009). Likewise, Veluri et al. (2004) reported an antimicrobial and antifungal effect of (+)-catechin to some soil strains (e.g., *Xanthomonas campestris, Agrobacterium radiobacter, Erwinia carotovora,* and *Erwinia amylovora*). Such activities of (+)-catechin on certain soil microbial isolates were not detected for the (-)-enantiomer of catechin, which was revealed to be phytotoxic (Bais et al., 2002).

Besides, it has been reported that epicatechin was able to inhibit lipoxygenase from *Colletotrichum gloeosporioides*, which inactivates antifungal dienes (1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene) formed during the ripening of avocado (Prusky et al., 1985). Later, Wattad et al. (1994) reported the infection-inhibiting activity of epicatechin against the

same fungus in the skin of avocado fruits and suggested that the flavan-3-ol, which is present in unripe fruit at high concentration, was may be involved in the resistance of avocado fruits by inhibiting the pectate lyase activity of *Colletotrichum gloeosporioides*. In addition, flavan-3-ols, including catechin and epicatechin, have been reported to show even better antioxidative and radical-scavenging activities than those of vitamins, ascorbic acid, α-tocopherol and other phenolics (Feucht and Treutter, 1999).

4.5.2.2 Caffeic acid

Contrary to the caffeic acid levels which significantly decreased in tomato roots upon *Arbuscular mycorrhizal* colonization (López-Ráez et al., 2010), levels of caffeic acid were induced in *F.* × *ananassa* infected roots particularly after 30 dpi in this study. Caffeic acid which is known to play a role in plant defense reactions, showed a strong *in vitro* antimicrobial activity against numerous fungus including *V. dahliae*. This antioxidant compound was shown to be increased in *Verticillium*-infected cotton plants (Wiese and Devay, 1970) and also in potato with a differential accumulation in roots and stems (El-Bebany et al., 2013). In pepper roots, Veloso et al. (2016) pointed out that the fungus *Fusarium oxysporum* stimulated the biosynthesis of caffeic acid and primed that of chlorogenic acid.

4.5.2.3 Citric acid

Similarly, accumulation of citric acid was induced at late stage in infected roots (30 dpi) supporting the hypothesis that this antioxidant affects growth and health of the plant host. Liu et al. (2014) reported that root secretion of citric acid was enhanced in *Fusarium oxysporum f. sp. cucumerinum*-infected cucumber plants and that citric acid acts as a chemoattractant in this process. Analysis of tomato, cucumber and sweet pepper root exudates from plants grown under gnotobiotic conditions showed that the exudates contained higher total amounts of organic acids such as citric acids than of sugars (Kamilova et al., 2006b). Kamilova et al. (2006a) also demonstrated that the root exudate composition is influenced by the rhizosphere microflora itself and that the application of the bacterial biocontrol strain *Pseudomonas fluorescens* on tomato roots resulted in increased levels of total citric acid, whereas the amount of succinic acid decreased.

4.5.2.4 Tryptophan

In this work, the levels of tryptophan, another strawberry plant constituent, in infected tissues exceeded the level in control plants mainly after 1 dpi in leaves and stems, and 20-30 dpi in

roots. It was obvious that strawberry have evolved various defense mechanisms against biotic stress including the production of metabolites that act as defense compounds. In *F.* × *ananassa* leaves inoculated with the angular leaf spot bacterium, *Xanthomonas fragariae*, the level of tryptophan as precursor of aromatic secondary metabolites was increased (Kim et al., 2016). It should also be noted that the tryptophan pathway was revealed to be involved in the defense responses against pathogenic infection in rice (Ishihara et al., 2008) as well as in *Arabidopsis* root (Iven et al., 2012).

4.5.2.5 Glutathione

The accumulation of glutathione upon fungal infection was exclusively observed in this work in infected leaves after 1 dpi revealing a quick response to *Verticillium* infection. Many researchers suggested that glutathione may play a role in mediating the response of plant cells to biological as well as physical stresses. Ball et al. (2004) emphasized that glutathione, as a major cellular antioxidant, may have an influence on stress defense in plants and that, according to the physiological state of the plant, both glutathione metabolism and levels can be considered to influence cellular defenses. In most plant tissues, glutathione is predominantly present in its reduced form (GSH) but the oxidized disulphide form of glutathione (GSSG) can also be detected. Due to the thiol group, GSH and its homologous are considered as essential components in plant cell by participating in the elimination of reactive oxygen species (ROS) via thiol-disulphide redox reactions and in detoxification of various xenobiotics by conjugation reactions (Mauch and Dudler, 1993, Noctor et al., 1998). The reduced form of glutathione is known to be highly involved in the stress response to oxidative and abiotic stress (Noctor et al., 1998) which rapidly accumulated after fungal attack. Winterbourn and Metodiewa (1999) confirmed also that the participation of GSH in antioxidative defense reactions and detoxification is the principal role of GSH in plant tissues either by direct reactions with reactive oxygen species or through the ascorbate-GSH cycle. Moreover, Edwards et al. (1991) underlined that both reduced glutathione and oxidized glutathione elicited the phytoalexin response in cell-suspension cultures of bean but had no effect in those of alfalfa. In a later work, Gönner and Schlösser (1993) analyzed endogenous GSH levels in oat leaves infected by the fungal pathogen *Drechslera*. A decline in GSH content was observed approximately 2 days post-inoculation indicating an increased demand for detoxification of reactive oxygen species in the early stages of pathogenesis. Similarly, except an accumulation peak at 1 dpi in infected leaves, lower levels of GSH were detected in this work in infected tissues in comparison with the controls leading to conclude that decrease

of GSH levels showed a progressively diminishing capacity of the leaf tissue to resist oxidative stress and to repair damage by regenerating protein sulphydryl groups. In a subsequent study, May et al. (1996) reported also changes in GSH content in tomato leaves carrying either the resistance genes *Cf-2* or *Cf-9* against the fungal pathogen *Cladosporium fulvum*. Results revealed that total GSH levels began to increase 2 to 8 hours after the injection of elicitor into the *Cf-2* and *Cf-9* leaves and that a large proportion (87%) of this accumulation was observed in the GSSG form. Substantial increase in foliar GSH levels was also observed in oat lines (*Avena sativa*) 24 hours after infection with *B. graminis f. sp. Avenae* (Vanacker et al., 1999) and in resistant tobacco (*Nicotiana tabacum L cv. Xanthi-nc*) plants infected with Tobacco mosaic virus (TMV) (Fodor et al., 1997).

4.5.2.6 Phytohormones

Phytohormones such as abscisic acid (ABA), salicylic acid (SA), gibberellic acid (GA), indole acetic acid (IAA) and jasmonic acid (JA) are major signaling molecules in plants during stress response and their levels have been investigated in *Verticillium* infected *F.* × *ananassa in vitro* plants. A critical step in plant defense is the perception of the stress in order to respond in a quick and efficient manner. The sensing of biotic and abiotic stress induces signaling cascades that activate ion channels, kinase and reactive oxygen species (ROS) and lead also to the accumulation of phytohormones.

In the present study, the ABA concentration in infected tissues exceeded the level in controls in leaves at 5 dpi and in stems during the first 10 dpi with a peak at 30 days post inoculation revealing a crucial role in defense response especially after attack by pathogens. Such findings suggested that the ABA signaling pathway is a candidate for the control of systemic colonization by *V. dahliae* in strawberry. While Flors et al. (2008) and Ton et al. (2009) established that ABA has a prominent role during pathogen infection, Cramer et al. (2011) considered that this phytohormone is just involved in the perception of environmental stresses, particularly osmotic stresses. Besides, a study conducted by Schmidt et al. (2008) revealed that the concentration of ABA increases in sugar beet leaves during *Cercospora beticola* fungus infection and that elevated ABA concentrations were detected during colonization at 3 to 9 dpi. Many researchers considered that the role of ABA in disease resistance depends on a numerous factors, such as the attacking pathogen, its specific way of gaining entry into the host, the timing of the defense response, and the type of plant tissue that is under attack. This makes ABA a controversial phytohormone with multiple effects

depending on the environmental conditions and its exact role in plant pathogen interaction is still a matter of debate.

Another major hormone produced by plants is gibberellic acid (GA) which is also known to participate mainly in the abiotic stress (Colebrook et al., 2014). In the 19th century, GA was identified as being responsible for the excessive growth of rice seeds infected with the fungal pathogen *Gibberella fujikuroi* in Japan and only recently GA has been considered to be implicated in plant responses to pathogen attack and the role of GA in plant immunity has been studied. Zhu et al. (2005) established that the outer capsid protein P2 of the rice dwarf virus (RDV) interacts with plant ent-kaurene oxidases, affecting the production of GA and that the RDV infected rice plants showed a significant reduction in GA. Those finding are consistent with the present study which revealed that GA accumulated in comparison with non-infected tissues at 5 dpi in leaves and stems and decreased during all the following infection days while in infected roots no significant GA level was detected during the 30 days of *Verticillium* infection confirming that GA is part of the hormonal system used by plants in defense signaling pathways during pathogen colonization. Aubé and Sackston (1965) investigated the production of gibberellin by *Verticillium* species using dwarf mutant seedlings of *Zea mays*. From the 114 isolates of *Verticillium* grown on potato-carrot dextrose medium for 15 to 25 days, only 27 isolates produced "gibberellins" and 12 were identified as *V. dahliae*. The same group demonstrated that treatment of sunflower with gibberellic acid was able to prevent the stunting of plants seedlings inoculated with *Verticillium*, indicating that it may result from interference with normal gibberellin activity. Many researchers reported that GA promoted plant growth by stimulating degradation of negative regulators of growth called DELLA proteins. In *Arabidopsis*, Navarro et al. (2008) reported that those plant growth repressors, which act as negative regulators of GA signaling, control plant immune responses by modulating SA and JA dependent defense responses. The *Arabidopsis* quadruple-Della mutant that lacks four DELLA genes (*rgl1-1, rgl2-1, gai-t6 and rga-t2*) was very susceptible to the fungal necrotrophic fungus *Botrytis cinerea* but more resistant to the biotrophic pathogens Pto DC3000 and *Hyaloperonospora arabidopsis* (Navarro et al., 2008). Also, necrotrophic pathogens such as *G. fujikuroi* might synthetize GA as a virulence factor to degrade DELLAs and develop host susceptibility, not only by interfering with SA/JA-dependent defenses but also by tilting the ROS-controlled balance towards death. Thus, the mechanism of action of GA in response to pathogen attack is still a topic of debate which needs to be further studied especially in strawberry.

While ABA and GA are predominantly involved in abiotic stress, SA and JA are more responsible for the plant's reaction to biotic stress. In this study, SA accumulated in comparison with non-infected strawberry plants exclusively in leaves at 5 dpi whereas JA exceeded the levels in the same infected tissue at 5 and 30 dpi. In stems, JA accumulated, in comparison with non-infected plants during the first 10 days post *Verticillium* infection and SA during the first 5 days while in infected roots only JA was identified at late stages (20-30 dpi). These results clearly demonstrate that both SA and JA defense signaling pathways are activated in strawberry plants during *Verticillium* colonization. Also in strawberry, Amil-Ruiz et al. (2016) measured the contents of SA and JA in *F.* × *ananassa* cv. Camarosa plantlets infected with *Colletotrichum acutatum*: the most significant SA and JA concentration in aerial tissues was also obtained after 5 dpi. This research group found out that a number of known components of both SA and JA-dependent defense pathways such as *FaPR1-1* and *FaGST1* (oxidative stress-responsive defense genes) are not significantly accumulated in strawberry during interaction with *C. acutatum* suggesting that this pathogen might be interfering with only certain branches of both SA and JA-dependent defense pathways in strawberry. This result supported also the hypothesis of a putative molecular strategy used by this pathogen to overcome strawberry plant defense. Song et al. (2003) reported that plants have evolved numerous complex defense mechanisms to survive fungal and microbial pathogen attacks and that hypersensitivity, which is associated with the accumulation of SA and certain PR proteins, is the most powerful mode of plants resistance against pathogen attack. High levels of SA were also found in the xylem sap from root and hypocotyl of *Verticillium longisporum*-infected *Brassica napus* and were correlated to the degree of stunting and the amount of pathogen DNA (Ratzinger et al., 2009).

The plant hormone SA is generally involved in the activation of defense responses against biotrophic and hemibiotrophic pathogens as well as the establishment of systemic acquired resistance (SAR, Grant and Lamb, 2006). This phytohormone was also proved to be implicated in the protection of plant tissues against *Verticillium* toxins (Zhen and Li, 2004).

In contrast, JA is usually associated with defense against necrotrophic pathogens and herbivorous insects (Glazebrook, 2005). Plant pathogenic *Verticillium* species are regarded as hemibiotrophs with a necrotrophic phase during the late stages of infection. Therefore and as confirmed in this work, the plant SA defense signaling pathway is activated during the first biotrophic stage of *Verticillium* infection while the JA signaling pathway is activated in leaves, stems and roots also at the late phases. Briefly, the success of pathogen infection and growth depends on the balance of how fast the pathogen escapes the plant defenses and how

quick the plant adapts the defense response. Besides, possible manipulations of the host's hormone status by the pathogen for its own benefit should be regarded.

Because of the availability of suitable mutants in *Arabidopsis thaliana*, most of the experiments on phytohormones were conducted in this plant. Dong (2004) established that one of the important regulatory components of SA signaling is the non-expressor of PR genes 1 (NPR1) which interacts with TGA (TGACG motif-binding protein family) transcription factors that are involved in the activation of SA-responsive PR genes. Downstream of NPR1, several WRKY transcription factors (containing one or two WRKY protein domains) play important roles in the regulation of SA-dependent defense responses in plants (Wang et al., 2006; Eulgem and Somssich, 2007).

Interestingly, Truman et al. (2007) observed a rapid accumulation of JA namely during the first 11 hours post inoculation (hpi), but not of SA, in phloem exudates of leaves challenged with an avirulent strain of *Pseudomonas syringae* and increased expression levels of JA biosynthetic genes suggesting that JA could act as a mobile signal in *Arabidopsis* pathogen immunity. Likewise, other researchers established that concentrations of JA increase in response to pathogen infection or tissue damage and that the expression of defense related genes is also induced (Lorenzo and Solano, 2005; Wasternack, 2007). JA-responsive gene expression is mainly mediated by a transcription factor Jasmonate insensitive 1/MYC2 (JIN1/MYC2) (Lorenzo et al., 2004), several members of the Apetala2/Ethylene-responsive factor (AP2/ERF) family (Mizoi et al., 2012), an F-box protein coronatine insensitive 1 (COI1) (Xie et al., 1998), a plant defensing 1.2 (PDF 1.2) (Brown et al., 2003) and a repressor protein Jasmonate-ZIM-Domain (JAZ) (Chini et al., 2007). Recently, a GbWRKY1 transcription factor has been shown to be a critical regulator mediating the plant defense to development transition during *Verticillium dahliae* infection by activating JAZ1 expression in cotton (Li et al., 2014).

Furthermore, the interaction between defense signaling pathways of SA and JA is an important mechanism for regulating defense responses against various types of pathogens. The *Arabidopsis* WRKY70 (transcription factor) (Li et al., 2004; Li et al., 2006), MPK4 (Mitogen activated protein kinase 4) (Brodersen et al., 2006) and GRX480 (glutaredoxin) (Meyer et al., 2008) have been found to regulate the antagonistic interaction between SA and JA-mediated defenses. In this work, JA levels in roots increased at late stages and those of SA were insignificant at this phase. This finding suggests an antagonistic relationship between the two pathways in strawberry. Also evidence of synergistic interactions between SA and JA defense pathways has been reported. Activation of the expression of some defense related

genes (Mur et al., 2006) and the transcription factor WRKY62 has been shown to be induced by MeJA and SA synergistically (Mao et al., 2007) after pathogen inoculation.

Besides the aforementioned phytohormones, IAA was also analyzed in this work. Significant levels of IAA were detected in all infected samples. In leaves, IAA concentration increased gradually in comparison with the non-infected controls with a peak at 10 dpi and then decreased until 30 dpi. IAA accumulated in comparison with the controls in stems during the first 5 days post *Verticillium* infection and exclusively in roots at 30 dpi. Such results suggest that IAA played also a crucial role in strawberry defense response toward *Verticillium* infection. A significant increase in IAA accumulation was also demonstrated in plant leaves infected with either Xcc (*Arabidopsis–Xanthomonas campestris* pv. Ccampestris) or Pto (*Pseudomonas syringae* pv. tomato) with a maximal level occurring 3 to 4 days after infection (O'Donnell et al., 2003). Moreover, auxin responsive *GH3* genes have been shown to play roles in plant defense responses in *Arabidopsis* and rice. Zhang et al. (2007) reported that, in *Arabidopsis*, *GH3.5* acts as a bifunctional modulator in both SA and auxin signaling during pathogen infection. A similar observation was made in rice where overexpression of *GH3.8* ensued in enhanced disease resistance to the pathogen *Xanthomonas oryzae* pv *oryzae* (Xoo). This gene activated disease resistance in a SA and JA signaling–independent pathway (Ding et al., 2008). Results of this group suggested that *GH3-8* inhibited expansin production, which was induced by IAA and that the suppression of expansin genes may be important for the resistance against *Xoo*. It was also hypothesized that *Xoo*-induced auxin-stimulated local expansin production was may be one of the mechanisms used by this pathogen to infect rice plants. Besides, auxin has been shown to interact antagonistically with SA, thereby increasing susceptibility to biotrophic pathogens (Kazan and Manners, 2009). It has been also demonstrated that transport inhibitor response 1 (TIR1), which is repressed by SA, is an auxin receptor that interacts with Aux/IAA proteins (Kepinski and Leyser, 2005; Dharmasiri et al., 2005).

In summary, the early induction of the accumulation of phytohormones such ABA, SA, GA and JA in leaves at 5 dpi and IAA at 10 dpi and also in stems and roots demonstrate the fast response of the *in vitro F.* × *ananassa* plants after pathogen infection. This work provides evidence of the involvement of phytohormones in plant defense signaling pathways and represents the first study of the hormonal implication in strawberry *F.* × *ananassa* in response to *Verticillium* infection. Nevertheless, the fundamental molecular mechanisms and the role of those compounds toward pathogen attack in strawberry are not fully understood. How precisely the machinery of phytohormone signaling differs between *Verticillium* strains in

strawberry, how does the plant modulate the level of phytohormones in response to numerous simultaneous pathogens attacks including *Verticillium* and which mechanisms may underlie a suitable control of signaling response to *Verticillium* infection, remain challenging questions for future investigations.

4.6 Fra a 1 interaction analysis

Analysis of proteins and their interactions with other molecules is a headstone in understanding their biological functions. As for the work of Langer et al. (2013) and Franz-Oberdorf et al. (2017a), time-resolved DNA switching measurement were also utilized in the present study to determine the physical interaction of five Fra a 1 proteins with a putative protein partner FaAP whose orthologue in apple (*Malus domesctica*) MdAP has been shown to interact with Mal d 1. Furthermore, the FaAP protein has an orthologue protein in the model plant *Arabidopsis thaliana* with a protein sequence identity of 46%. The orthologue protein AtAP (AAD21467.1) is for the present specified as a hypothetical protein. A high-quality binary protein-protein interactome map showed that AtAP putatively interacts with two proteins of the autophagy pathway: Atg8d and Atg8f (Autophagy-related proteins). The Atg8 gene family encodes ubiquitin-like proteins and Atg8 family members are expressed in various tissues, where they participate in multiple cellular processes, such as intracellular membrane trafficking and autophagy (Shpilka et al., 2011).

Moreover, in the current work association and dissociation kinetics were measured in real time allowing the determination of the switching dynamics. Interactions between Fra a 1.04-1.08 proteins and FaAP were successufully obtained. Fra a 1.04, Fra a 1.05, Fra a 1.06, Fra a 1.07 and Fra a 1.08 bind to FaAP with K_D of 27.5 nM, 2,181 nM, 72.05 nM, 51.5 nM, and 53.1 nM, respectively. Likewise, Franz-Oberdorf et al. (2017a) demonstrated that Fra a 1.01, Fra a 1.02 and Fra a 1.03 bind to FaAP with lower K_D values ranging from 4.5 nM to 15 nM. As the expression of Fra a 1 proteins is directly linked to flavonoid biosynthesis (Muñoz et al., 2010) and *Arabidopsis* AtAP (FaAP orthologue protein) is associated with Atg8d/f (Franz-Oberdorf et al., 2017a), the interaction between the Fra a 1.01-08 proteins and FaAP suggested that this binding might be crucial for the biological functions of Fra a 1 proteins. It was also supposed that the protein association facilitates flavonoid transport through membranes and that PR-10 are consequently incorporated in a protein-protein interaction network.

4.7 Fra a 1 expression and RNA-seq

Taking into consideration that *F. vesca* is an excellent model for the cultivated strawberry, RNA-seq analysis was carried out on receptacle and achene tissues of three *F. vesca* botanical forms (Yellow Wonder "YW", Hawaii 4 "HW4" and Reine des Valleés "RdV") at three different ripening stages (green, white and ripe). The transcript levels of Fra a 1 orthologues in *F. vesca* genotypes were represented in a heat map. Botanically, the receptacle of flowering plants is the area at the stem tip that bears flower organs or groups of flowers. Contrary to other plants, this organ can be consumed as fruit in strawberry. In the current work, *Fra a 1.06*, *Fra a 1.02*, *Fra a 1.07*, *07084*, *07087* and *07085* genes were mainly expressed in receptacles of ripe fruits. Courtney et al. (2014) reported that the transcriptome of a developing receptacle is most similar to that of young floral (stages 1 to 4) and that the woodland strawberry homologs of a number of meristem regulators including LOST MERSITEM (FveLOM) and WUSCHEL (FveWUS) genes were identified by their large numbers of connecting edges within the receptacle network. Also in strawberry, Härtl et al. (2017) found out that the transcript levels of the flavonoid genes were particularly abundant in green receptacle and that MYB10 transcripts (encoded by *gene31413* and characterized as positive regulator of anthocyanin biosynthesis in *F.* × *ananassa* and *F. vesca*) were more abundant in ripe receptacle of the white strawberries.

Furthermore, in achenes, which are distributed spirally across the epidermis of the pulp, *Fra a 1.06*, *Fra a 1.02*, *Fra a 1.01*, *Fra a 1.03*, *07085* and *07077* genes were equally expressed throughout the three fruit ripening stages. Developing fruit of the three genotypes (green, white and ripe) showed a distinctive pattern of *Fra a 1* gene expression in the two tissues. In receptacles, *Fra a 1* gene expression was generally higher in ripe fruit than in white and green fruit while in achenes *Fra a 1* transcripts were less abundant in green immature fruit. In *F. vesca*, Courtney et al. (2014) emphasized that *FveAP2* (*gene23876*) was the unique expressed gene in achenes (seeds). Besides, Härtl et al. (2017) reported that key polyphenol genes in *F. vesca* showed considerably lower transcript levels in the receptacle and achenes of white genotypes, compared to the red genotype and that their expression profile suggested a coordinated transcriptional regulation in both tissues during fruit ripening.

4.8 Basophil activation test (BAT)

Although Fra a 1.01-1.08 have protein sequence similarities of 69.4 to 99.4% **(Table 8)**, the basophil activation mediated by the eight Fra a 1 proteins differed considerably. Contrary to Fra a 1.02 and Fra a 1.03, which seem to be major allergenic proteins in strawberry with

respectively 66 and 73% of total basophils, Fra a 1.01 and Fra a 1.04-1.08 showed a low capacity to activate basophils (15% to 40% of total basophils). The BAT is considered as a valuable tool for the determination of sensitization to food and is routinely used for allergy diagnosis (Erdmann et al., 2005; Sabato et al., 2011; Franz-Oberdorf et al., 2017b). Erdmann et al. (2005) reported that sensitivity of the BAT for PR-10 proteins such as Mal d 1 (apple), Dau c 1 (carrot) and Api g 1 (celery) was 75, 65 and 75% with corresponding specificities of 68, 100 and 77%. The major birch pollen allergen Bet v 1 has been reported to be responsible for IgE binding in more than 95% of birch pollen allergic patients (Breiteneder et al., 1989) and is therefore the main cause of type I allergies (Faber et al., 1996). Thus, reduced IgE binding was suggested to be the main cause of low allergenic potential of some PR-10 proteins despite the high sequence similarities. Kofler et al. (2012) proposed that the iDXC (inner deoxycholate) binding site in high IgE binding isoforms such as Bet v 1a is considerably constricted by the characteristic Phe_{31} as compared to low IgE binding Bet v 1 isoforms or Pru av 1 with Val_{31}. At this position, Fra a 1.01-08 proteins contain all an isoleucine residue (Ile_{31}) **(Figure 42)**. In another study, antibodies were raised against Bet v 1 derived peptides and were used to compete with allergic patients' IgE binding to Bet v 1 to search for sequences involved in IgE recognition. Strong inhibitions of patients' IgE binding to Bet v 1 (52–75%) were obtained with two peptides comprising amino acids P2 (30–59) and amino acids P6 (74–104), of Bet v 1 (Gieras et al., 2011). Surprisingly, Fra a 1.02, Fra a 1.04 and Fra a 1.05 have identical P2 sequences so this putative binding site cannot explain the contrasting immunological effect. The data clearly proved that even small changes in the Fra a 1 protein sequences can change orthologues in their allergenic characteristics. Indeed, birch pollen allergic patients may react differently to different fruit varieties since different isoforms of allergens can be detected within a single fruit (Holm et al., 2001; Reuter et al., 2005; Son et al., 1999). The difference in allergenicity can be due either to minor sequence variations between the isoforms leading to variation in IgE binding or to differences in expression levels in the tested tissue (Ferreira et al., 1996). Many investigations and researches have proved that very small changes in amino acid sequence of the birch pollen orthologues can change a protein from being hyperallergenic to hypoallergenic (Spangfort et al., 1996; Ferreira et al., 1996). Moreover, Ferreira et al. (1998) demonstrated that point mutations in six amino acids were sufficient to change Bet v 1 from an allergenic protein to a hypoallergenic protein and suggested that Fra a 2 might be also a hyperallergenic isoform.

5 Conclusion

According to the sequences provided by the *F. vesca* genome, putative *Fra a 1* isoforms were identified in *F.* × *ananassa*, heterologously expressed in *Escherichia coli* and functionally characterized. Quantitative PCR analyses revealed the expression pattern of 21 *Fra a 1* genes with a predominance of expression in roots. Nineteen *Fra a 1* genes were clearly upregulated after infection of *in vitro* cultivated *F.* × *ananassa* plants with *Verticillium dahliae*. Also, Fra a 1.06 was shown to possess ribonuclease activity while Fra a 1.04, Fra a 1.05, Fra a 1.07 and Fra a 1.08 were phosphorylated and unable to hydrolyze RNA. Association of FaAP with five Fra a 1 proteins was demonstrated by a novel time resolved DNA-switching system. Altogether, the findings of this work increase our knowledge on the biological significance of the strawberry *Fra a 1* genes, which is a key to understand the functions of PR-10 proteins.

6 Literature

Ackaert C, Kofler S, Horejs-Hoeck J, Zulehner N, Asam C, von Grafenstein S, Fuchs J E, Briza P,1 Liedl K R, Bohle B, Ferreira F, Brandstetter H, Oostingh G J, Duschl A (2014) The Impact of Nitration on the Structure and Immunogenicity of the Major Birch Pollen Allergen Bet v 1.0101. *PLoS One* 9:e104520.

Agarwal P, Bhatt V, Singh R, Das M, Sopory SK, Chikara J (2013) Pathogenesis-related gene, JcPR-10a from Jatropha curcas exhibit RNase and antifungal activity. *Molecular Biotechnology* 54:412-425.

Agarwal P, Agarwal PK (2014) Pathogenesis related-10 proteins are small, structurally similar but with diverse role in stress signaling. *Molecular Biology Reports* 41: 599-611.

Amlot PZ, Kemeny DM, Zachary C, Parkes P, Lessof MH (1987) Oral allergy syndrome (OAS) symptoms of IgE mediated hypersensitivity to foods. *Clinical & Experimental Allergy* 17:33-42.

Amil-Ruiz F, Blanco-Portales R, Muñoz-Blanco J, Caballero J L (2011) The strawberry plant defence mechanism:a molecular review. *Plant & Cell Physiology* 52:1873–1903.

Amil-Ruiz F, Garrido-Gala J, Blanco-Portales R, Folta K M, Muñoz-Blanco J, Caballero J L (2013) Identification and validation of reference genes for transcript normalization in strawberry (*Fragaria* × *ananassa*) defense responses. *PLoS One* 8:e70603.

Amil-Ruiz F, Garrido-Gala J, Gadea J, Blanco-Portales R, Muñoz-Mérida A, Trelles O, de Los Santos B, Arroyo FT, Aguado-Puig A, Romero F, Mercado JÁ, Pliego-Alfaro F, Muñoz-Blanco J, Caballero JL (2016) Partial Activation of SA- and JA-Defensive Pathways in Strawberry upon Colletotrichum acutatum Interaction. *Frontiers in Plant Science* 7:1036.

Andrade LB, Oliveira AS, Ribeiro JK, Kiyota S, Vasconcelos IM, de Oliveira JT, de Sales MP (2010) Effects of a novel pathogenesis-related class 10 (PR- 10) protein from Crotalaria pallida Roots with papain inhibitory activity against root-knot nematode Meloidogyne incognita. *Journal of Agricultural and Food Chemistry* 58:4145–4152.

Asanaka M, T Kurimura, R Kobayashi, T Okuda, M Mori, H Yokoi (1988) Anti-HIV activity of ellagitannins. *Fourth International Conference on Immunopharmacology* Osaka, Japan Abstract p.47.

Atalay M, Gordillo G, Roy S, Rovin B, Bagchi D, Bagchi M, Sen CK (2003) Antiangiogenic property of edible berry in a model of hemangioma. *FEBS Letters* 544:252−257.

Atkinson RG, Perry J, Matsui T, Ross GS, MacRae EA (1996) A stress-, pathogenesis-, and allergen-related cDNA in apple fruit is also ripening-related. *New Zealand Journal of Crop and Horticultural Science* 24:103–107.

Aubé C, Sackston WE (1965) Distribution and prevalence of *Verticillium* species producing substances with gibberellin-like biological. *Canadian Journal of Botany* 43:1335-1342.

Ayala-Zavala JF, Wang SY, Wang CY, González-Aguilar GA (2004) Effect of storage temperatures on antioxidant capacity and aroma compounds in strawberry fruit. *LWT - Food Science and Technology* 37:687-695.

Baer CE, Iavarone A T, Alber T, Sassetti CM (2014) Biochemical and spatial coincidence in the provisional Ser/Thr protein kinase interaction network of *Mycobacterium tuberculosis*. *Journal of Biologcal Chem*istry 289: 20422–20433.

Bais HP, Walker TS, Stermitz FR, Hufbauer RA, Vivanco JM (2002) Enantiomeric-dependent phytotoxic and antimicrobial activity of (±)-catechin. A rhizosecreted racemic mixture from spotted knapweed. *Plant Physiology* 128:1173-1179.

Bais HP, Vepachedu R, Lawrence CB, Stermitz FR, Vivanco JM (2003) Molecular and biochemical characterization of an enzyme responsible for the formation of hypericin in St. John's wort (Hypericum perforatum L.). *Journal of Biological Chemistry* 278: 32413–32422.

Bakal CJ, Davies JE (2000) No longer an exclusive club: eukaryotic signalling domains in bacteria. *Trends in Cell Biology* 10:32-38.

Ball L, Accotto GP, Bechtold U, Creissen G, Funck D, Jimenez A, Kular B, Leyland N, Mejia-Carranza J, Reynolds H, Karpinski S, Mullineaux PM (2004) Evidence for a direct link between glutathione biosynthesis and stress defense gene expression in *Arabidopsis*. *Plant Cell* 16: 2448-2462.

Bantignies B, Seguin J, Muzac I, Dedaldechamp F, Gulick P, Ibrahim R (2000) Direct evidence for ribonucleolytic activity of a PR-10-like protein from white lupin roots. *Plant Molecular Biology* 42:871–881.

Basharat Z (2016) Computational characterization and epitope prediction for Bet-v1 like protein of Cannabis sativa. *Peer*: CC BY 4.0.

Basu A, Rhone M, Lyons TJ (2010) Berries: emerging impact on cardiovascular health. *Nutrition Reviews* 68:168–77.

Beck I, Jochner S, Gilles S, McIntyre M, Buters JT, Schmidt-Weber C, Behrendt H, Ring J, Menzel A, Traidl-Hoffmann C (2013) High Environmental Ozone Levels Lead to Enhanced Allergenicity of Birch Pollen. *PLoS One* 8:e80147.

Benítez-Burraco A, Blanco-Portales R, Redondo-Nevado J, Bellido ML, Moyano E, Caballero JL, Muñoz-Blanco J (2003) Cloning and characterization of two ripening-related strawberry (*Fragaria* × *ananassa* cv. Chandler) pectate lyase genes. *Journal of Experimental Botany* 54:633–645.

Beuning LL, Bowen JH, Persson HA, Barraclough D, Bulley S, Macrae EA (2004) Characterisation of Mal d 1-related genes in *Malus*. *Plant Molecular Biology* 55:369-388.

Bhargava UC, Westfall BA, Siehr DJ (1968) Preliminary pharmacology of ellagic acid from *Juglans nigra* (black walnut). *Journal of Pharmaceutical Sciences* 57:1728-1732.

Biesiadka J, Bujacz G, Sikorski MM & Jaskolski M (2002) Crystal structures of two homologous pathogenesis-related proteins from yellow lupine. *Journal of Molecular Biology* 319:1223–1234.

Bircher AJ, Van Melle G, Haller E, Curty B, Frei PC (1994) IgE to food allergens are highly prevalent in patients allergic to pollens, with and without symptoms of food allergy. *Clinical & Experimental Allergy* 24:367–374.

Bishop CD, Cooper RM (1983) An ultrastructural study of root invasion in three vascular wilt diseases. *Physiological Plant Pathology* 22:15–27.

Blair AC, Hanson BD, Brunk GR, Marrs RA, Westra P, Nissen SJ, Hufbauer RA (2005) New techniques and findings in the study of a candidate allelochemical implicated in invasion success. *Ecology Letters* 8:1039–1047.

Breiteneder H, Pettenburger K, Bito A, Valenta R, Kraft D, Rumpold H, Scheiner O, Breitenbach M (1989) The gene coding for the major birch pollen allergen Betv1, is highly homologous to a pea disease resistance response gene. *The EMBO Journal* 8:1935-1938.

Breiteneder H, Ebner C (2000) Molecular and biochemical classification of plant-derived food allergens. *The Journal of Allergy and Clinical Immunology* 106:27–36.

Breiteneder H, Radauer C (2004) A classification of plant food allergens. *The Journal of Allergy and Clinical Immunology* 113:821-30.

Breiteneder H, Mills EN (2005) Molecular properties of food allergens. The *Journal of Allergy and Clinical Immunology* 115:14-23.

Brodersen P, Petersen M, Bjørn Nielsen H, Zhu S, Newman MA, Shokat KM, Rietz S, Parker J, Mundy J (2006) *Arabidopsis* MAP kinase 4 regulates salicylic acid- and jasmonic acid/ethylene-dependent responses via EDS1 and PAD4. *Plant Journal* 47:532–546.

Brown RL, Kazan K, McGrath KC, Maclean DJ, Manners JM (2003) A role for the GCC-box in jasmonate-mediated activation of the PDF1.2 gene of *Arabidopsis*. *Plant Physiology* 132:1020–32.

Bufe A, Spangfort MD, Kahlert H, Schlaak M, Becker WM (1996) The major birch pollen allergen, Bet v 1, shows ribonuclease activity. *Planta* 199:413–415.

Canonica GW, Ansotegui IJ , Pawankar R , Schmid-Grendelmeier P , van Hage M , Baena-Cagnani CE , Melioli G , Nunes G, Passalacqua G , Rosenwasser L, Sampson H, Sastre J, Bousquet J, Zuberbier T and WAO-ARIA-GA2LEN Task Force: Allen K, Asero R, Bohle B, Cox L, de Blay F, Ebisawa M, Maximiliano-Gomez R, Gonzalez-Diaz S, Haahtela T, Holgate S, Jakob T, Larche M, Matricardi PM, Oppenheimer J, Poulsen LK, Renz HE, Rosario N, Rothenberg M, Sanchez-Borges M, Scala E, Valenta R (2013) A Wao-Aria-Galen consensus document on molecular-based allergy diagnostics. *World Allergy Organization Journal* 6:17.

Carpin S, Laffer S, Schoentgen F, Valenta R, Chenieux JC, Rideau M, Hamdi S (1998) Molecular characterization of a cytokinin-inducible periwinkle protein showing sequence

homology with pathogenesis-related proteins and the Bet v 1 allergen family. *Plant Molecular Biology* 36:791–798.

Casañal A, Zander U, Dupeux F, Valpuesta V, Marquez JA (2013a) Purification, crystallization and preliminary X-ray analysis of the strawberry allergens Fra a 1E and Fra a 3 in the presence of catechin. *Crystallization communications*. *Acta Crystallographica section F* 69:510-514.

Casañal A, Zander U, Muñoz C, Dupeux F, Luque I, Botella MA, Schwab W, Valpuesta V, Marquez JA (2013b) The Strawberry Pathogenesis-related 10 (PR-10) Fra a Proteins Control Flavonoid Biosynthesis by Binding Metabolic Intermediates. *Journal of Biological Chemistry 288*:35322–35332.

Cerdá B, Tomás-Barberán FA, Espín JC (2005) Metabolism of antioxidant and chemopreventive ellagitannins from strawberries, raspberries, walnuts, and oak-aged wine in humans: identification of biomarkers and individual variability. *Journal of Agricultural and Food Chemistry* 53:227–235.

Chadha P, Das RH (2006) A pathogenesis related protein, AhPR-10 from peanut: an insight of its mode of antifungal activity. *Planta* 225:213–222.

Chakrabortya J, Dutta TK (2011) From lipid transport to oxygenation of aromatic compounds: evolution within the bet v1-like superfamily. *Journal of Biomolecular structure & Dynam*ics 29: 67-78.

Chalavi V, Tabaeizadeh Z (2003) Enhanced Resistance to *Verticillium dahliae* in Transgenic Strawberry Plants Expressing a Lycopersicon chilense Chitinase Gene. *Journal of the American Society for Horticultural Science* 128: 747-753.

Chen L, Zhang S, Illa E, Song L, Wu S, Howard W, Arús P, van de Weg E, Chen K, Gao Z (2008) Genomic characterization of putative allergen genes in peach/almond and their synteny with apple. *BMC Genomics* 9:543.

Chen P, Lee B, Robb J (2004) Tolerance to a non-host isolate of *Verticillium dahliae* in tomato. *Physiological and Molecular Plant Pathololog*y 64:283–291.

Chen ZY, Brown RL, Rajasekaran K, Damann KE, Cleveland TE (2006) Identification of a maize kernel pathogenesis-related protein and evidence for its involvement in resistance to Aspergillus flavus infection and aflatoxin production. *Phytopathology* 96:87–95.

Chini A, Fonseca S, Fernandez G, Adie B, Chico JM, Lorenzo O, García-Casado G, López-Vidriero I, Lozano FM, Ponce MR, Micol JL, Solano R (2007) The JAZ family of repressors is the missing link in jasmonate signalling. *Nature* 448:666–671.

Choi DS, Hwang IS, Hwang BK (2012) Requirement of the Cytosolic Interaction between PATHOGENESIS-RELATED PROTEIN10 and LEUCINE-RICH REPEAT PROTEIN1 for Cell Death and Defense Signaling in Pepper. *Plant Cell* 24:1675–1690.

Cliffton EE (1967) The effects of ellagic acid on the coagulation mechanisms and on traumatic bleeding. *The Americain Journal of the Medical Sciences* 254:483-490.

Colebrook EH, Thomas SG, Phillips AL, Hedden P (2014) The role of gibberellin signalling in plant responses to abiotic stress. *Journal of Experimental Biology* 217:67–75.

Courtney AH, Chunying K, Omar D, Aviva G, Benjamin FM, Janet S, Nadim A, Zhongchi L (2014) Floral Transcriptomes in Woodland Strawberry Uncover Developing Receptacle and Anther Gene Networks. *Plant Physiolgy* 165:1062-1075.

Cramer GR, Urano K, Delrot S, Pezzotti M, Shinozaki K (2011) Effects of abiotic stress on plants: A systems biology perspective. *BMC Plant Biology 11*:163–177.

Cregeen S, Radisek S, Mandelc S, Turk B, Stajner N, Jakse J, Javornik B (2015) Different Gene Expressions of Resistant and Susceptible Hop Cultivars in Response to infection with a Highly Aggressive Strain of *Verticillium albo-atrum. Plant Molecular Biology Reporter* 33:689-704.

Damiano C, Monticelli S, Frattarelli A, Nicolini S, Corazza L (1997) Somaclonal variability and *in vitro* regeneration of strawberry. *Acta Horticulturae* 447:87-94.

Das DK (1994) Naturally occurring flavonoids: structure, chemistry, and high-performance liquid chromatography methods for separation and characterization. *Methods in Enzymology* 234:411-420.

Das M, Bickers DR, Mukhtar H (1985) Effect of ellagic acid on hepatic and pulmonary xenobiotic metabolism in mice: studies on the mechanism of its anticarcinogenic action. *Carcinogenesis* 6:1409-1413.

Despres C, Subramaniam R, Matton DP, Brisson N (1995) The activation of the potato PR-10a gene requires the phosphorylation of the nuclear factor PBF1. *Plant Cell* 7:589–598.

Dharmasiri N, Dharmasiri S, Estelle M (2005) The F-box protein TIR1 is an auxin receptor. *Nature* 435:441–445.

Ding X, Cao Y, Huang L, Zhao J, Xu C, Li X, Wang S (2008) Activation of the indole-3-acetic acid-amido synthetase GH3-8 suppresses expansin expression and promotes salicylate- and jasmonate-independent basal immunity in rice. *Plant Cell 20*:228–240.

Dong X (2004) NPR1, all things considered. *Current Opinion in Plant Biology* 7:547–552.

Ebner C, Birkner T, Valenta R, Rumpold H, Breitenbach M, Scheiner O, Kraft D (1991) Common epitopes of birch pollen and apples—Studies by Western and Northern blot. *The Journal of Allergy and Clinical Immunology* 88:588-94.

Ebner C, Hirschwehr R, Bauer L, Breiteneder H, Valenta R, Ebner H, Kraft D, Scheiner O (1995) Identification of allergens in fruits and vegetables: IgE cross-reactivities with the important birch pollen allergens Bet v 1 and Bet v 2 (birch profilin). *The Journal of Allergy and Clinical Immunology* 95:962–969.

Edwards R, Blount JW, Dixon RA (1991) Glutathione and elicitation of the phytoalexin response in legume cell cultures. *Planta* 184:403-409.

El-Bebany, Adam LR, Daayf F (2013) Differential accumulation of phenolic compounds in potato in response to weakly and highly aggressive isolates of *Verticillium dahliae. Canadian Journal of Plant Pathology* 35:232-240.

Erdmann S, Sachs B, Schmidt A, Merk H, Scheiner O, Moll-Slodowy S, Sauer I, Kwiecien R, Maderegger B, Hoffmann- Sommergruber K (2005) *In vitro* analysis of birch-pollen-associated food allergy by use of recombinant allergens in the basophil activation test. *International Archives of Allergy and Immunology* 136:230−238.

Eulgem T, Somssich IE (2007) Networks of WRKY transcription factors in defence signaling. *Current Opinion in Plant Biology* 10:366–371.

Faber C, Lindemann A, Sticht H, Ejchart A, Kungl A, Susani M, Frank RW, Kraft D, Breitenbach M, Rösch P (1996) Secondary structure and tertiary fold of the birch pollen allergen Bet v 1 in solution. *Journal of Biological Chemistry* 271:19243-19250.

Fahlbusch B, Rudeschko O, Müller W.D, Schlenvoigt, G, Vettermann S, Jäger L (1995) Purification and characterization of the major allergen from apple and its allergenic cross-reactivity with Bet v 1. *International Archives of Allergy and Immunology* 108:119–126.

Fan S, Jiang L, Wu J, Dong L, Cheng Q, Xu P, Zhang S (2015) A Novel Pathogenesis-Related Class 10 Protein Gly m 4l, Increases Resistance upon Phytophthora sojae Infection in Soybean (Glycine max [L.] Merr.). *PLoS One* 10:e0140364.

FAOSTAT (2012) http://faostat.fao.org/ (Status of December 2012).

Fernandes H, Bujacz A, Bujacz G, Jelen F, Jasinski M, Kachlicki P, Otlewski J, Sikorski M M, Jaskolski M (2009) Cytokinin-induced structural adaptability of a *Lupinus luteus* PR-10 protein. *FEBS Journal* 276:1596–1609.

Fernandes H, Michalska K, Sikorski M, Jaskolski M (2013) Structural and functional aspects of PR-10 proteins. *FEBS Journal* 280:1169-99.

Fernandez-Rivas M, Gonzalez-Mancebo E, Rodriguez-Perez R, Benito C, Sanchez-Monge R, Salcedo G, Alonso MD, Rosado A, Tejedor MA, Vila C, Casas ML (2003) Clinically relevant peach allergy is related to peach lipid transfer protein, Pru p 3, in the Spanish population. *The Journal of Allergy and Clininical Immunology* 112:789-795.

Ferreira F, Hirtenlehner K, Jilek A, Godnik-Cvar J, Breiteneder H, Grimm R, Hoffmann-Sommergruber K, Scheiner O, Kraft D, Breitenbach M, Rheinberger HJ, Ebner C (1996) Dissection of immunoglobulin E and T lymphocyte reactivity of isoforms of the major birch pollen allergen Bet v 1: potential use of hypoallergenic isoforms for immunotherapy. *The Journal of Experimental Medicine* 183:599–609.

Ferreira F, Ebner C, Kramer B, Casari G, Briza P, Kungl AJ, Grimm R, Jahn-Schmid B, Breiteneder H, Kraft D, Breitenbach M, Rheinberger HJ, Scheiner O (1998) Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy. *The FASEB Journal* 12:231-242.

Feucht W, Treutter D (1999) The role of flavan-3-ols and proanthocyanidins in plant defense. In: Principles and Practices in Plant Ecology: Allelochemical Interactions. Ed. by Inderjit, Dakshini KMM, Foy CL. Boca Raton: *CRC Press* 307–338.

Finkler C, Giacomet C, Muschner VC, Salzano FM, Freitas LB (2005) Molecular investigations of pathogenesis-related Bet v 1 homologues in Passiflora (Passifloraceae). *Genetica* 124:117-125.

Floerl S, Druebert C, Majcherczyk A, Karlovsky P, Kues U, Polle A (2008) Defence reactions in the apoplastic proteome of oilseed rape (Brassica napus var. napus) attenuate *Verticillium longisporum* growth but not disease symptoms. *BMC Plant Biology* 8:129.

Flores T, Alape-Giron A, Flores-Diaz M, Flores HE (2002) Ocatin. A novel tuber storage protein from the Andean tuber crop oca with antibacterial and antifungal activities. *Plant Physiology* 128:1291- 1302.

Flors, V, Ton J, van Doorn R, Jakab G, García Agustín P, Mauch-Mani B (2008) Interplay between JA, SA and ABA signalling during basal and induced resistance against *Pseudomonas syringae* and *Alternaria brassicicola*. *Plant Journal 54*:81–92.

Fodor J, Gullner G, Adam AL, Barna B, Komives T, Kiraly Z (1997) Local and systemic responses of antioxidants to tobacco mosaic virus infection and to salicylic acid in tobacco. (Role in systemic acquired resistance). *Plant Physiology* 114:1443-1451.

Folta KM, Davis TM (2006) Strawberry genes and genomics. *Critical Reviews in Plant Sciences* 25:399–415.

Food Allergy & Anaphylaxis Public Declaration (EAACI) http://www.eaaci.org/attachments/FoodAllergy&AnaphylaxisPublicDeclarationCombined.pdf

Fradin EF, Thomma BP (2006) Physiology and molecular aspects of *Verticillium* wilt diseases caused by *V. dahliae* and *V. albo-atrum*. *Molecular Plant Pathology* 7:71–86.

Fradin EF, Zhang Z, Ayala JCJ, Castroverde CDM, Nazar RN, Robb J, Liu CM, Thomma B (2009) Genetic dissection of *Verticillium* wilt resistance mediated by tomato Ve1. *Plant Physiology* 150:320–332.

Franz-Oberdorf K, Eberlein B, Edelmann K, Hücherig S, Besbes F, Darsow U, Ring J, Schwab W (2016) Fra a 1.02 Is the Most Potent Isoform of the Bet v 1-like Allergen in Strawberry Fruit. *Journal of Agricultural and Food Chemistry* 18:3688-3696.

Franz-Oberdorf K, Langer A, Strasser R, Isono E, Ranftl QL, Wunschel W, Schwab W (2017a) Physical Interaction between the Strawberry Allergen Fra a 1 and an Associated Partner FaAP. *Proteins: Structure, Function, and Bioinformatics* 85:1891-1901.

Franz-Oberdorf K, Eberlein B, Edelmann K, Bleicher P, Kurze E, Helm D, Olbricht K, Darsow U, Ring J, Schwab W (2017b) White-fruited strawberry genotypes are not per se hypoallergenic. *Food Research International* 100: 748-756.

Gajhede M, Osmark P, Poulsen FM, Ipsen H, Larsen JN, Joost van Neerven, RJ, Schou C, Løwenstein H, Spangfort MD (1996) Xray and NMR structure of Bet v 1, the origin of birch pollen allergy. *Nature Structural and Molecular Biology* 3:1040–1045.

Galiana E, Bonnet P, Conrod S, Keller H, Panabières F, Ponchet M, Poupet A, Ricci P (1997) RNase activity prevents the growth of a funga1 pathogen in tobacco leaves and increases upon induction of systemic acquired resistance with elicitin. *Plant Physiology* 115:1557-1567.

Gams W, Zare R, Summerbell RC (2005) (1654) Proposal to conserve the generic name *Verticillium* (anamorphic Ascomycetes) with a conserved type. *Taxon* 54:179.

Gao ZS, van de Weg WE, Schaart JG, Schouten HJ, Tran DH, Kodde LP, van de Meer IM, van der Geest AHM, Kodde J, Breiteneder H, Hoffmann- Sommergruber K, Bosch D, Gilissen LJ (2005) Genomic cloning and linkage mapping of the Mal d 1 (PR-10) gene family in apple (Malus domestica). *Theoretical and Applied Genetics* 111:171-183.

Giampieri F, Tulipani S, Alvarez-Suarez JM, Quiles JL, Mezzetti B, Battino M (2012) The strawberry: Composition, nutritional quality, and impact on human health. *Nutrition* 28:9-19.

Giampieri F, Alvarez-Suarez JM, Battino M (2014) Strawberry and Human Health: effects beyond Antioxidant Activity. *Journal of Agricultural and Food Chemistry* 62:3867-76.

Gieras A, Cejka P, Blatt K, Focke-Tejkl M, Linhart B, Flicker S, Stoecklinger A, Marth K, Drescher A, Thalhamer J, Valent P, Majdic O, Valenta R (2011) Mapping of conformational IgE epitopes with peptide-specific monoclonal antibodies reveals simultaneous binding of different IgE antibodies to a surface patch on the major birch pollen allergen, Bet v 1. *Journal of Immunology* 186:5333− 5344.

Girolami A, Agostino D, Cliffton, EE (1966) The effect of ellagic acid on coagulation in vivo. *Blood* 27:93-102.

Glazebrook J (2005) Contrasting mechanisms of defence against biotrophic and necrotrophic pathogens. *Annual Review of Phytopathology* 43:205–227.

Gold J, Robb J (1995) The role of the coating response in Craigella tomatoes infected with *Verticillium dahliae* races 1 and 2. *Physiological and Molecular Plant Pathology* 47:141-157.

Gomez-Gomez L, Rubio-Moraga A, Ahrazem O (2011) Molecular cloning and characterisation of a pathogenesis-related protein CsPR-10 from Crocus sativus. *Plant Biolology* 13:297–303.

Gönner Mv, Schlösser E (1993) Oxidative stress in interactions between *Avena sativa* L. and *Drechslera spp*. *Physiological and Molecular Plant Pathology* 42:221 -234.

Goodman T, Schulenberg B, Steinberg TH, Patton WF (2004) Detection of phosphoproteins on electroblot membranes using a small-molecule organic fluorophore. *Electrophoresis* 25:2533–2538.

Goulas E, Richard-Molard C, Le Dily F, Le Dantec, Ozouf J, Ourry A (2007) A cytosolic vegetative storage protein (TrVSP) from white clover is encoded by a cold-inducible gene. *Physiologia Plantarum* 129:567–577.

Grangeasse C, Obadia B, Mijakovic I, Deutscher J, Cozzone AJ, Doublet P (2003) Autophosphorylation of the Escherichia coli protein kinase Wzc regulates tyrosine phosphorylation of Ugd, a UDP-glucose dehydrogenase. *Journal of Biological Chemistry* 278:39323-39329.

Grant M, Lamb C (2006) Systemic immunity. *Current Opinion in Plant Biology* 9:414–420.

Grundner C, Gay LM, Alber T (2005) *Mycobacterium tuberculosis* serine/threonine kinases PknB, PknD, PknE, and PknF phosphorylate multiple FHA domains. *Protein Science* 14:1918–1921.

Hancock JF, Luby JJ (1993) Genetic Resources at Our Doorstep: The Wild Strawberries. *BioScience* 43:141-147.

Hancock JF, Sjulin TM, Lobos GA (2008) Strawberries. In: Temperate Fruit Crop Breeding: Germplasm to Genomics. *Springer* 45:393-437.

Handschuh L, Femiak I, Kasperska A, Figlerowicz M, Sikorski MM (2007) Structural and functional characteristics of two novel members of pathogenesisrelated multigene family of class 10 from yellow lupine. *Acta Biochimica Polonica* 54:783-796.

Hanhineva K, Kärenlampi S, Aharoni A (2011) Recent advances in strawberry metabolomics. *Genes, Genomes and Genomics* 5:65–75.

Hawksworth DL, Talboys PW (1970) *Verticillium dahliae*. *CMI Descriptions of Pathogenic Fungi and Bacteria* 255-259.

Härtl K, Denton A, Franz-Oberdorf K, Hoffmann T, Spornraft M, Usadel B, Schwab W (2017) Early metabolic and transcriptional variations in fruit of natural white-fruited *Fragaria vesca* genotypes. *Scientific Reports* 7:45113.

Heber D (2008) Multitargeted therapy of cancer by ellagitannins. *Cancer Letters* 269:262–268.

Heinz R, Lee SW, Saparno A, Nazar RN, Robb J (1998) Cyclical systemic colonization in *Verticillium*-infected tomato. *Physiological and Molecular Plant Pathology* 52:385–396.

Henning SM, Seeram NP, Zhang Y, Li L, Gao K, Lee RP, Wang DC, Zerlin A, Karp H, Thames G, Kotlerman J, Li Z, Heber D (2010) Strawberry Consumption Is Associated with Increased Antioxidant Capacity in Serum. *Journal of Medicinal Food* 13:116-22.

Hjerno K, Alm R, Canbäck B, Matthiesen R, Trajkovski K, Björk L, Roepstorff P, Emanuelsson C (2006) Down-regulation of the strawberry Bet v 1-homologous allergen in concert with the flavonoid biosynthesis pathway in colorless strawberry mutant. *Proteomics* 6:1574-1587.

Hoch JA (2000) Two-component and phosphorelay signal transduction. *Current Opinion in Microbiology* 3:165-170.

Hoffmann-Sommergruber K (2002) Pathogenesis-related (PR)- proteins identified as allergens. *Biochemical Society Transactions* 30: 930– 935.

Holm J, Baerentzen G, Gajhede M, Ipsen H, Larsen JN, Løwenstein H, Wissenbach M, Spangfort MD (2001) Molecular basis of allergic crossreactivity between group 1 major allergens from birch and apple. *Journal of Chromatography B: Biomedical Sciences and Applications* 756:307–313.

Horticulture Germany
https://www.bmel.de/SharedDocs/Downloads/EN/Publications/HorticultureGermany-Brochure.html

Huisman OC (1982) Interrelations of root growth dynamics to epidemiology of root-invading fungi. *Annual Review of Phytopathology* 20:303–327.

Husberg C, Agnetti G, Holewinski RJ, Christensen G, Van Eyk JE (2012) Dephosphorylation of cardiac proteins *in vitro* – a matter of phosphatase specificity. *Proteomics* 12:973–978.

Inderbitzin P, Bostock RM, Davis RM, Usami T, Platt HW, Subbarao KV (2011) Phylogenetics and taxonomy of the fungal vascular wilt pathogen *Verticillium*, with the descriptions of five new species. *PLoS One* 6:e28341.

Inderjit, Kaur R, Kaur S, Callaway RM (2009) Impact of (±)-catechin on soil microbial communities. *Communicative & Integrative Biology* 2:127-129.

Ishihara A, HashimotoY, Tanaka C, Dubouzet JG, Nakao T, Matsuda F, Nishioka T, Miyagawa H and Wakasa K (2008) The tryptophan pathway is involved in the defense responses of rice against pathogenic infection via serotonin production. *Plant Journal* 54: 481-495.

Iven T, Stefanie König S, Singh S, Braus-Stromeyer SA, Matthias Bischoff M, Tietze LF, Gerhard H. Braus GH, Lipka V, Feussner I and Dröge-Laser W (2012) Transcriptional Activation and Production of Tryptophan-Derived Secondary Metabolites in *Arabidopsis* Roots Contributes to the Defense against the Fungal Vascular Pathogen *Verticillium* longisporum. *Molecular Plant* 5:1389-402.

Jain S, Srivastava S, Sarin NB, Kav NNV (2006) Proteomics reveals elevated levels of PR-10 proteins in saline-tolerant peanut (*Arachis hypogaea*) calli. *Plant Physiology and Biochemistry* 44:253-259.

Jain S, Kumar A (2015) The Pathogenesis Related Class 10 proteins in Plant Defense against Biotic and Abiotic Stresses. *Advances in Plants & Agriculture Research* 3:00077.

Jain SM (2001) Tissue culture-derived variation in crop improvement. *Euphytica* 118:153-166.

Johansson A, Staal J, Dixelius C (2006) Early responses in the *Arabidopsis–Verticillium longisporum* pathosystem are dependent on NDR1, JA- and ET-associated signals via cytosolic NPR1 and RFO1. *Molecular Plant–Microbe Interactions* 19:958–969.

Kamilova F, Kravchenko LV, Shaposhnikov AI, Makarova N, Lugtenberg B (2006a) Effects of the tomato pathogen *Fusarium oxysporum* f. sp *radicis-lycopersici* and of the biocontrol bacterium *Pseudomonas fluorescens* WCS365 on the composition of organic acids and sugars in tomato root exudate. *Molecular Plant-Microbe Interactions* 19:1121–1126.

Kamilova F, Kravchenko LV, Shaposhnikov AI, Azarova T, Makarova N, Lugtenberg B (2006b) Organic acids, sugars, and L-tryptophane in exudates of vegetables growing on stonewool and their effects on activities of rhizosphere bacteria. *Molecular Plant-Microbe Interactions* 19:250–256.

Karlsson AL, Alm R, Ekstrand B, Fjelkner-Modig S, Schiott A, Bengtsson U, Bjork L, Hjerno K, Roepstorff P, Emanuelsson CS (2004) Bet v 1 homologues in strawberry identified as IgE-binding proteins and presumptive allergens. *Allergy* 59:1277-84.

Kazan K, Manners JM (2009) Linking development to defense: Auxin in plant–pathogen interactions. *Trends in Plant Science* 14: 373–382.

Kepinski S, Leyser O (2005) The *Arabidopsis* F-box protein TIR1 is an auxin receptor. *Nature* 435:446–451.

Kim MS, Jin JS, Kwak YS, Hwang GS (2016) Metabolic Response of Strawberry (*Fragaria X Ananassa*) Leaves Exposed to the Angular Leaf Spot Bacterium (Xanthomonas Fragariae). *Journal of Agricultural and Food Chemistry* 64:1889-98.

Klebahn H (1913) Beiträge zur Kenntnis der Fungi imperfecti. 1. Eine *Verticillium*-Krankheit auf Dahlien. *Mycologisches Centralblatt* 3:49–66.

Klocke JA, VanWagenen B, Balandrin MF (1986) The ellagitannin geraniin and its Hydrolysis products isolated as insect growth inhibitors from semi-arid land plants. *Photochemistry* 25:85-91.

Klosterman SJ, Atallah ZK, Vallad GE, Subbarao KV (2009) Diversity, pathogenicity, and management of *Verticillium* species. *Annual Review of Phytopathology* 47:39–62.

Klosterman SJ, Anchieta A, Garcia-Pedrajas MD, Maruthachalam K, Hayes RJ, Subbarao KV (2011) SSH reveals a linkage between a senescence-associated protease and *Verticillium* wilt symptom development in lettuce (Lactuca sativa). *Physiological and Molecular Plant Pathology* 76:48–58.

Knox B, Suphioglu C (1996) Environmental and molecular biology of pollen allergens. *Trends in Plant Science* 1:156–164.

Kofler S, Asam C, Eckhard U, Wallner M, Ferreira F, Brandstetter H (2012) Crystallographically mapped ligand binding differs in high and low IgE binding isoforms of birch pollen allergen Bet v 1. *Journal of Molecular Biology* 422:109–123.

Koistinen KM, Kokko HI, Hassinen VH, Tervahauta AI, Auriola S, Kärenlampi SO (2002a) Stress-related RNase PR-10c is post-translationally modified by glutathione in birch. *Plant, Cell and Environement* 25:707–715.

Koistinen KM, Hassinen VH, Gynther PAM, Lehesranta SJ, Keinänen SI, Kokko HI, Oksanen EJ, Tervahauta AI, Seppo Auriola S, Kärenlampi SO (2002b) Birch PR-10c is induced by factors causing oxidative stress but appears not to confer tolerance to these agents. *New Phytologist* 155:381–391.

Koistinen KM, Soininen P, Venalainen TA, Hayrinen J, Laatikainen R, Perakyla M, Tervahauta AI & Kärenlampi SO (2005) Birch PR-10c interacts with several biologically important ligands. *Phytochemistry* 66:2524–2533.

Kosuth J, Katkovcinova Z, Olexova P, Cellarova E (2007) Expression of the hyp-1 gene in early stages of development of Hypericum perforatum L. *Plant Cell Reports* 26:211–217.

Krishnaswamy S, Baral PK, James MN, Kav NNV (2011) Site-directed mutagenesis of histidine 69 and glutamic acid 148 alters the ribonuclease activity of pea ABR17 (PR-10.4). *Plant Physiology and Biochemistry* 49:958–962.

Lam SK, Ng TB (2001) Isolation of a novel thermolabile heterodimeric ribonuclease with antifungal and antiproliferative activities from roots of the sanchi ginseng *Panax notoginseng*. *Biochemical and Biophysical Research Communications* 285:419–423.

Langer A, Hampel PA, Kaiser W, Knezevic J, Welte T, Villa V, Maruyama M, Svejda M, Jähner S, Fischer F, Strasser R and Rant U (2013) Protein analysis by time-resolved measurements with an electro-switchable DNA chip. *Nature Communications* 4:2099.

Lebel S, Schellenbaum P, Walter B, Maillot P (2010) Characterisation of the Vitis vinifera PR-10 multigene family. *BMC Plant Biology* 10:184.

Lee EJ, Facchini P (2010) Norcoclaurine synthase is a member of the pathogenesis-related 10/Bet v1 protein family. *Plant Cell* 22:3489–3503.

Lee OR, Pulla RK, Kim YJ, Balusamy SR, Yang DC (2011) Expression and stress tolerance of PR-10 genes from Panax ginseng C. A. Meyer. *Molecular Biology Reports* 39:2365–2374.

Li C, He X, Luo X, Xu L, Liu L, Min L, Jin L, Zhu L, Zhang X (2014) Cotton WRKY1 mediates the plant defense-to-development transition during infection of cotton by *Verticillium dahliae* by activating JASMONATE-ZIM-DOMAIN1 expression. *Plant Physiol*ogy 166:2179–2194.

Li J, Brader G, Palva ET (2004) The WRKY70 transcription factor: a node of convergence for jasmonate-mediated and salicylatemediated signals in plant defence. *Plant Cell* 16:319–331.

Li J, Brader G, Kariola T, Palva ET (2006) WRKY70 modulates the selection of signaling pathways in plant defence. *Plant Journal* 46:477–491.

Liao Z, Chen M, Guo L, Gong Y, Tang F, Sun X, Tang K (2004) Rapid isolation of high-quality total RNA from *Taxus* and *Ginkgo*. *Preparative Biochemistry and Biotechnology* 34: 209-214.

Liu JJ, Ekramoddoullah AK (2003) Root-specific expression of a western white pine PR-10 gene is mediated by different promoter regions in transgenic tobacco. *Plant Molecular Biology* 52:103–120.

Liu JJ, Ekramoddoullah AK, Piggott N, Zamani A (2005) Molecular cloning of a pathogen/wound-inducible PR-10 promoter from Pinus monticola and characterization in transgenic *Arabidopsis* plants. *Planta* 221:159-69.

Liu JJ, Ekramoddoullah AKM (2006) The family 10 of plant pathogenesis-related proteins: their structure, regulation, and function in response to biotic and abiotic stresses. *Physiological and Molecular Plant Pathology* 68:3-13.

Liu X, Huang B, Lin J, Fei J, Chen Z, Pang Y, Sun X, Tang K (2006) A novel pathogenesis-related protein (SsPR-10) from Solanum surattense with ribonucleolytic and antimicrobial activity is stress- and pathogeninducible. *Journal of Plant Physiology* 163:546–556.

Liu Y, Zhang N, Qiu M, Feng H, Vivanco JM, Shen Q, Zhang R (2014) Enhanced rhizosphere colonization of beneficial Bacillus amyloliquefaciens SQR9 by pathogen infection. *FEMS Microbiol Letters* 353:49–56.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2 (-Delta Delta C (T)) method. *Methods* 25:402-408.

López-Ráez JA, Flors V, García JM,1 and Pozo MJ (2010) AM symbiosis alters phenolic acid content in tomato roots. *Plant Signaling & Behaviour* 5:1138–1140.

Lorenzo O, Chico JM, Sánchez-Serrano JJ, Solano R (2004) JASMONATE-INSENSITIVE1 encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defence responses in *Arabidopsis*. *Plant Cell* 16:1938–1950.

Lorenzo O, Solano R (2005) Molecular players regulating the jasmonate signaling network. *Current Opinion in Plant Biology* 8:532–540.

Lu Y, Du J, Tang J, Wang F, Zhang J, Huang J, Liang W, Wang L (2009) Environmental regulation of floral anthocyanin synthesis in Ipomoea purpurea. *Molecular Ecology* 18:3857-3871.

Lüttkopf D, Muller U, Skov PS, Ballmer-Weber BK, Wuthrich B, Skamstrup Hansen K, Poulsen LK, Kastner M, Haustein D, Vieths S (2002) Comparison of four variants of a major allergen in hazelnut (Corylus avellana) Cor a 1.04 with the major hazel pollen allergen Cor a 1.01. *Molecular Immunolology* 38:515–525.

Maas JL, Galletta GJ, Stoner GD (1991a) Ellagic acid, an anticarcinogen in fruits, especially in strawberries: A Review. *HortScience* 26:10-14.

Maas JL, Wang SY, Galletta GJ (1991b) Evaluation of strawberry cultivars for ellagic acid content. *HortScience* 26:66–68.

Maas JL, Zhong L, Galletta GJ (1993) *In vitro* screening of strawberry plant and root cultures for resistance to *Phytophthora fragariae* and *P.cactorum*. *Acta Horticuturae* 348: 496-499.

Macek B, Gnad F, Soufi B, Kumar C, Olsen JV, Mijakovic I, Mann M (2008) Phosphoproteome of *Escherichia coli* reveals high conservation of bacterial serine/threonine/ tyrosine phosphorylation. *Molecular & Cellular Proteomics* 7:299-307.

Mandal S, Ahuja A, Shivapurkar NM, Cheng SJ, Groopman JD, Stoner GD (1987) Inhibition of aflatoxin B, mutagenesis in Salmonella typhimurium and DNA damage in cultured rat and human tracheobronchial tissues by ellagic acid. *Carcinogenesis* 8:1651-1656.

Mao P, Duan M, Wei C, Li Y (2007) WRKY62 transcription factor acts downstream of cytosolic NPR1 and negatively regulates jasmonate-responsive gene expression. *Plant & Cell Physiology* 48:833–842.

Marković-Housley Z, Degano M, Lamba D, von Roepenack-Lahaye E, Clemens S, Susani M, Ferreira F, Scheiner O, Breiteneder H (2003) Crystal structure of a hypoallergenic isoform of the major birch pollen allergen Bet v 1 and its likely biological function as a plant steroid carrier. *Journal of Molecular Biology* 325:123–133.

Marzban G, Mansfeld A, Hemmer W, Stoyanova E, Katinger H, da Câmara Machado M (2005) Fruit cross-reactive allergens: a theme of uprising interest for consumers' health. *Biofactors* 23:235-241.

Marzban G, Maghuly F, Herndl A, Katinger H, Laimer M (2008) Screening and identification of putative allergens in berry fruits of the *Rosaceae* family: technical challenges. *BioFactors* 34:37-46.

Marzban G, Maghuly F, Laimer M, Hemmer W, Nestby R (2012) Allergomics of Berry Fruits. *Acta Horticulturae* 926:663-668.

Matton DP, Brisson N (1989) Cloning, expression, and sequence conservation of pathogenesis-related gene transcripts of potato. *Molecular Plant-Microbe Interactions* 2:325–331.

Mauch F, Dudler R (1993) Differential induction of distinct glutathione-S-transferases of wheat by xenobiotics and by pathogen attack. *Plant Physiology* 102:1193-1201.

May MJ, Hammond-Kosack KE, Jones J (1996) Involvement of reactive oxygen species, glutathione metabolism, and lipid peroxidation in the (Cf-gene-dependent defense response of tomato cotyledons induced by race-specific elicitors of Cladosporium fulvum. *Plant Physiology* 110:1367-1379.

Meyer Y, Siala W, Bashandy T, Riondet C, Vignols F, Reichheld JP (2008) Glutaredoxins and thioredoxins in plants. *Biochimica et Biophysica Acta* 1783:589–600.

Michalska K, Fernandes H, Sikorski M & Jaskolski M (2010) Crystal structure of Hyp-1, a St. John's wort protein implicated in the biosynthesis of hypericin. *Journal of Structural Biology* 169:161–171.

Millet YA , Danna CH, Clay NK, Songnuan W, Simon MD, Werck-Reichhart D, Ausubel FM (2010) Innate immune responses activated in *Arabidopsis* roots by microbe-associated molecular patterns. *Plant Cell* 22:973–990.

Mizoi J, Shinozaki K, Yamaguchi-Shinozaki K (2012) AP2/ERF family transcription factors in plant abiotic stress responses. *Biochimica and Biophysica Acta* 1819:86–96.

Mogensen JE, Wimmer R, Larsen JN, Spangfort MD, Otzen DE (2002) The Major Birch Allergen, Bet v 1, Shows Affinity for a broad spectrum of physiological ligands. *Journal of Biological Chemistry* 277:23684-23692.

Mogensen JE, Ferreras M, Wimmer R, Petersen SV, Enghild JJ, Otzen DE (2007) The major allergen from birch tree pollen, Bet v 1, binds and permeabilizes membranes. *Biochemistry* 46:3356-3365.

Moiseyev GP, Beintema JJ, Fedoreyeva LI, Yakovlev GI (1994) High sequence similarity between a ribonuclease from ginseng calluses and funguselicited proteins from parsley indicates that intracellular pathogenesis-related proteins are ribonucleases. *Planta* 193: 470–472.

Moiseyev GP, Fedireyeva LI, Zhuravlev YN, Yasnetskaya E, Jekel PA, Beintema JJ (1997) Primary structures of two ribonucleases from ginseng calluses. New members of the PR-10 family of intracellular pathogenesis-related plant proteins. *FEBS Letters* 407:207–210.

Mol L (1995) Effect of plant roots on the germination of microsclerotia of *Verticillium dahliae*. *European Journal of Plant Pathology* 101:679-685.

Muñoz C, Hoffmann T, Escobar NM, Ludemann F, Botella MA, Valpuesta V, Schwab W (2010) The Strawberry Fruit Fra a Allergen Functions in Flavonoid Biosynthesis. *Molecular Plant* 3:113-124.

Mur LAJ, Kenton P, Atzorn R, Miersch O, Wasternack C (2006) The outcomes of concentration specific interactions between salicylate and jasmonate signaling include synergy, antagonism, and oxidative stress leading to cell death. *Plant Physiology* 140:249–262.

Murashige T, Skoog F (1962) A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiologia Plantarum* 15:473–497.

Musidlowska-Persson A, Alm R, Emanuelsson C (2007) Cloning and sequencing of the Bet v 1-homologous allergen Fra a 1 in strawberry (*Fragaria ananassa*) shows the presence of an intron and little variability in amino acid sequence. *Molecular Immunology* 44:1245–1252.

Navarro L, Bari R, Achard P, Lison P, Nemri A, Harberd NP, Jones JD (2008) DELLAs control plant immune responses by modulating the balance of jasmonic acid and salicylic acid signaling. *Current Biology* 18:650–655.

Neudecker P, Schweimer K, Nerkamp J, Scheurer S, Vieths S, Sticht H, Rösch P (2001) Allergic cross-reactivity made visible: solution structure of the major cherry allergen Pru av 1. *Journal of Biological Chemistry* 276:22756–22763.

Noctor G, Arisi ACM, Jouanin L, Kunert KJ, Rennenberg H, Foyer CH (1998) Glutathione: biosynthesis, metabolism and relationship to stress tolerance explored in transformed plants. *Journal of Experimental Botany* 49:623-647.

Okubara PA, Paulitz TC (2005) Root defense responses to fungal pathogens: a molecular perspective. *Plant and Soil* 274:215–226.

Olsson S, Nordbring-Hertz B (1985) Microsclerotial germination of *Verticillium dahliae* as affected by rape rhizosphere. *FEMS Microbiology Letters* 31:293–299.

Olsson ME, Andersson SC, Berglund RH, Gustavsson K-E, Oredsson S (2006) Extracts from organically and conventionally cultivated strawberries inhibit cancer cell proliferation *in vitro*. *Acta Horticulturae* 744:189–194.

Orlando R, Magro P, Rugini E (1997) Pectic enzymes as a selective pressure for *in vitro* recovery of strawberry plants with fungal disease resistance. *Plant Cell Reports* 16:272-276.

Osterballe M, Hansen TK, Mortz CG, Bindslev-Jensen C (2005) The clinical relevance of sensitization to pollen-related fruits and vegetables in unselected pollen-sensitized adults. *Allergy 60*:218-225.

O'Donnell PJ, Schmelz EA, Moussatche P, Lund ST, Jones JB, Klee HJ (2003) Susceptible to intolerance—a range of hormonal actions in a susceptible *Arabidopsis* pathogen response. *Plant Journal* 33:245–257.

Pantelides IS, Tjamos SE, Paplomatas EJ (2010) Ethylene perception via ETR1 is required in *Arabidopsis* infection by *Verticillium dahliae*. *Molecular Plant Pathology* 11:191–202.

Park CJ, Kim KJ, Shin R, Park JM, Shin YC & Paek KH (2004) Pathogenesis-related protein 10 isolated from hot pepper functions as a ribonuclease in an antiviral pathway. *Plant Journal* 37:186–198.

Parthibane V, Iyappan R, Vijayakumar A, Venkateshwari V, Rajasekharan R (2012) Serine/Threonine/Tyrosine Protein Kinase Phosphorylates Oleosin, a Regulator of Lipid Metabolic Functions. *Plant Physiology* 159:95–104.

Pastorello EA, Ortolani C, Farioli L, Pravettoni V, Ispano M, Borga A, Bengtsson A, Incorvaia C, Berti C, Zanussi C (1994) Allergenic cross-reactivity among peach, apricot, plum, and cherry in patients with oral allergy syndrome: an in vivo and *in vitro* study. *Journal of Allergy and Clinical Immunology* 94:699-707.

Peace C, Norelli JL (2009) Genomics Approaches to Crop Improvement in the Rosaceae. *Plant Genetics and Genomics* 6:19-53.

Pegg GF, Brady BL (2002) *Verticillium* Wilts. Wallingford, UK: *CABI Publishing* 552.

Pietack N, Becher D, Schmidl SR, Saier MH, Hecker M, Commichau FM, Stülke J (2010) *In vitro* phosphorylation of key metabolic enzymes from *Bacillus subtilis*: PrkC phosphorylates enzymes from different branches of basic metabolism. *Journal of Molecular Microbiology and Biotechnology* 18:129–140.

Pinto M da S, de Carvalho JE, Lajolo FM, Genovese MI, Shetty K (2010) Evaluation of antiproliferative, anti-Type 2 diabetes, and antihypertension potentials of ellagitannins from strawberries (*Fragaria* × *ananassa* Duch.) using *in vitro* models. *Journal of Medicinal Food* 13:1027–1035.

Podgornaia AI, Laub MT (2013) Determinants of specificity in two component signal transduction. *Current Opinion in Microbiology* 16:156–162.

Poupard P, Brunel N, Leduc N, Viemont JD, Strullu DG, Simoneau P (2001) Expression of a Bet v 1 homologue gene encoding a PR 10 protein in birch roots: induction by auxin and localization of the transcripts by in situ hybridization. *Australian Joiurnal of Plant Physiology* 28:57–63.

Prusky D, Kobiler I, Jacoby B, Sims JJ, Midland SL (1985) Inhibitors of avocado lipoxygenase: their possible relationship with the latency of Colletotrichum gloeosporioides. *Physiological Plant Pathology* 27:269–279.

Puhl I, Treutter D (2008) Ontogenetic variation of catechin biosynthesis as basis for infection and quiescence of Botrytis cinerea in developing strawberry fruits. *Journal of Plant Diseases and Protection* 115:247–251.

Pungartnik C, da Silva AC, de Melo SA, Gramacho KP, de Mattos Cascardo JC, Brendel M, Micheli F, da Silva Gesteira A (2009) High-affinity copper transport and Snq2 export permease of Saccharomyces cerevisiae modulate cytotoxicity of PR-10 from Theobroma cacao. *Molecular Plant-Microbe Interactions* 22:39–51.

Pühringer H, Moll D, Hoffmann-Sommergruber K, Watillon B, Katinger H, Laimer da Camara Machado M (2000) The promoter of an apple YPR-10 gene, encoding the major allergen Mal d 1, is stress- and pathogen-inducible. *Plant Science* 152:35–50.

Pühringer HM, Zinoecker I, Marzban G, Katinger H, Laimer M (2003) MdAP, a novel protein in apple, is associated with the major allergen Mal d 1. *Gene 321*:173-183.

Radauer C, Lackner P, Breiteneder H (2008) The Bet v 1 fold: an ancient, versatile scaffold for binding of large, hydrophobic ligands. *BMC Evolutionary Biology* 8:286.

Ratzinger A, Riediger N, von Tiedemann A, Karlovsky P (2009) Salicylic acid and salicylic acid glucoside in xylem sap of *Brassica napus* infected with *Verticillium longisporum*. *Journal of Plant Research* 122:571-579.

Ravikumar V, Shi L, Krug K, Derouiche A, Jers C, Cousin C, Kobir A, Mijakovic I, Macek B (2014) Quantitative phosphoproteome analysis of Bacillus subtilis reveals novel substrates of the kinase PrkC and phosphatase PrpC. *Molecular & Cellular Proteomics* 13:1965–1978.

Reuter A, Fortunato D, Perono Garoffo L, Napolitano L, Scheurer S, Giuffrida MG, Vieths S, Conti A (2005) Novel isoforms of Pru av 1 with diverging IgE properties identified by a synergistic combination of molecular biology and proteomics. *Proteomics* 5:282-289.

Ring J, Krämer U, Schäfer T, Behrendt H (2001) Why are allergies increasing? *Current Opinion in Immunology* 13:701–708.

Robb J, Powell DA, Street PFS (1987) Time course of wall-coating secretion in *Verticilium*-infected tomatoes. *Physiological and Molecular Plant Patho*logy 31:217-226.

Robb J, Lee SW, Mohan R, Kolattukudy P E (1991) Chemical Characterization of Stress-Induced Vascular Coating in Tomato. *Plant Physiol*ogy 97:528-536.

Robb J, Lee B, Nazar RN (2007) Gene suppression in a tolerant tomato-vascular pathogen interaction. *Planta* 226: 299–309.

Sabato V, van Hengel AJ, De Knop KJ, Verweij MM, Hagendorens MM, Bridts CH, De Clerck LS, Schiavino D, Stevens WJ, Ebo DG (2011) Human basophils: a unique biological instrument to detect the allergenicity of food. *Journal of Investigational Allergology and Clinical Immunology* 21:179-184.

Samanani N, Liscombe DK & Facchini PJ (2004) Molecular cloning and characterization of norcoclaurine synthase, an enzyme catalyzing the first committed step in benzylisoquinoline alkaloid biosynthesis. *Plant Journal* 40:302–313.

Saraste M, Sibbald PR, Wittinghofer A (1990) The P-loop—a common motif in ATP- and GTP-binding proteins. *Trends in Biochemical Sciences* 15:430–434.

Saxon A, Diaz-Sanchez D (2005) Air pollution and allergy: you are what you breathe. *Nature Immunolology* 6:223–226.

Scalzo J, Politi A, Pellegrini N, Mezzetti B, Battino M (2005) Plant genotype affects total antioxidant capacity and phenolic contents in fruit. *Nutrition* 21:207–13.

Schenk MF, Cordewener JHG, America AHP, van't Westende WPC, Smulders MJM, Gilissen LJWJ (2009) Characterization of PR-10 genes from eight Betula species and detection of Bet v I isoforms in birch pollen. *BMC Plant Biology* 9:24.

Schmidt K, Pflugmacher M, Klages S, Mäser A, Mock A, Stahl DJ (2008) Accumulation of the hormone abscisic acid (ABA) at the infection site of the fungus Cercospora beticola supports the role of ABA as a repressor of plant defence in sugar beet. *Molecular Plant Pathology* 9:661-73.

Schmittgen TD, Zakrajsek BA (2000) Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RTPCR. *Journal of Biochemical and Biophysical Methods* 46:69-81.

Seeram NP, Adams LS, Zhang Y, Lee R, Sand D, Scheuller HS, Heber D (2006) Blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry extracts

inhibit growth and stimulate apoptosis of human cancer cells *in vitro*. *Journal of Agricultural and Food Chemistry* 54:9329–9339.

Seutter von Loetzen C, Hoffmann T, Hartl MJ, Schweimer K, Schwab W, Rösch P, Hartl-Spiegelhauer O (2014) Secret of the major birch pollen allergen Bet v 1: identification of the physiological ligand. *Biochemical Journal* 457:379-390.

Shaw DV, Gubler WD, Larson KD, Hansen J (1996) Genetic variation for field resistance to *Verticillium dahliae* evaluated using genotypes and segregating progenies of California strawberries. *Journal of the American Society for Horticultural Science* 121:625–628.

Shaw DV, Gordon TR, Hansen J, Kirkpatrick SC (2010) Relationship between the extent of colonization by *Verticillium dahliae* and symptom expression in strawberry (*Fragaria* × *ananassa*) genotypes resistant to *verticillium* wilt. *Plant Pathology* 59:376–381.

Shpilka T, Weidberg H, Pietrokovski S, Elazar Z (2011) Atg8: an autophagy-related ubiquitin-like protein family. *Genome Biology* 12:226.

Shulaev V, Korban SS, Sosinski B, Abbott AG, Aldwinckle HS, Folta KM, Iezzoni A, Main D, Arús P, Dandekar AM, Lewers K, Brown SK, Davis TM, Gardiner SE, Potter D, Veilleux RE (2008) Multiple models for rosaceae genomics. *Plant Physiology* 147:985–1003.

Shulaev V, Sargent DJ, Crowhurst RN, Mockler TC, Folkerts O, Delcher AL, Jaiswal P, Mockaitis K, Liston A, Mane SP, Burns P, Davis TM, Slovin JP, Bassil N, Hellens RP, Evans C, Harkins T, Kodira C, Desany B, Crasta OR, Jensen RV, Allan AC, Michael TP, Setubal JC, Celton JM, Rees DJ, Williams KP, Holt SH, Ruiz Rojas JJ, Chatterjee M, Liu B, Silva H, Meisel L, Adato A, Filichkin SA, Troggio M, Viola R, Ashman TL, Wang H, Dharmawardhana P, Elser J, Raja R, Priest HD, Bryant DW Jr, Fox SE, Givan SA, Wilhelm LJ, Naithani S, Christoffels A, Salama DY, Carter J, Lopez Girona E, Zdepski A, Wang W, Kerstetter RA, Schwab W, Korban SS, Davik J, Monfort A, Denoyes-Rothan B, Arus P, Mittler R, Flinn B, Aharoni A, Bennetzen JL, Salzberg SL, Dickerman AW, Velasco R, Borodovsky M, Veilleux RE, Folta KM (2011) The genome of woodland strawberry (*Fragaria vesca*). *Nature Genetics* 43:109-118.

Somssich IE, Schmelzer E, Kawalleck P & Hahlbrock K (1988) Gene structure and in situ transcript localization of pathogenesis-related protein 1 in parsley. *Molecular and General Genetics* 213:93–98.

Son DY, Scheurer S, Hoffmann A, Haustein D, Vieths S (1999) Pollen-related food allergy: cloning and immunological analysis of isoforms and mutants of Mal d 1, the major apple allergen, and Bet v 1, the major birch pollen allergen. *European Journal of Nutrition* 38:201–215.

Song J, Bradeen JM, Naess SK, Raasch JA, Wielgus SM, Haberlach GT, Liu J, Kuang H, Austin-Phillips S, Buell CR, Helgeson JP, Jiang J (2003) Gene RB cloned from Solanum bulbocastanum confers broad spectrum resistance to potato late blight. *Proceedings of the National Academy of Sciences of the United States of America* 100:9128–9133.

Sowik I, Bielenin A, Michalczuk L (2001) *In vitro* testing of strawberry resistance to *Verticillium dahliae* and *Phytophthora cactorum*. *Scientia Horticulturae* 88:31-40.

Sowik I, Michalczuk L, Wójcik D (2008) A method for *in vitro* testing strawberry susceptibility to *Verticillium*. *Journal of Fruit and Ornamental Plant Research* 16:111-121.

Spangfort MD, Ipsen H, Sparholt SH, Aasmul-Olsen S, Osmark P, Poulsen FM, Larsen M, Mortz E, Roepstorff P, Larsen JN (1996) Characterisation of recombinant isoforms of birch pollen allergen Bet v 1. *Advances in Experimental Medicine and Biology* 409:251–254.

Spangfort MD, Gajhede M, Osmark P, Poulsen FM, Ipsen H, Larsen JN, van Neerven RJ, Schou C, Løwenstein H (1997) Three-dimensional structure and epitopes of Bet v 1. *Internaional Archives of Allergy* and *Immunology* 113:243–245.

Srivastava S, Emery RJN, Kurepin LV, Reid DM, Fristensky B, Kav NNV (2006) Pea PR 10.1 is a ribonuclease and its transgenic expression elevates cytokinin levels. *Plant Growth Regulation* 49:17–25.

Srivastava S, Emery RJN, Rahman MH, Kav NNV (2007) A crucial role for cytokinins in pea ABR17- mediated enhanced germination and early seedling growth of *Arabidopsis thaliana* under saline and lowtemperature stresses. *Journal of Plant Growth Regulation* 26:26–37.

Stock JB, Stock AM, Mottonen JM (1990) Signal transduction in bacteria. *Nature* 344:395-400.

Street PFS, Robb J, Ellis BE (1986) Secretion of vascular coating components by xylem parenchyma cells of tomatoes infected with *Verticillium albo-atrum*. *Protoplasma* 132:1-11.

Subbarao KV, Kabir Z, Martin FN, Koike ST (2007) Management of soilborne diseases in strawberry using vegetable rotations. *Plant Disease* 91:964–972.

Sun L, Ghosh I, Barshevsky T, Kochinyan S, Xu MQ (2007) Design, preparation and use of ligated phosphoproteins: A novel approach to study protein phosphatases by dot blot array, ELISA and Western blot assays. *Methods* 42:220-226.

Swoboda I, Jilek A, Ferreira F, Engel E, Hoffmann-Sommergruber K, Scheiner O, Kraft D, Breiteneder H, Pittenauer E, Schmid E, Vicente O, Heberle-Bors E, Ahorn H, Breitenbach M (1995a) Isoforms of Bet v 1, the major birch pollen allergen, analyzed by liquid chromatography, mass spectrometry, and cDNA cloning. *Journal of Biological Chemistry* 270:2607–2613.

Swoboda I, Scheiner O, Heberle-Bors E, Vicente O (1995b) cDNA cloning and characterization of three genes in the Bet v 1 gene family that encode pathogenesis-related proteins. *Plant, Cell and Environment* 18:865-874.

Swoboda I, Hoffman-Sommergruber K, O'Riordain G, Scheiner O, Heberle-Bors E, Vicente O (1996) Bet v 1 proteins, the major birch pollen allergens and members of a family of conserved pathogenesis-related proteins, show ribonuclease activity *in vitro*. *Physiologia Plantarum* 96:433–438.

Take Y, Inouye Y, Nakamura S, Allaudeen HS, Kubo A (1989) Comparative studies of the inhibitory properties of antibiotics on human immunodeficiency virus and avian

myeloblastosis virus reverse transcriptases and cellular DNA polymerases. *Journal of Antibiotics* 42:107-115.

Takeuchi K, Gyohda A, Tominaga M, Kawakatsu M, Hatakeyama A, Ishii N, Shimaya K, Nishimura T, Riemann M, Nick P, Hashimoto M, Komano T, Endo A, Okamoto T, Jikumaru Y, Kamiya Y, Terakawa T, Koshiba T (2011) RSOsPR-10 expression in response to environmental stresses is regulated antagonistically by jasmonate/ethylene and salicylic acid signaling pathways in rice roots. *Plant & Cell Physiology* 52:1686-1696.

Tewari S, Brown SM, Kenyon P, Balcerzak M, Fristensky B (2003) Plant defense multigene families: II evolution of coding sequence and differential expression of PR-10 genes in Pisum. https://arxiv.org/abs/q-bio/0310038.

Tjamos SE, Flemetakis E, Paplomatas EJ, Katinakis P (2005) Induction of resistance to *Verticillium dahliae* in *Arabidopsis thaliana* by the biocontrol agent K-165 and pathogenesis-related proteins gene expression. *Molecular Plant–Microbe Interactions* 18:555–561.

Ton J, Flors V, Mauch-Mani B (2009) The multifaceted role of ABA in disease resistance. *Trends in Plant Science 14*:310–317.

Toyoda H, Horikoshi K, Yamano Y, Ouchi S (1991) Selection of *Fusarium* wilt disease resistance from regenerants derived from callus of strawberry. *Plant Cell Reports* 10:167-170.

Truman W, Bennett MH, Kubigsteltig I, Turnbull C, Grant RM (2007) *Arabidopsis* systemic immunity uses conserved defence signaling pathways and is mediated by jasmonates. *Proceedings of the National Academy of Sciences* 104:1075–1080.

Tulipani S, Mezzetti B, Capocasa F, Bompadre S, Beekwilder J, de Vos CH, Capanoglu E, Bovy A, Battino M (2008) Antioxidants, phenolic compounds, and nutritional quality of different strawberry genotypes. *Journal of Agricutural and Food Chemistry* 56:696-704.

Valério M, Gomes RS, Almeida M, Gomes AM, Neves AM (2015) Food Allergens - an Overview on Molecular Properties and Diagnosis. *Journal of Advances in Medical and Pharmaceutical Sciences* 3:52-60.

van Esse HP, Fradin EF, de Groot PJ, de Wit PJ, Thomma BP (2009) Tomato transcriptional responses to a foliar and a vascular fungal pathogen are distinct. *Molecular Plant-Microbe Interactions Journal* 22:245-258.

van Loon LC, Rep M, Pieterse CM (2006) Significance of inducible defense-related proteins in infected plants. *Annual Review of Phytopathoogy* 44:135-162.

Vanacker H, Foyer Ch, Carver TLW (1999) Changes in apoplast antioxidants induced by powdery mildew attack in oat genotypes with race non-specific resistance. *Planta* 208:444-452.

Vander Molen GE, Beckman CH, Rodehorst E (1977) Vascular gelation: A general response phenomenon following infection. *Physiological Plant Patholo*gy 11:95-100.

Vanek-Krebitz M, Hoffmann-Sommergruber K, Laimer da Camara Machado M, Susani M, Ebner C, Kraft D, Scheiner O, Breiteneder H (1995) Cloning and sequencing of

Mal d 1, the major allergen from apple (Malus domestica), and its immunological relationship to Bet v 1, the major birch pollen allergen. *Biochemical and Biophysical Research Communications* 214:538-551.

Vattem DA, Shetty K (2005) Biological functionality of ellagic acid: a review. *Journal of Food Biochemistry* 29:234–266.

Veloso J, Alabouvette C, Olivain C, Flors V, Pastor V, Garcia T, Diaz J (2016) Modes of action of the protective strain Fo47 in controlling *verticillium* wilt of pepper. *Plant Pathology* 65: 997–1007.

Veluri R, Weir TL, Bais HP, Stermitz FR, Vivanco JM (2004) Phytotoxic and antimicrobial activities of catechin derivatives. *Journal of Agricultural and Food Chemistry* 52: 1077-1082.

Vieths S, Schöning B, Peterson A (1994) Characterization of the 18-kDa apple allergen by two-dimensional immunoblotting and microsequencing. *International Archives of Allergy and Immunology* 104:399–404.

Vieths S, Jankiewicz A,Wuthrich B, Baltes W (1995) Immunoblot study of IgE binding allergens in celery roots. *Annals of Allergy, Asthma and Immunology* 75:48-55.

Vieths S, Scheurer S, Ballmer-Weber B (2002) Current understanding of cross-reactivity of food allergens and pollen. *Annals of the New York Academy of Sciences* 964:47-68.

Wang CS, Huang JC, Hu JH (1999) Characterization of two subclasses of PR- 10 transcripts in lily anthers and induction of their genes through separate signal transduction pathways. *Plant Molecular Biology* 40:807-814.

Wang D, Amornsiripanitch N, Dong X (2006) A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. *PLoS Pathogens* 2:e123.

Wang LS, Kuo CT, Cho SJ, Seguin C, Siddiqui J, Stoner K, Weng YI, Huang TH, Tichelaar J, Yearsley M, Stoner GD, Huang YW (2013) Black raspberry-derived anthocyanins demethylate tumor suppressor genes through the inhibition of DNMT1 and DNMT3B in colon cancer cells. *Nutrition and Cancer* 65:118−125.

Wang H, Cao G, Prior RL (1996) Total antioxidant capacity of fruits. *Journal of Agricultural and Food Chemistry* 44:701–705.

Wang SY, Jiao H (2000) Scavenging capacity of berry crops on superoxide radicals, hydrogen peroxide, hydroxyl radicals, and singlet oxygen. *Journal of Agricultural and Food Chemistry 48*:5677–5684.

Wang SY, Lin HS (2000) Antioxidant activity in fruits and leaves of blackberry, raspberry, and strawberry varies with cultivar and developmental stage. *Journal of Agricultural and Food Chemistry* 48:140-146.

Wang SY, Feng R, Lu Y, Bowman L, Ding M (2005) Inhibitory effect on activator protein-1, nuclear factor-kappaB, and cell transformation by extracts of strawberries (*Fragaria* × *ananassa* Duch.). *Journal of Agricultural and Food Chemistry* 53:4187–4193.

Wasternack C (2007) Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. *Annals of Botany* 100:681–697.

Wattad C, Dinoor A, Prusky D (1994) Purification of pectate lyase produced by *Colletotrichum gloeosporioides* and its inhibition by epicatechin: A possible factor involved in the resistance of unripe avocado fruits to anthracnose. *Molecular Plant-Microbe Interactions* 7:293-297.

Wen J, Vanek-Krebitz M, Hoffmann-Sommergruber K, Scheiner O, Breiteneder H (1997) The potential of Betv1 homologues, a nuclear multigene family as phylogenetic markers in flowering plants. *Molecular Phylogenetics and Evolutions* 8:317-333.

Wiese MV, Devay JE (1970) Growth Regulator Changes in Cotton Associated with Defoliation Caused by *Verticillium albo-atrum. Plant Physiology 45:304-309.*

Wilhelm S (1955) Longevity of the *Verticillium* wilt fungus in the laboratory and in the field. *Phytopathology* 45:180–181.

Winterbourn CC, Metodiewa D (1999) Reactivity of biologically important thiol compounds with superoxide and hydrogen peroxide. *Free Radical Biology and Medicine* 27:322-328.

Wu F, Li Y, Chang S, Zhou Z, Wang F, Song X, Lin Y, Gong W (2002) Purification, characterization and preliminary crystallographic studies of a PR-10 protein from Pachyrrhizus erosus seeds. *Acta Crystallographica Section D* 58:2165–2167.

Wu F, Yan M, Li Y, Chang S, Song X, Zhou Z, Gong W (2003) cDNA cloning, expression, and mutagenesis of a PR-10 protein SPE-16 from the seeds of Pachyrrhizus erosus. *Biochemical and Biophysical Research Communications* 312:761–766.

Xie DX, Feys BF, James S, Nieto-Rostro M, Turner JG (1998) COI1: an *Arabidopsis* gene required for jasmonate-regulated defense and fertility. *Science* 280:1091–1094.

Xie YR, Chen ZY, Brown RL & Bhatnagar D (2010) Expression and functional characterization of two pathogenesis-related protein 10 genes from Zea mays. *Journal of Plant Physiology* 167:121–130.

Yamaguchi-Shinozaki K, Shinozaki K (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. *Annual Review of Plant Biology* 57:781-803.

Yamamoto M, Nakatsuka, S, Otani H, Kohmoto K,Nishimura S (2000) (+)-Catechin acts as an infection-inhibiting factor in strawberry leaf. *Phytopathology* 90:595–600.

Yan Q, Qi X, Jiang Z, Yang S & Han L (2008) Characterization of a pathogenesis-related class 10 protein (PR-10) from Astragalus mongholicus with ribonuclease activity. *Plant Physiology and Biochemistry* 46:93–99.

Yen Y, Green PJ (1991) Identification and Properties of the Major Ribonucleases of *Arabidopsis thaliana*. *Plant Physiology* 97:1487-1493.

Yin Y, Li W, Son YO, Sun L, Lu J, Kim D, Wang X, Yao H, Wang L, Pratheeshkumar P, Hitron AJ, Luo J, Gao N, Shi X, Zhang Z (2013) Quercitrin protects skin from UVB-induced oxidative damage. *Toxicology and Applied Pharmacology* 269:89−99.

Yu XS, Ekramoddoullah AK, Misra S (2000) Characterization of Pin m III cDNA in western white pine. *Tree Physiology* 20:663-71.

Zare R, Gams W, Starink-Willemse M, Summerbell RC (2007) Gibellulopsis, a suitable genus for *Verticillium nigrescens*, and Musicillium, a new genus for V. theobromae. *Nova Hedwigia* 85:463–489.

Żebrowska J, Hortyński J, Cholewa T, Honcz K (2006) Resistance to *Verticillium dahliae* (Kleb.) in the strawberry breeding lines. *Communications in Agricultural and Applied Biological Sciences* 71:1031- 1036.

Żebrowska J (2011) Efficacy of resistance selection to *Verticillium* wilt in strawberry (*Fragaria x ananassa* Duch.) tissue. *Acta Agrobotanica* 64: 3–12.

Zhen XH, Li YZ (2004) Ultrastructural changes and location of beta-1,3-glucanase in resistant and susceptible cotton callus cells in response to treatment with toxin of *Verticillium dahliae* and salicylic acid. *Journal of Plant Physiology* 161:1367–1377.

Zhang Y, Seeram NP, Lee R, Feng L, Heber D (2008) Isolation and identification of strawberry phenolics with antioxidant and human cancer cell antiproliferative properties. *Journal of Agricultural and Food Chemistry* 56:670–675.

Zhang Z, Li Q, Li Z, Staswick PE, Wang M, Zhu Y, He Z (2007) Dual regulation role of GH3.5 in salicylic acid and auxin signaling during *Arabidopsis*-Pseudomonas syringae interaction. *Plant Physiology* 145:450–464.

Zhou XJ, Lu S, Xu YH, Wang JW & Chen XY (2002) A cotton cDNA (GaPR-10) encoding a pathogenesisrelated 10 protein with *in vitro* ribonuclease activity. *Plant Science* 162:629–636.

Zhu S, Gao F, Cao X, Chen M, Ye G, Wei C, Li Y (2005) The rice dwarf virus P2 protein interacts with ent-kaurene oxidases *in vivo*, leading to reduced biosynthesis of gibberellins and rice dwarf symptoms. *Plant Physiology* 139:1935–1945.

Ziadi S, Poupard P, Brisset MN, Paulin JP, Simoneau P (2001) Characterization in apple leaves of two subclasses of PR-10 transcripts inducible by acibenzolar-S-methyl, a functional analogue of salicylic acid. *Physiological and Molecular Plant Pathology* 59:33–43.

Zubini P, Zambelli B, Musiani F, Ciurli S, Bertolini P & Baraldi E (2009) The RNA hydrolysis and the cytokinin binding activities of PR-10 proteins are differently performed by two isoforms of the Pru p 1 peach major allergen and are possibly functionally related. *Plant Physiology* 150:1235–1247.

7 Appendix

>***Fra a 1.04***
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GAGGGAGGATCCATTGTTAAGAGCATCAGTAACTACTACACCATTGGTGATATTG
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>***gene07084***
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>***gene07087***
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>***gene32299***
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Figure 40. DNA sequences of the *Fra a 1* isoforms.

Fra a 1.04
Met G V F T Y K T E F T S V I P P P R L F K A F I L D A D N L I P E I A P Q A V K C A
E I L E G D G G V G T I K K I T F G E G S Q F G S V T H K I D G I D K E N F V Y S Y S
L V E G D A L S D K I E K I S Y E T K L V A S S D G G S V I K S T S N Y H T K G D V
E I K E E H V K A G K E K A S H L F K L V E D Y L L A N P N E Y C G S R S

Fra a 1.05
Met G V F T Y E T E F T S V I P P P R L F K A F I L D A D N L I P K I A P Q A V K C A
E I I E G D G G V G T I K K I T F G E G S Q F G S V T H K I D G I D K E N F V Y S Y S
L V E G D A L S D K I E K I S Y E T K L V S S S D G G S I I K S T S N Y H T K G D V E
I K E E H V K A G K E K A S H L F K L V E G Y L L A N P N E Y C R S

Fra a 1.06
Met G V F T Y E T E F T S V I P P P R L F K A F I L D A D N L I P K I A P Q A V K C A
E I V E G D G G V G T I T K I T F G E G S Q F G S V T H K I D G I D K E N F V Y S Y S
L V E G D A L S D K I E K I S Y E T K L V A S S D G G S V I K S T S N Y H T K G D V
E I K E E H V K A G K E K A S H L F K L V E D Y L L A N P N E Y C G S R S

Fra a 1.07
Met G V F T Y E T E F T S V I P P P R L Y K A F V L D A D N L I P K I A P Q A V K S T
E L V Q G D G G V G T I K K I H L G E G S E Y S Y V K H Q I D G L D K D N F V Y N
Y S I I E G D A I G D K V E K I S Y E I K L V A S P S G G S I I K S T S H Y H C K G E V
E I K E E H V K A G K E R A A G L F K I I E N Y L L G N P D A Y N G S R S

Fra a 1.08

Met G V F T Y E T E F T S V I P P P R L Y K A F V L D A D N L I P K I A P Q A V K S A
E I V Q G D G G V G T I K K I H L G E G S E Y S Y V K H Q I D G L D K D N F V Y N
Y S I I E G D A I G D K V E K I S Y E I K L V A S P S G G S I I K S T S H Y H C K G E V
E I K E E H V K A G K E K A A G L F K I I E N H L L A N P E A Y N G S R S

Figure 41. Amino acid sequences of Fra a 1.04-1.08.

Figure 42. Alignment of amino acid sequences of Fra a 1.04-1.08. Red underline indicate the P-loop motif and blue underline indicate the Bet v 1 motif.

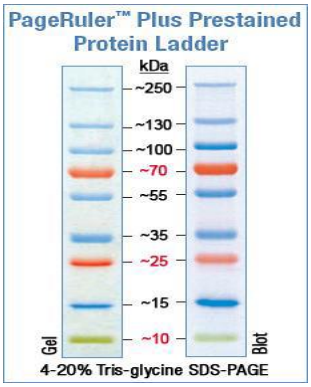


Figure 43. PageRuler Plus Prestained Protein Ladder (Fermentas, Germany).

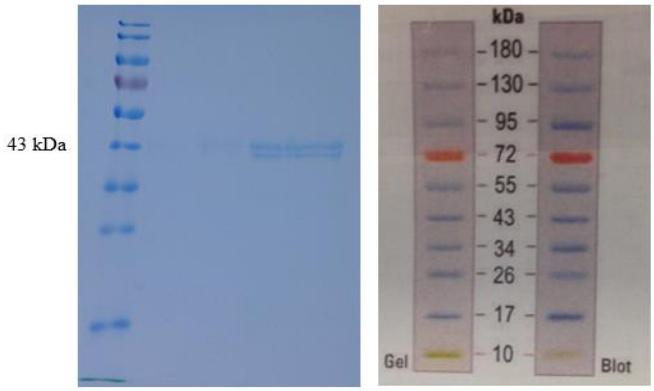


Figure 44. SDS-PAGE of pepsin showing a band at 43 kDa.

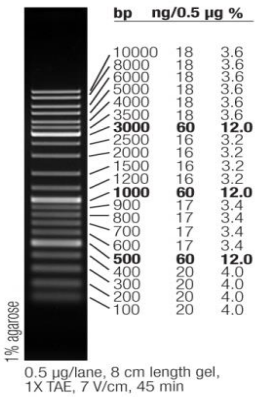


Figure 45. GeneRuler DNA Ladder Mix (Thermo scientific).

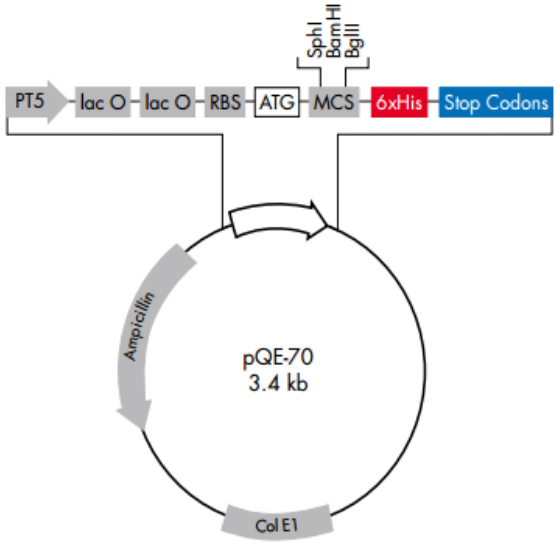
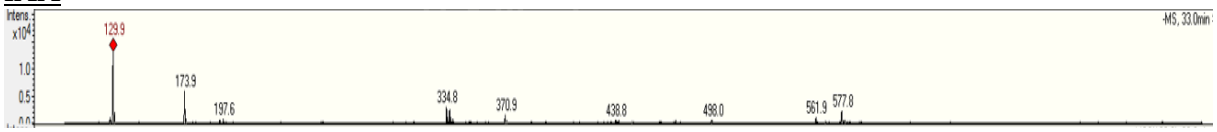


Figure 46. pQE70 vector.

GA

IAA



SA

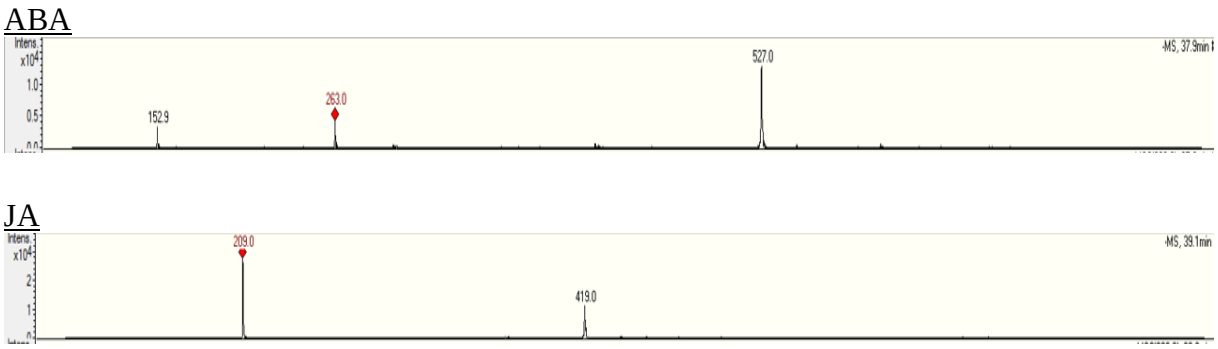


Figure 47. LC-MS analysis: Retention time of the five phytohormones GA, IAA, SA, ABA and JA.

Table 9. Relative expression values (± SD) of the 21 *Fra a 1* isoforms in leaves, young leaves, stems, roots, flowers and fruits (green, white and red).

	Leaves	**Young leaves**	**Stems**	**Roots**	**Flowers**	**Green fruits**	**White fruits**	**Red fruits**
Fra a 1.04	3,88 ±0,69	1	5,47 ±0,2	52,33 ±4,04	5,3 ±0,41	0,9 ±0,61	3,76 ±0,08	13,83 ±0,14
Fra a 1.05	4,53 ±3	1	2,91 ±0,59	42,11 ±3,76	4,21 ±3,1	1,12 ±0,4	4,71 ±0,75	80,1 ±8,32
Fra a 1.06	1,13 ±0,17	1	8,83 ±5,77	97,11 ±0,9	14,95 ±0,78	1,01 ±0,002	2,39 ±0,11	42,35 ±7,48
Fra a 1.07	2,92 ±0,75	1	3,84 ±1,08	69,1 ±4,75	1,52 ±0,19	2,58 ±0,725	3,31 ±2,52	24,71 ±4,43
Fra a 1.08	3,83	1	3,99 ±3,73	347, ± 0,96	5,53 ±0,61	1,39	0,79 ±0,24	82,96 ±3,39
07063	6,99 ±0,23	1	0,95 ±0,55	106,95 ±14,1	1,42 ±1,25	8,34 ±6,76	19,39 ±12,57	28,622 ±1,32
07084	12,53 ±6,35	1	2,27 ±0,02	50,24 ±7,33	4,07 ±0,87	3,42 ±0,11	0,98 ±0,16	11,89 ±5,37
11094	163,96 ±17,1	1	21,09 ±6,71	283,85 ±36,78	40,05 ±4,95	5,25 ±1,83	6,49 ±2,93	44,99 ±3,7
07085	109,47 ±16,59	1	60,69 ±2,60	275,31 ±8,63	20,89 ±5,46	8,76 ±0,42	11,76 ±8,17	33,46 ±4,14
07076	7,2 ±4,32	1	1,25 ±0,01	37,08 ±0,5	10,78 ±3,53	1,02 ±0,03	0,57 ±0,18	15,06 ±1,26
04962	5,37 ±0,45	1	3,17 ±0,77	20,68 ±2,86	4,44 ±0,53	0,12 ±0,06	2,39 ±1,15	3,15 ±0,67
05185	66,86 ±86	1	999,77 ±104,25	182670,36 ±29222	49 ,44± 5,7944	33,54 ±0,94	100,5 ±71,98	18,67 ±6,57
Fra a 1.01	108,39 ±15,4	1	212,48 ±5,18	287,42 ±16,36	18,50 ±7,82	13,12 ±2,46	26,9 ±2,48	2,5 ±0,04
Fra a 1.02	2,49 ±0,3	1	4,51 ±0,5	52,09 ±4,67	7,37 ±1,81	3,21 ±0,73	81,53 ±14,93	231,7 ±15,3
Fra a 1.03	1875,93 ±566,87	1	14,23 ±3,86	59,86 ±0,52	62,51 ±5,87	6,82 ±3,94	3,25 ±0,69	2,37 ±0,17
32299	30,58 ±6,38	1	7,5 ±0,65	25,45 ±15,96	136,6 ±9,23	6,49 ±0,36	1,43 ±0,19	3,19 ±0,9
07077	8,61 ±0,64	1	11,33 ±0,06	4,81 ±0,07	1,56 ±0,07	2,17 ±0,39	3,32 ±0,18	3,22 ±0,91

07078	1,35 ± 0,19	1	1,61± 0,006	0,39± 0,02	1,46± 0,14	1,14± 0,16	0,31± 0,05	0,17± 0,004
07087	0,67 ±0,2	1	2,11 ±0,1	1,02 ±0,06	0,62 ±0,13	0,53 ±0,2	0,6 ±0,05	0,34 ±0,02
07081	1,56 ±0,001	1	5,92 ±0,002	3,01 ±0,28	76,7 ±19,14	16,22 ±5,5	0,5 ±0,006	0,5 ±0,01
07083	9,85 ±1,06	1	0,69 ±0,001	4,15 ±0,06	0,61 ±0,25	5,2 ±0,02	0,1 ±0,005	017 ±0,008

Table 10. Relative content (± SD) of epicatechin, catechin, caffeic acid, citric acid, tryptophan and glutathione in infected (i) (with *V. dahliae*) and control (c) leaves (L), stems (S) and roots (R) at day 1, 5, 10, 20, and 30.

	Epicatechin	**Catechin**	**Caffeic acid**	**Citric acid**	**Tryptophan**	**Glutathione**
L1i	0,38 ±0,29	0,13 ±	0,17 ±0,02	0,09 ±0,009	0,23 ±	0,05 ± 0,0009
L1c	1,14 ± 0,27	2,48 ±	0,52 ±0,02	0,13 ±0,13	0,03 ±0,008	0,02 ±0,003
L5i	0,12 ±	0,07 ±	0	0	0	0
L5c	0,87 ± 0,21	0,17 ±	0,20 ±0,05	0,05 ±0,01	0,02 ±0,001	0,0008 ±4,2E-05
L10i	0,04 ±	0,17 ±	0,07 ±0,005	0	0,001 ±0,0002	0
L10c	1,02 ±0,92	0,9 ±	0,52 ±0,04	0,22 ±0,01	0,03 ±0,001	0,008 ±0,0005
L20i	3,67 ±3,37	2,76 ±	0,11 ±0,02	0,11 ±0,01	0,09 ±0,001	0,005 ±0,003
L20c	1,95 ±1,51	0,65 ±	0,2 ±0,0005	0,08 ±0,005	0,03 ±0,004	0,004 ±0,0003
L30i	6,70 ±2,63	3,01 ±	0,12 ±0,069	0,1 ±0,001	0,09 ±0,003	0
L30c	1,69 ±1,08	0,46 ±	0,2 ±0,03	0,2 ±0,006	0,005 ±0,0004	0,008 ±0,0008
S1i	0,31±0,04	11,58 ± 1,39	0,11 ±0,005	0,15 ±0,007	0,13 ±0,001	0
S1c	1,77 ±1,32	1,09 ±0,69	0,19 ±0,02	0,14 ±0,07	0,003 ±0,0003	0,002 ±0,002
S5i	3,02 ±2,04	2,22 ±0,46	0,06 ±0,03	0	0,009 ±0,0002	0,01 ±0,001
S5c	2,18 ±1,89	0,84 ±0,75	0,14 ±0,02	0,08 ±0,01	0,006 ±0,001	0,03 ±0,001
S10i	15,88 ±1,54	13,75 ±2,13	0,03 ±0,004	0,07 ±0,006	0,008 ±0,003	0,01 ±0,002
S10c	1,85 ±1,71	1,30 ±1,31	0,03 ±0,02	0,62 ± 0,02	0,03 ±0,009	0,01 ±0,0005
S20i	31,35 ±6,08	15,51 ±1,72	0,03 ±0,007	0,13 ±0,02	0,01 ±0,008	0,01 ±0,001
S20c	12,94 ±1,95	6,11 ±2,05	0,65 ±0,02	0,11 ±0,005	0,03 ±0,002	0,05 ±0,002

S30i	44,41 ±4,86	22,64 ±1,66	0,04 ±0,03	0	0,02 ±0,001	0
S30c	17,03 ±1,13	9,53 ±1,67	0,79 ±0,04	0,17 ±0,04	0,05 ±0,01	0,05 ±0,004
R1i	31,26 ±3,51	31,8 ±2,22	0,08 ±0,07	0	0,002 ±5E-05	0,003 ±0,0009
R1c	17,95 ±4,82	11,5 ±2,48	0,04 ±0,03	0,2 ±0,01	0,0003 ± 5,4E-05	0,03 ±0,0005
R5i	39,29 ±2,01	25,78 ±2,89	0,16 ±0,07	0	0,002 ±0,001	0,002 ±0,0002
R5c	21,66 ±0,82	16,47 ± 0,4	0,07 ±0,05	0,19 ±0,01	0,004 ±0,0005	0,003 ±0,002
R10i	38,44 ± 4,84	20,34 ±2,79	0,11 ±0,02	0,38 ±0,07	0,004 ±0,003	0,01 ±0,0005
R10c	14,05 ±1,72	7,25 ±0,38	0,1 ±0,02	0,12 ±0,01	0,002 ±0,001	0,06 ±0,0003
R20i	20,38 ±0,08	9,57 ±0,08	0,04 ±0,001	0,05 ±0,04	0,01 ±0,005	0,02 ±0,0006
R20c	26,86 ±0,06	14,94 ±0,99	0,1 ±0,02	0,11 ±0,09	0,01 ±0,007	0,02 ±0,004
R30i	90,59 8,85	36,83 ±2,39	0,42 ±0,03	4,51 ±0,31	0,04 ± 0,004	0
R30c	2,77 ±1,74	1,91 ±0,97	0,02 ±0,01	0,02 ±0,004	0,0005 ±1,91E-05	0,002 ±0,001

Table 11. Relative content (± SD) of abscisic acid (ABA), indole acetic acid (IAA), salicylic acid (SA), jasmonic acid (JA) and gibberellic acid (GA) in infected (i) (with *V. dahliae*) and control (c) leaves (L), stems (S) and roots (R) at day 1, 5, 10, 20, and 30.

	ABA	**IAA**	**SA**	**JA**	**GA**
L1i	0,001 ±8,2656E-05	0,89 ±0,05	0	0,004 ±0,00001	0,006 ±0,0002
L1c	0,0007 ±4,2186E-05	0,82 ±0,38	0	0,0016 ±0,0002	0,001 ±0,0001
L5i	0,09 ±0,004	2,79 ±0,31	0,09 ±0,004	0,15	0,20240867
L5c	0,002 ±0,0004	0,96 ±0,31	0	0	0
L10i	0,003 ±0,0001	12,42 ±0,9	0	0,001	0
L10c	0,01 ±0,0001	3,5 ±0,32	0,001 ±0,002	0,02 ±0,003	0,01 ±0,006
L20i	0,001 ±6,0399E-05	0,37 ±0,32	0,0002 ±0,0004	0	0,006 ±0,002
L20c	0,001 ±0,0002	6,67 ±1,26	0	0,0005 ±0,0008	0,002 ±0,0001
L30i	0,008 ±0,0006	1,47 ±0,44	0,001 ±0,001	0,04	0,002 ±0,001

L30c	0,01 ±0,008	0,71 ±0,27	0,0004 ±0,0006	0,005 ±0,004	0,001 ±0,0001
S1i	0	2,72 ±0,62	0,01 ±0,001	0,03	0
S1c	0,01 ±0,007	0,18 ±0,08	0,0016 ±0,002	0,002 ±0,002	0,004 ±0,0004
S5i	0,05 ±0,009	2,47 ±0,41	0,01 ±0,001	0,11 ±0,003	0,06 ±0,008
S5c	0,03 ±0,007	0,47 ±0,28	0,004 ±0,003	0,03 ±0,001	0,01 ±0,007
S10i	0,05 ±0,0002	0,42 ±0,23	0,003 ±0,003	0,06 ±0,001	0,004 ±0,002
S10c	0,02 ±0,005	1,88 ±0,89	0	0,005 ±0,005	0,003 ±0,0004
S20i	0,0007 ±4,0026E-05	0,49 ±0,53	0,0004 ±0,0007	0,0004 ±0,0006	0,0004 ±0,0003
S20c	0,07 ±0,01	0,42 ±0,19	0,003 ±0,0001	0,007 ±0,003	0,002 ±0,0004
S30i	0,18 ±0,01	0,55 ±0,58	0,0004 ±0,0006	0,02 ±0,004	0,01 ±0,0002
S30c	0,09 ±0,006	0,51 ±0,21	0,003 ±0,002	0,004 ±0,003	0,002 ±0,0002
R1i	0,004 ±0,002	0,15 ±0,07	0	0,005 ±0,0001	0,0006 ± 0,0006
R1c	0,03 ±0,0008	0,07 ±0,05	0,0004 ±0,0003	0,002 ±0,002	0,004 ±0,00004
R5i	0	0,37 ±0,35	0	0,02 ±0,005	0,0003 ±0,0005
R5c	0,0006 ±0,0003	0,07 ±0,06	0	0,004 ±0,0009	0,0016 ±0,0001
R10i	0,0018 ± 0,0004	0,44 ±0,4	0	0,0007 ±0,0006	0,0002 ±0,0003
R10c	0,0007 ±7,9463E-05	0,18 ±0,04	0,0004 ±0,0006	0,001 ±0,0004	0,0005 ±0,00006
R20i	0,006 ±0,003	0,42 ±0,46	0,001 ±0,001	0,05 ±0,004	0,004 ±0,00005
R20c	0,004 ±0,003	0,18 ±0,1	0	0,001 ±0,001	0,002 ±0,0002
R30i	0,005 ±0,002	2,55 ±0,32	0,002 ±0,0004	0,05 ±0,005	0,007 ±0,00007
R30c	0,0017 ± 0,001	0,17 ±0,14	0	0,005 ±0,005	0,0001 ±0,0002