Impact of biotic factors on the allergenic potential of tomato and identification of cyclophilin as a new putative tomato allergen

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There is no such thing as a failed experiment,
only experiments with unexpected outcomes.

Richard Buckminster Fuller

ZUSAMMENFASSUNG

Die Tomate ist weltweit eines der am meist konsumierten Gemüse, doch der hohe Verzehr erhöht auch das Risiko einer allergischen Reaktion. Zu den Allergenen zählen viele Proteine der Pflanzenabwehr, welche durch verschiedenste biotische und abiotische Stressoren induziert werden. Allerdings ist bis heute der Einfluss biotischer Faktoren auf die Allergenität von Obst und Gemüse weitestgehend unbekannt. Diese Dissertation befasst sich mit den Auswirkungen der symbiontischen Mykorrhiza und des pathogenen Pepino Mosaikvirus auf die Allergenität von Tomaten.

Früchte von mykorrhizierten Tomatenpflanzen wiesen eine höhere Expression allergenkodierender Gene auf als die nicht mykorrhizierten Kontrollen, allerdings übertrug sich dies nicht auf die Allergenität der Tomaten. Pepino Mosaikvirus-infizierte Tomatenpflanzen zeigten keine generelle Aktivierung der Allergenexpression und auch keine erhöhte Allergenität. Aufgrund großer individueller Reaktionsunterschiede der Probanden in den klinischen Allergietests konnten einzeln auftretende Unterschiede nicht über die Gesamtheit der getesteten Allergiker abgesichert werden. Die individuellen Reaktionen der Probanden konnten wiederum nicht auf die Anzahl und Identität der Allergene von Tomatenpflanzen mit unterschiedlichen genetischen Hintergründen oder aus verschiedenen Anbauweisen zurückgeführt werden. Während der Experimente wurden 13 neue putative Tomatenallergene identifiziert unter denen das Cyclophilin rekombinant in Escherichia coli hergestellt und seine allergene Aktivität bestätigt wurde.

Schlussfolgernd nahmen biotische Faktoren keinen nennenswerten Einfluss auf die Allergenität von kommerziell angebauten Tomaten. Über dies hinaus wurden neue Erkenntnisse über persistente Virus-Pflanzen Interaktionen gewonnen. Schließlich konnte die Liste der putativen Tomatenallergene um einige Kandidaten erweitert werden, von denen das Cyclophilin als Allergen bestätigt wurde.

Schlagwörter: Solanum lycopersicum, Pepino Mosaikvirus, Mycorrhiza, Tomatenallergie, Allergene, Cyclophilin

ABSTRACT

Tomatoes are now among most consumed vegetables worldwide; unfortunately accompanied by an increasing risk of allergic reactions. Pathogenesis-related proteins can act as allergens and are induced by various biotic and abiotic stresses. Up to now nearly nothing is known about the impact of biotic factors on allergenic potentials. This thesis investigates the allergenicity of symbiotically mycorrhized and Pepino mosaic virus-infected tomato fruits.

Although induced allergen-encoding gene expression was detected in the fruits of mycorrhizal tomato plants, there was no impact on allergenicity. In contrast, general induction of defence-related allergens in Pepino mosaic virus-infected tomato fruits was not observed. Consequently, clinical allergy tests did not reveal any generally increased allergenic potential of Pepino mosaic virus-infected tomato fruits. High inter-individual differences in clinical allergy tests made it difficult to make statistically confirmed statements about the allergenicity of colonised tomato fruits. However, the hypothesis that such individual variability is based on differential reactions of individual subjects to particular allergens in tomato fruits, from plants with certain genetic backgrounds or cultivated under distinct conditions, had to be rejected. During these investigations, 13 new putative tomato allergens were identified. One of the candidates, cyclophilin, was recombinantly produced in Escherichia coli and its allergenic activity was confirmed in different clinical allergy tests with tomato-allergic subjects.

In conclusion, this study demonstrates that biotic factors are only of minor importance for the allergenic potential of commercially produced tomatoes. Moreover, the experiments revealed new insights into persistent plant-virus interactions. In particular, these extended the list of putative tomato allergens to include new candidates, and confirmed the allergenic activity of one of these, namely cyclophilin.

Keywords: Solanum lycopersicum, Pepino mosaic virus, mycorrhiza, tomato allergy, allergens, cyclophilin

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ABBREVIATIONS

allergen/protein name – respective protein

allergen/protein name – gene coding for respective allergen/protein

1D – 1-dimensional

2D – 2-dimensional

2DE – 2-dimensional electrophoresis

AC_{30} – value which indicates the activation of 30% of basophils

AM – arbuscular mycorrhizal fungus

ANOVA – analysis of variance

AP – alkaline phosphatase

ATP – adenosine triphosphate

BAT - basophil activation test

BDT – basophil degranulation test

bp – base pair

BSA – bovine serum albumin

CCD – cross reactive carbohydrates

cDNA – copy deoxyribonucleic acid

CDS – coding sequence

CNRQ – calibrated, normalised, relative quantity (relative expression unit of the qBase^{+} software)

Ct – cycle threshold

CTP – cytidine triphosphate

CV – coefficient of variance

CYC - cyclophilin

DNA – deoxyribonucleic acid

DBPCFC – double blind placebo controlled food challenge (provocation test)

DC – value which indicates the degranulation of 30% of basophils

DEPC – Diethylpyrocarbonate

dH_2O – distilled water

dNTP – deoxyribonucleic acid triphosphates

DTT – Dithiothreitol

E. coli – *Escherichia coli*

EAACI – European Academy of Allergology and Clinical Immunology

EC – electric conductivity

ECL – enhanced chemilumescent

EDTA – Ethylenediaminetetraacetic acid

ELISA – enzyme-linked immunosorbent assay

ET – Ethylene

f – female

for – forward

GTP – guanosine triphosphate

Gu – Guanidine

HRP – horse radish peroxidase

IgE – Immunoglobulin E
IGZ – Leibinz-Institute of Vegetable and Ornamental Crops
IL – interleukin (cytokines)
IPTG – Isopropyl-β-D-thiogalactopyranosid
ISR –induced systemic resistance
iTRAQ – isobaric tag for relative and absolute quantification
IUIS – International Union of Immunological Societies
JA – jasmonate
kDa – kilo Dalton
LB – lysogeny broth
M_{ES} – gene stability measure
mRNA – messenger ribonucleic acid
MS – mass spectrometry
MW – molecular weight
NA – non-allergic subject
NC – negative control
NCBI – National Center for Biotechnology Information
nd – not determined
NiNTA – Nickel nitrilotriacetic acid
NP40 – anionic detergent
OD – optical density
org N – organic nitrogen
PBS – phosphate buffered saline
PCR – polymerase chain reaction
PEG – Polyethylene glycol
PepMV – *Pepino mosaic virus*
PepMV CH2 – *Pepino mosaic virus* Chilean isolate
PepMV EU – *Pepino mosaic virus* European isolate
PepMVCP – *Pepino mosaic virus* coat protein
pI – isoelectric point
POD – peroxidase
PR-protein – pathogenesis-related protein
qRT-PCR – quantitative real-time reverse transcriptase polymerase chain reaction
rCYC – recombinant cyclophilin
rev – reverse
Rn – raw fluorescence (qBase^{+})
RNA – ribonucleic acid
rRNA – ribosomal ribonucleic acid
SA – salicylic acid
SAR – systemic acquired resistance
SDS – Sodium dodecyl sulphate
SDS-PAGE – Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SDT – Sodium thioglycolat
SOC – super optimal broth

Sola l – *Solanum lycopersicum*
SPT – skin prick test
TAE – Tris-acetate-EDTA
TBS – Tris buffered saline
TCA – Tris-citric acid
TEMED – Tetramethylethylenediamine
Th cell – T-helper cell
Tris – Tris(hydroxymethyl)aminomethane
TTP – thymidine triphosphate
U – unit
UV – ultra violet
VEGAL – project: Vegetable Allergies
v/v – volume/volume
WPI – weeks post PepMV inoculation
WS – week storage

1. INTRODUCTION

1.1 Tomato (*Solanum lycopersicum, Lycopersicon esculentum*)

Tomato belongs to the nightshade family (Solanaceae) and therefore is closely related to potato, pepper, and eggplant, but also to e.g. tobacco and petunia. As an Angiosperm, tomato was first described in the *Solanum* genus as *Solanum lycopersicum* by Linnaeus in 1753 (Linnaeus, 1753), but later classified into the genus *Lycopersicon* as *Lycopersicon esculentum* (Miller, 1768). To date the classification of tomato is still discussed. Previous studies referring to taxonomic and genomic data again put the tomato into the *Solanum* genus (Heiser and Anderson, 1999;Peralta and Spooner, 2000). Tomato originates from the Andean region and is a native of Ecuador and Peru. The first extensive domestication probably started in Mexico and tomato was brought to Europe in the 16th century (Sims, 1980;Costa and Heuvelink, 2005). Through extensive breeding a huge selection of varieties and cultivars, producing tomato fruits with a wide range of shapes, sizes, and colours, has been developed (Dorais *et al.*, 2001;Foolad, 2007). In addition to morphological traits cultivars with high productivity, improved fruit quality, resistances, and tolerances to biotic and abiotic stressors, were selected.

The cultivated tomato is a perennial plant, but today mostly cultivated annually. It is grown worldwide in open fields or greenhouses, depending on the respective climate conditions and on its further use as fresh-market fruits or for additional processing. Most of commercially greenhouse grown tomatoes are produced in soilless hydroponic systems with perlite or rockwool. In hydroponic cultivation plants are constantly supplied with an optimised nutrient solution. Staking is normally used and fruits are mainly harvested by hand as green or red-ripe fruits, depending on further processing (Heuvelink *et al.*, 2005).

Tomato belongs to the most important vegetables worldwide, also because of the various popular tomato products, like pastes, juices, sauces, dried tomatoes, ketchup, and many others. The global tomato production in 2011 was almost reaching 160 million tonnes (159,023,383.3 t) and tomato was globally one of the most consumed vegetables in 2009 (http://faostat.fao.org). In Germany tomato consumption reached almost 25 kg/capita/year, and therefore, was the German's most favourite vegetable in 2011 (www.aid.de).

1.1.1 A healthy fruit - Tomato fruit composition

Red-ripe tomatoes consist of 93% water and 5 - 8% dry matter. The dry matter of tomato compromises glucose and fructose (each around 22%, but together can reach up to 50%), a small amount of sucrose (~1%), about 25% of proteins, pectins, hemicelluloses and celluloses, and 8% minerals, mainly potassium, calcium, magnesium, and phosphate. Titratable and organic acids account for approximately 14% of the dry matter (Saltveit, 2005). The tomato flavour mainly depends on the amount and ratio of sugars to organic acids and also on the composition of aroma volatiles (Krumbein and Auerswald, 1998;Saltveit, 2005).

Tomato fruits are a good source of vitamins, especially for vitamin A and C. Vitamin A is provided from the fruit in the form of β-carotene, a precursor of vitamin A. The most abundant carotenoid in

tomato fruits is lycopene (Krumbein *et al.*, 2006), and the predominant source of lycopene in human diet are tomatoes (Nishino *et al.*, 2002). Lycopene is known to protect cells against reactive oxygen species which might damage lipids, DNA, and proteins in human cells. The protection of DNA is probably the reason for the anti-carcinogenic effect which has been described for lycopene (Nishino *et al.*, 2002;Stahl and Sies, 2005).

1.1.2 Tomato as a model species

In contrast to other model species such as e.g. *Arabidopsis*, tomato is a fruit producing crop with a sympodial shoot. Tomato plants are easy to cultivate nearly everywhere on earth. They have a high self-fertility, a great reproductive potential, a short life cycle, and can be easily manipulated through e.g. grafting, hybridisation, or genetic transformation (Heuvelink *et al.*, 2005;Foolad, 2007). A lot of mutants are available and the genome has recently been sequenced (Sato *et al.*, 2012). Altogether, these features describe the tomato plant as an ideally suited model organism to study on and to transfer this knowledge to other commercially important plants in the Solanaceae family.

Transfer to this study

As outlined above the tomato fruit is one of the most important vegetables worldwide, therefore, it is imperative to further investigate its allergenic potential. Tomato represents a suitable model organism and many genes are known, simplifying the work on molecular basis, like e.g. RNA accumulation analyses. Many patho-systems are described for tomato and also the mutualistic symbiosis with arbuscular mycorrhizal fungi is well-investigated; this put our work into a well-established context and led us to our working hypotheses.

1.2 Fungal symbionts of tomato - Mycorrhizal fungi

One of the most wide-spread and best described symbiosis is that of plant roots with mycorrhizal fungi. 80% of all land plant species are living in such a symbiosis (Smith and Read, 2008) and also tomato is able to form and to profit from the mutualistic interaction with arbuscular mycorrhizal fungi (AM, Glomeromycota). In contrast to ectomycorrhizas, AM belong to the endomycorrhizas: AM fungal hyphae penetrate cell walls of root cortex cells and form tree-like structures, so-called arbuscules inside the apoplast (Parniske, 2008).

Mutualism implies that two different species are cohabiting and both benefiting from each other. In the AM symbiosis the plant is feeding the obligate biotrophic mycorrhiza with photosynthetically produced carbohydrates and in exchange the fungus helps the plant with its fine and extensive hyphal network to take up mineral nutrients from soil (George *et al.*, 1995;Solaiman and Saito, 1997;Bago *et al.*, 2003;Finlay, 2008).

The environmental pollution with fertilisers from agriculture is an increasing problem. The application of mycorrhizal fungi could be an alternative to conventional fertilisers since they support the plants to take up nutrients also from meagre soils (Gianinazzi *et al.*, 2010). Different mycorrhiza inocula are on the market and already used in modern agri- and horticulture. However, the successful interaction between mycorrhizal fungi and their host species depends on, and is influenced by genetic constrains of the partners and the respective environmental conditions.

Therefore, costs versus benefits have to be carefully calculated when using mycorrhizal fungi in agri- and horticultural practice (Sawers *et al.*, 2008).

Transfer to this study

Mycorrhiza is a widely distributed symbiosis all over the world and also crop plants profit from these interactions. Mycorrhized tomato plants are quite well characterised, even if tomato is often commercially cultivated in soilless culture where mycorrhiza only plays a minor role. The whole metabolism of the tomato plant is impacted and also fruit composition can be influenced through mycorrhizal fungi (Salvioli *et al.*, 2012). Therefore, it is essential to also investigate the influence of mycorrhization on the allergenic potential of tomato fruits.

1.3 Viral diseases of tomato

Viral pathogens are quite dangerous because they can quickly evolve and adapt. Due to their large population size, short generation times, and their high mutation and recombination rate, it is difficult to confine viral diseases (Moya *et al.*, 2004). No antiviral products are available on the market for commercial plant cultivation systems. Besides abolishment through proper hygienic control, the only reliable method to protect plants from viral disease is to introduce resistances (Hanssen *et al.*, 2010b). Many natural existing resistances probably got lost through extensive breeding. Already 136 viral species are described just for tomato (Brunt *et al.*, 1996). This big number of viruses probably exists due to monocultural tomato production in greenhouses under controlled conditions. These conditions perfectly facilitate viral survival and spread, and therefore, many viral species became endemic. The two major tomato-infesting viruses are the begomoviruses and the *Pepino mosaic virus* (Hanssen *et al.*, 2010b).

1.3.1 *Pepino mosaic virus* - Characteristics and genome diversity

The *Pepino mosaic virus* (PepMV), a Potexvirus, belongs to the family of Flexiviridae. Flexiviridae are single-stranded positive RNA plant viruses. The genome of PepMV is 6.4 kB in size and possesses five open reading frames, encoding a RNA-dependent RNA polymerase, three triple gene block proteins, and a coat protein (Aguilar *et al.*, 2002). PepMV particles perform non-enveloped flexuous rods from 508 nm (Jones *et al.*, 1980). Today four PepMV genotypes are described:

- The Peruvian genotype (LP), the original isolate from Pepino (Jones *et al.*, 1980) and also called the Pepino isolate, is distinct from all other following tomato isolates (Mumford and Metcalfe, 2001;van der Vlugt *et al.*, 2002;Verhoeven *et al.*, 2003).
- The European genotype (EU), first isolated in European tomatoes (Mumford and Metcalfe, 2001;Verhoeven *et al.*, 2003;Pagan *et al.*, 2006).
- The American genotype (US1), first isolated from tomatoes of the US (Maroon-Lango *et al.*, 2005).
- The Chilean genotype (CH2), first isolated in Chilean tomatoes (Ling, 2007).

1.3.2 *Pepino mosaic virus* - Occurrence and transmission

PepMV was first isolated in 1973 from Pepino (*Solanum muricatum,*(Jones et al., 1980)). It mainly affects plants from the Solanaceae family, like e.g.: tomato, eggplant, potato, pepper, but also tobacco and thornapple (Jones *et al.*, 1980;Martin and Mousserion, 2002;Salomone and Roggero,

2002;Soler *et al.*, 2002;Verhoeven *et al.*, 2003). In addition, PepMV infection was described in non-solanaceaeous species, like e.g. in basil (Davino *et al.*, 2009) and in weeds (Cordoba *et al.*, 2004). Tomato is still its main host and PepMV was first detected on tomato plants in 1999 in the Netherlands (van der Vlugt *et al.*, 2000). Henceforward, PepMV successfully spreads through all major tomato-producing countries in Europe, North-, Central-, and South America, Africa, and Asia. Today PepMV infection is globally one of the major diseases of greenhouse tomatoes worldwide (Hanssen *et al.*, 2010b).

Highly infectious PepMV virus particles are easily, mechanically transmittable (Jones *et al.*, 1980). Insects, like e.g. bumblebees, which are used for pollination in commercial tomato production, can be transmitters of PepMV (Shipp *et al.*, 2008). PepMV can be passed from plant to plant through normal production procedures and handlings, and even transmission through nutrient solution was observed (Schwarz *et al.*, 2010a;Spence *et al.*, 2006). In contrast, seed transmission was shown to be comparably low (below 2%, (Hanssen and Thomma, 2010)).

1.3.3 *Pepino mosaic virus* - Symptoms on tomato plants and virus control

Symptoms of PepMV disease on tomatoes can be observed on the whole plant and, depending on the viral isolate, a wide range of symptoms can occur (figure 1.1). Plants infected with mild isolates sometimes show no symptoms at all; on the contrary infection with aggressive isolates leads to serious modifications in vegetative growth and yield losses of up to 50% (Hanssen *et al.*, 2008;Hanssen *et al.*, 2009;Hasiow-Jaroszewska *et al.*, 2009). PepMV symptoms on the plants are nettle-heads, leaf bubbling, and premature leaf senescence. Fruits from infected plants can show marbling, flaming, open fruits, and the necrosis of sepals (Hanssen *et al.*, 2009). The occurrence of symptoms after PepMV infection depends not only on the isolate, but might also be enhanced under inappropriate environmental conditions (Jorda *et al.*, 2001;Soler-Aleixandre *et al.*, 2005;Spence *et al.*, 2006). Infection in a later developmental status of the plant also revealed to influence the symptom characteristics and often showed more severe symptoms on fruits (Spence *et al.*, 2006;Hanssen *et al.*, 2008).

Figure 1.1: Differential symptomology of tomato plants after infection with *Pepino mosaic virus*: Open, flamed, and marbled fruits, nettle-heads and leaf bubbling.

Risk exposure of PepMV infection can be minimised by following proper hygienic standards. Another strategy to prevent high plant and yield loss through PepMV infection is a strategy called cross-protection. It was first described by McKinney in 1929. He showed that pre-infection of tobacco plants with a mild isolate of *Tobacco mosaic virus* prevented the plants from disease symptoms of a subsequent inoculated aggressive isolate (Mckinney, 1929). Today, this cross-protection is applied to vaccinate plants and to protect them from aggressive viral isolates (Lecoq *et al.*, 1991). However, vaccination has to be strictly controlled and applied with care, because vaccination with the wrong genotype can result in even higher symptom severity (Hanssen *et al.*, 2010a). Besides unofficial

treatments with mild PepMV isolates, occurring since a few years, controlled vaccination is already officially approved and applied in commercial cultivation in Belgium and probably soon in the Netherlands (personal contact to Inge Hanssen, Scientia Terrae, Belgium, (Jones and Lammers W., 2005)).

Transfer to this study

PepMV is one of the major diseases in greenhouse tomato crops worldwide. For this reason and due to cross-protection strategies to control viral disease, tomatoes naturally or intentionally infected with PepMV are commercially available in the supermarkets (Schwarz *et al.*, 2010a). Since little is known about the reaction of fruits to virus particle spread there is a certain need to investigate the impact of PepMV on the allergenic potential of tomato fruits.

1.4 Plant defence

Plants are sessile organisms, and therefore, had to evolve different strategies to react to their natural surroundings from which they cannot escape. This requires for example an elaborated protection against adverse environmental conditions, meaning abiotic stressors, like drought, osmotic, light, and temperature stress. In addition, numerous biological factors provoke defence responses of the plant, among those elicitors, e.g. attacks by viruses, fungi, bacteria, nematodes, or herbivores (biotic stressors) occur. These plant pathogens can be divided into biotrophic pathogens, describing those which require a living host, e.g. viruses and fungi; and necrotrophic pathogens, e.g. herbivores causing wounding and potentially even kill the plant (Walters, 2011).

Interactions between plants and pathogens can be compatible or incompatible, depending on host resistance and pathogen virulence. Plant pathogens might be:

- non-pathogenic, meaning that they are not able to cause a disease, because the plant is a non-host and therefore not susceptible. The interaction is incompatible.
- avirulent, meaning that the pathogens recognise the plant as host but with insignificant effects, because the host is resistant and therefore not susceptible. The interaction is incompatible.
- virulent, meaning that they have significant effects on the plant which is susceptible for the pathogen. The interaction is compatible (Walters, 2011).

Plants protect themselves through permanent barriers, e.g. thorns, hairs, waxes, or constitutively produced anti-microbial secondary metabolites. Additionally, they evolved inducible defence mechanisms. Activation of inducible defence mechanisms takes place when specific plant receptors recognise specific elicitors, coming from pathogens (pathogen- or microbe-associated molecular patterns) or recently damaged plant tissue (damage-associated molecular patterns). Upon pathogen recognition a signal transduction cascade activates the production of endogenous signalling compounds and leads to local and systemic activation of genes (Thomma *et al.*, 2001). Inducible mechanisms, appearing at restricted locations where the pathogen attack took place, include e.g. lignification of certain plant parts, cell wall rigidification, the emission of anti-microbial phytoalexins, and the accumulation of defence- and pathogenesis-related (PR) proteins (Somssich and Hahlbrock, 1998;Pieterse *et al.*, 2009;Walters, 2011). A particular defence mechanism is the hypersensitive response. After the recognition of biotrophic pathogens the plant reacts with the liberation of reactive oxygen species and forms local necrotic lesions, resulting in local programmed cell death in order to keep the pathogen isolated from the rest of the plant (Dewit, 1997). This hypersensitive

response is potentially not active against necrotrophic pathogens, as it would favour their growth (Murphy *et al.*, 1999;Glazebrook, 2005).

Plant reactions following pathogen attacks include a complex interaction of signal molecules, like phytohormones or bioactive peptides, such as systemin, and the induction and expression of PR-genes and -proteins (Thomma *et al.*, 2001;Kunkel and Brooks, 2002). The phytohormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are mainly regulating the effective defence response of plants. Auxins, abscisic acid, gibberellins, and cytokines seem to play minor roles and are less well studied (Koornneef and Pieterse, 2008;Bari and Jones, 2009;Pieterse *et al.*, 2009). However, the communication between plants and their environment do not only include interactions with pathogens but also with beneficial organisms, wherefore plants evolved strategies to distinguish whether the respective colonising organism is profitable or ill-natured (Pieterse and Dicke, 2007).

1.4.1 Induced resistance - The plant's immune system

Induced resistance describes the phenomenon that plants infected with a pathogen or colonised with a symbiont became resistant to a secondary infection with a pathogenic organism. Plants can achieve enhanced resistance through virulent, avirulent, and non-pathogenic microorganisms or even through chemical agents (Kavroulakis *et al.*, 2006).

After the first pathogen attacked a plant systemic acquired resistance (SAR) can be established (van Loon *et al.*, 1998;Pieterse and van Loon, 1999). SAR is associated with an increase of SA which has shown to be a key player in SAR and, in turn, activates the expression of anti-microbial PR-proteins (Malamy *et al.*, 1990;Metraux *et al.*, 1990;Gaffney *et al.*, 1993). SAR and plant immunity can last several weeks and protect the plant against further biotic or abiotic stresses (Ryals *et al.*, 1996;Thomma *et al.*, 2001;Hull, 2002).

It has been shown that plants colonised by adjuvant plant growth-promoting bacteria or fungi possess an enhanced resistance against certain pathogens through systemically activated defence mechanisms (AzconAguilar and Barea, 1996;van Driesche and Bellows, 1996;Ongena *et al.*, 1999;Pozo *et al.*, 2002;Pozo *et al.*, 2004). This induced systemic resistance (ISR), occurring after colonisation of non-pathogenic organisms, is mainly controlled by JA and ET (Pieterse *et al.*, 1998). In contrast to SAR, ISR is SA-independent and PR-proteins are not necessarily accumulated (Hoffland *et al.*, 1996;Pieterse *et al.*, 1996;van Wees *et al.*, 1999). However, a so-called priming effect leads to faster and higher defence-related gene expression in ISR and upon later challenge inoculation with a pathogen (Conrath *et al.*, 2002). In a recent genome wide characterisation, differentially regulated genes in ISR were found not only from ET and JA pathways, but also from SA pathway, including PR-protein-encoding genes (Mathys *et al.*, 2012).

Altogether, plants evolved complex regulation systems to react to their outer environment. This includes negative and positive cross-talk not only between SA, JA, and ET, but also between other plant hormones. This became necessary because plants are normally exposed to a lot of different pathogenic or beneficial microorganisms simultaneously. So far it is not well-understood how hormone-regulated, developmental, and defence-related responses are regulated in the different plant organs (Bari and Jones, 2009).

1.4.2 Induced systemic resistance in plants after mycorrhization

Plants, living in a symbiosis with arbuscular mycorrhiza, can be more tolerant to drought stress and might have an increased resistance to pathogens (Hause and Fester, 2005). It is known that mycorrhizal colonisation starts with an increase of SA in the plant. In case of a compatible interaction between the mycorrhizal fungi and the plant SA decreases and after the fungus is performing arbuscules JA increases (Blilou *et al.*, 1999;Pozo and Azcon-Aguilar, 2007). Many studies showed local expression of defence-related hydrolytic enzymes and PR-proteins, like chitinases and glucanases, in mycorrhized plant roots. These enzymes are able to hydrolyse cell walls of fungi and might be part of a constitutive induced resistance against root pathogens (DumasGaudot *et al.*, 1996;Lambais and Mehdy, 1998;Pozo *et al.*, 1998;Pozo *et al.*, 1999;Pozo *et al.*, 2002). However, mycorrhiza-induced resistance has also been shown in upper plant parts and is possibly due to priming of defence- and PR-protein expression (Gernns *et al.*, 2001;Lingua *et al.*, 2002;Garmendia *et al.*, 2004;Fritz *et al.*, 2006). In a transcriptomic analysis the systemic induction of defence- and PR-protein-encoding genes, and a better resistance against the fungus *Magnaporthe oryzae*, was shown after mycorrhization in rice (Campos-Soriano *et al.*, 2012). This has been shown before in a *Medicago truncatula*-mycorrhiza symbiosis and more than 500 genes were differentially regulated even in shoots, at once being more resistant against the bacterial pathogen *Xanthomonas campestris* (Liu *et al.*, 2007). Interestingly, some PR-proteins seem to be induced even stronger in mycorrhiza defective mutants ('RMC') compared to the corresponding wild types after mycorrhiza colonisation, which might be due to certain defence mechanisms, restricting colonisation in mutants (Gao *et al.*, 2004).
Summarising, mycorrhization of plants can lead to local and systemic induced resistance, including the induction of defence- and PR-proteins (figure 1.2).

1.4.3 Systemic acquired resistance in plants after virus infection

Viruses can provoke a lot of alterations in host gene expression and metabolism (Whitham *et al.*, 2003;Whitham *et al.*, 2006). Changes in leaf protein pattern after virus infection of tobacco plants, showing a hypersensitive response, have already been discovered in 1970 by Gianinazzi and in parallel by van Loon and van Kammen (Gianinazzi *et al.*, 1970;van Loon and van Kammen, 1970). These virus-induced proteins were later termed as pathogenesis-related proteins (PR-proteins) by Antoniw (Antoniw *et al.*, 1980). Induction of PR-protein expression is part of SAR. SAR, as a response to virus attacks, was first described by Ross in 1961 and afterwards pathogen resistance after virus infestation has been shown many more times (Anfoka and Buchenauer, 1997;van Loon and van Strien, 1999;Jeun and Buchenauer, 2001;Laird *et al.*, 2004;Park *et al.*, 2004;van Loon *et al.*, 2006;Farrag *et al.*, 2007).
Virus infection leads to different host responses. Besides SA accumulation and the activation of defence-related genes, an accumulation of heat shock proteins and certain silencing suppressors also exist (Whitham *et al.*, 2006). The activation of PR-proteins protects the plant against further pathogen attacks from fungi or bacteria, whereas the other defence strategies decrease further viral replication and movement, thereby circumventing secondary virus infection (Pennazio and Roggero, 1998;Murphy *et al.*, 1999;Jameson and Clarke, 2002;Hanssen *et al.*, 2010a). Typical virus-induced PR-proteins are 1,3-β-glucanases, chitinases, osmotin-like proteins, and peroxidases (Bol *et al.*, 1990;Elvira *et al.*, 2008). Recently, it has been shown that also the infection of tomato plants with PepMV showed virus-enhanced expression of defence- and PR-genes (Hanssen *et al.*, 2011).

Concluding, virus infestation in plants can lead to systemic acquired resistance, including the induction of defence- and PR-proteins (figure 1.2).

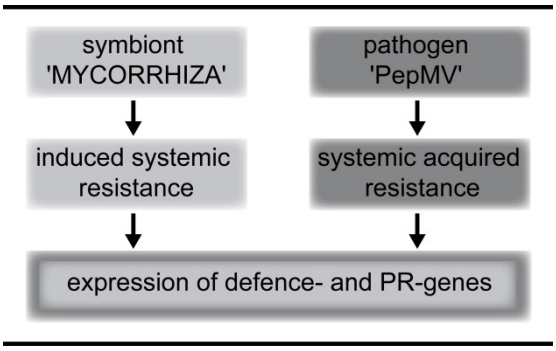

Figure 1.2: Illustration of the processes occurring in a tomato plant colonised with symbiotic mycorrhizal fungi or infected with the pathogen *Pepino mosaic virus*.

1.4.4 Pathogenesis-related proteins

Besides many other reported changes occurring in the defence response of a plant, the activation of many different defence-related genes might be of specific interest for this study (chapter 2). Numerous defence-related genes encode for so-called PR-proteins. PR-proteins are defined as proteins from host plants induced by various biotic and abiotic stresses (van Loon *et al.*, 2006). PR-proteins are known for a long time (Antoniw *et al.*, 1980) and today are grouped into 17 PR-protein families (table 1.1, (van Loon and van Strien, 1999;van Loon *et al.*, 2006)). The majority of the PR-proteins belong to multigenic families.

Table 1.1: PR-protein families and their major property. Table is modified from van Loon and colleagues (van Loon *et al.*, 2006).

property	PR-protein family
unknown	PR-1, PR-17
chitinases	PR-3, PR-4, PR-8, PR-11
1,3-β-glucanases	PR-2
thaumatin-like proteins	PR-5
proteinase inhibitors	PR-6
endoproteinases	PR-7
peroxidases	PR-9
ribonuclease-like, Bet v 1-related	PR-10
defensins	PR-12
thionins	PR-13
lipid transfer proteins	PR-14
oxalate oxidases, oxalate oxidase-like	PR-15, PR-16

PR-proteins differentially occur in certain species and also in an organ-specific distribution (van Loon *et al.*, 2006). They play important roles in SAR, especially the SA-induced proteins PR-1, PR-2 and PR-5. PR-3, PR-4 and PR-12 defensins (e.g. PDF1.2) become activated rather through ET and JA (Thomma *et al.*, 2001). PR-proteins can be partly responsible for resistance against further pathogenic attacks through their potential anti-microbial activity.

Transfer to this study

Symbionts, like mycorrhizal fungi and viral pathogens, like PepMV are able to differentially activate the defence response of a plant. As described above, this defence activation might be accompanied by the induction of defence- and PR-proteins. Some of these PR-proteins are known to act as allergens from pollen, fruits, and vegetables (Hoffmann-Sommergruber *et al.*, 2000;Ebner *et al.*, 2001;Midoro-Horiuti *et al.*, 2001;Asensio *et al.*, 2004). Therefore, it is necessary to evaluate the allergenic potential of affected plants.

1.5 Food allergy

Food allergy, also known as food hypersensitivity, becomes an increasing problem in industrialised countries. The enhanced use of food additives, the changing diet habits, increased hygiene standards, as well as changing environmental conditions may contribute to this rise (Gao *et al.*, 2012). No accurate data about the prevalence of food allergy are available, but approximately 20% of the population alter their diet because of adverse reactions to food (Sicherer and Sampson, 2006). Adverse reactions to food are not necessarily defined as food allergy; and due to the rise of occurrences the European Academy of Allergy and Clinical Immunology (EAACI) proposed a classification based on the underlying mechanisms (figure 1.3, (Bruijnzeelkoomen *et al.*, 1995)). Accordingly, food allergy is divided into IgE- and non-IgE-mediated immune mechanisms and responses. The IgE-dependent type I allergies, the immediate hypersensitivities, are the ones characterised best and probably the most important regarding allergic reactions to food. The three other types (type II, III, IV) are IgE-independent (Mills *et al.*, 2007). Food intolerances, including enzyme deficiencies and pharmacologic intolerances, do not involve the immune system, and therefore, cannot be regarded as an allergy (Bruijnzeelkoomen *et al.*, 1995;Gao *et al.*, 2012). Due to the overlap in the resulting allergic symptoms food intolerances are often misinterpreted and reported as food allergies by the allergic sufferers themselves. In contrast, it is reported that food allergies of adults are underdiagnosed, and therefore, might be underestimated (Diesner *et al.*, 2011).

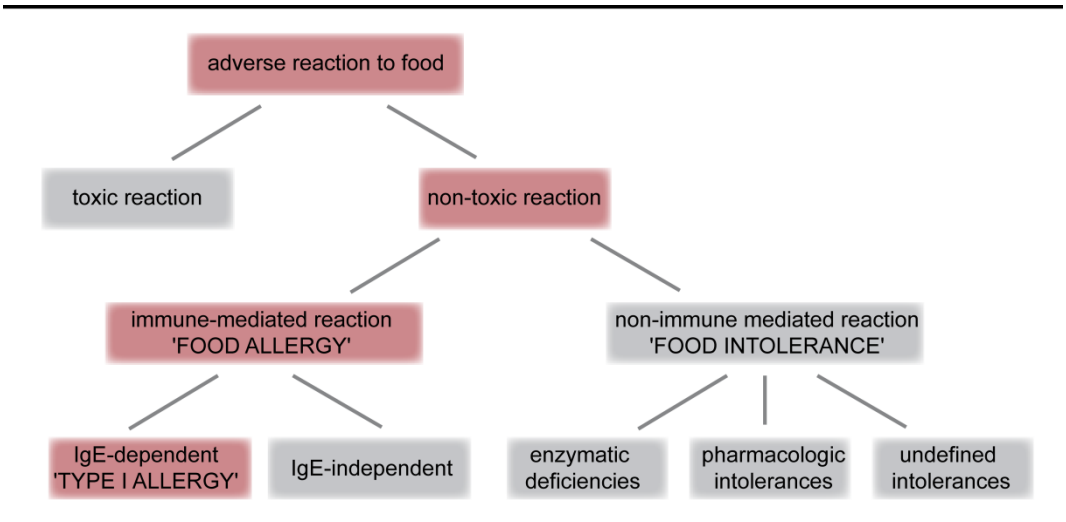

Figure 1.3: Overview about adverse reactions to food. This scheme is based on the classification of EAACI (Bruijnzeelkoomen *et al.*, 1995) and explains type I food allergy (red boxes**,** (Untersmayr and Jensen-Jarolim, 2006)).

As mentioned above, it is difficult to predict the prevalence of food allergy, because many studies only refer to self-reported and not to clinically proved food allergies. Symptoms of food intolerances and those of allergies are often indistinguishable. However, it is estimated that approximately 2 - 4% of the population might suffer from food allergy (Zuberbier *et al.*, 2004;Beyer and Teuber, 2005;Zuidmeer *et al.*, 2008). This percentage is even higher for children but also refers mainly to milk and egg allergy, whereas adults mainly suffer from food allergies caused by fruits and vegetables (Sicherer and Sampson, 2006). Up to now, no reliable therapy exists and the only option for food allergic people is to adapt their diet and to avoid the food they are allergic to.

Food allergic people might suffer from a wide range of local and systemic symptoms. The allergic reactions differ individually even when people are allergic to the same food. They can reach from local, oral allergic symptoms to systemic reactions of the cardiovascular system. Other locally appearing symptoms might be reactions of the gastrointestinal tract, like gastritis, vomitus, diarrhoea, and enteritis. The most dangerous systemic reaction is anaphylaxis that can also involve different organs, including the skin, the respiratory- and the gastrointestinal tract, and the cardiovascular system and might even cause death (Worm *et al.*, 2012). Urticaria, rhinitis, conjunctivitis, asthma, and atopic eczema might also appear as systemic reactions immediately after ingestion of a certain food composite. These life threatening systematic reactions to proteins from food are the reason for a labelling obligation for certain ingredients, e.g. nuts, milk, egg, and also celery, on convenient and processed food (EU Directive on labelling of foods).

1.5.1 Mechanism of type I allergy

Type I allergy is characterised through an acute onset of symptoms directly after ingestion of the respective food, containing specific allergens (Sicherer, 2002). Food allergens are non-toxic and normally harmless proteins, provoking an IgE-dependent allergic reaction (Bruijnzeelkoomen *et al.*, 1995). Figure 1.4 shows a schematic overview about the mechanism of type I allergy. Allergens enter via mucosal surfaces and get into contact with the immune system by passing through epithelial, dendritic or M-cells (Chehade and Mayer, 2005). The first contact with an allergen and the establishment of an immune response is called sensitisation. Allergens are taken up by antigen-presenting cells or are directly recognised by B-cells. In antigen-presenting cells the allergens are processed and presented to T-cells which, therefore, differentiate into Th2-cells. Th2-cells release special cytokines (interleukins, particularly IL-4 and IL-13) which provoke a class switch of respective B-cells. B-cells start to produce large amounts of specific IgE antibodies against the specific allergen, which are excreted to the serum. These antibodies have a high affinity and directly bind to their specific receptors (FcεRI), expressed on the surface of mast and basophil cells. If the specific allergen enters into the body of a sensitised person a second time, it is directly recognised by the specific IgE antibodies, localised on the surface of mast- and basophil cells. The cross-linking between IgE antibodies and allergens lead to the activation of mast- and basophil cells and trigger an internal signal transduction cascade, resulting in the degranulation and the release of histamine or other chemical mediators. Histamine is the principal and most important mediator and elicitor for immediate symptoms of allergy (Mills *et al.*, 2007;Gao *et al.*, 2012).

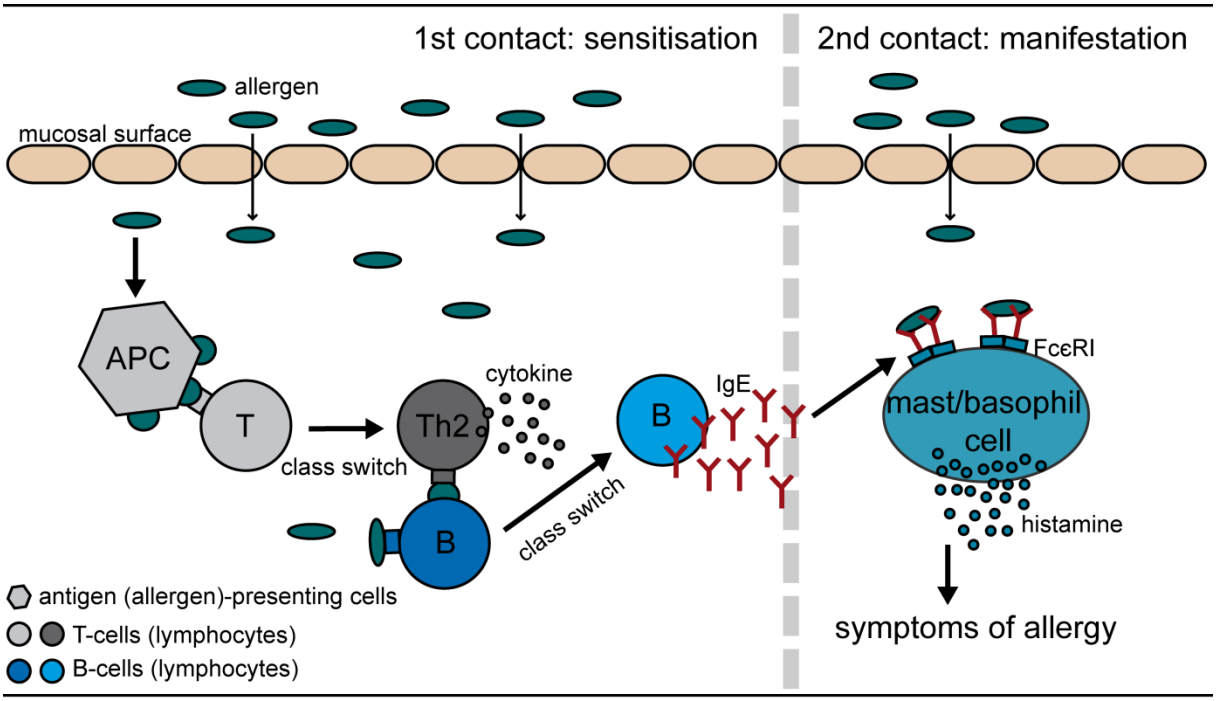

Figure 1.4: Scheme about type I allergic reaction: Sensitisation and manifestation. Explanations can be found in the text.

1.5.2 Allergens and cross-reactivity

Allergens are listed in different databases according to the organism they come from, e.g. in the 'International Union of Immunological Societies (IUIS) allergen nomenclature sub-committee' (www.allergen.org) and the 'Allergome' (www.allergome.org) database. The official IUIS nomenclature of allergens is based on the allergenic source they originate from, e.g. the first identified and confirmed allergen from tomato (***Lyc****opersicon* ***e****sculentum*) was named Lyc e 1 but previously was changed to Sola l 1 (***Sola****num* ***l****ycopersicum*). Other databases, like 'AllFam' (www.meduniwien.ac.at/allergens/allfam) classify allergens, based on their biological function, into certain allergen families. This clarifies that allergens belong to a small group of protein families and their biological functions are limited. Plant food allergens for example, mainly belong to prolamins, cupins, PR-proteins, profilins, and expansins (Radauer *et al.*, 2008). However, the underlying mechanisms, properties, and factors of proteins making them to allergens are not completely understood.

Food allergens can be classified into two categories, one group is able to directly sensitise in the gastrointestinal tract, the other one is only able to provoke allergy after an already established sensitisation through another allergen, e.g. from pollen. This so-called cross-reactivity requires sequence or structural similarities of both allergens, coming from different sources, to bind to the same specific IgE antibody. Cross-reactivity and the sensitisation through pollen allergens, often deriving from birch, is quite common among fruit and vegetable allergies (Garcia and Lizaso, 2011). Allergens requiring an already established sensitisation often possess conformational epitopes, resulting in a less stabile antibody binding site in regard to heat and enzymatic degradation (Sampson, 2004). The other group of plant food allergens is able to directly sensitise and additionally, often survives heat and protease exposure, thereby indicating that epitopes are based on sequences.

Those allergens are mainly seed storage proteins belonging to cupin and prolamin families but also proteins from the plant defence system (Radauer *et al.*, 2008). Interestingly, many major plant food allergens are in some respect related to the plant defence mechanisms and represent PR-proteins, proteases, and protease inhibitors (Sampson, 2004).

1.5.3 Diagnosis - Clinical allergy tests

The clinical diagnosis starts with a detailed anamnesis, in which the allergic subjects report about their abnormal reactions to food and any other potential allergies. Afterwards and depending on the anamnesis, specific sensitisations are screened by means of skin tests, eventually different *in vitro* tests, and double blind placebo controlled food challenges (DBPCFC).

The skin prick test (SPT) is routinely used and the most common tool to determine whether it concerns an IgE-mediated allergy. Within this test a lot of different food stuffs can be screened simultaneously. Corresponding food stuff (prick-to-prick) or respective food extracts, a positive (histamine), and a negative control (saline solution) are applied on the forearm and invaded through pricking. Afterwards, the diameters of the emerging wheals are measured. Considering the positive and negative control the SPT is meant to be positive with a wheal diameter ≥ 3 mm. Positive SPT to a specific food stuff means that the corresponding person is sensitised, but clinical relevance still has to be considered (e.g. by DBPCFC). Additionally, false-positive reactions, like unspecific reactions to the test material or overreaction of the skin, and false-negative reactions, e.g. through inactivated test material, medication, or missing reaction of the skin, can never be completely excluded (Henzgen *et al.*, 2008).

Many different *in vitro* tests for the evaluation of food allergies exist. Among those the most important and the one used in daily, clinically practice is the determination of specific IgE. For this purpose commercial products with a selection of specific food or even specific single allergens are available (e.g. Phadia-Immuno CAP system). Detected specific IgE values can be an indication for IgE-mediated sensitisation to a specific food under consideration of clinical relevance and confirmation with e.g. DBPCFC. The use of other *in vitro* tests, like histamine release or basophil activation tests, can be a matter of choice in individual cases, but are not routinely used because of their complexity. Today these tests are mainly used for research (Kleine-Tebbe *et al.*, 2009).

The DBPCFC is the most reliable clinical allergy test, because people are directly provoked with the food they are allergic to (Bindslev-Jensen *et al.*, 2004). After ingestion, objective and subjective symptoms are recorded in a double blind manner, meaning that neither the physician, recording the symptoms, nor the allergic subject knows whether he/she was provoked with the potential allergic foodstuff or a placebo. The DBPCFC is relatively time consuming and due to the risk of severe systemic allergic reactions carried out only in specific cases.

1.5.4 Tomato allergy and known allergens of fruits

Among the food-allergic population 1.5 - 16% suffer from tomato allergy (Ortolani *et al.*, 1989;Petersen *et al.*, 1996). Interestingly, there is a North to South gradient in the occurrence of tomato allergy, correlating with the frequency of consumption. Therefore, tomato allergy is more common in the Mediterranean area than in the Northern parts of Europe. Tomato is often associated with pollen allergy and results from cross-reactivity, therefore, more people react to fresh in contrast

to processed tomatoes (Foetisch *et al.*, 2001;Ferrer *et al.*, 2007;Dölle *et al.*, 2011a). As many other food allergies tomato allergy depends on geographical sensitisation patterns through pollen but also on local dietary habits. Tomato cultivars with a lower allergenic potential have recently been identified (Dölle *et al.*, 2011a;Lopez-Matas *et al.*, 2011a); and also Sola l 1- and Sola l 3-silenced transgenic tomatoes have shown reduced allergenicity (Le *et al.*, 2006;Lorenz *et al.*, 2006). Tomato-allergic subjects react to a lot of different proteins. In contrast to other vegetables, a high number of known and putative tomato allergens are described and today twelve allergens and their additional isoforms are listed in the 'Allergome' database (www.allergome.org). Tomato allergens, as most of the plant food allergens, belong to a small group of protein families. According to their function they can be grouped to proteins related to plant defence, proteins integrated in cell organisation, proteins involved in metabolic and developmental processes, and storage proteins mainly occurring in seeds.

Four tomato allergens are officially confirmed and named by IUIS (www.allergen.org), namely a profilin, Sola l 1 (Foetisch *et al.*, 2001;Westphal *et al.*, 2004), a β-fructofuranosidase, Sola l 2 (Petersen *et al.*, 1996;Kondo *et al.*, 2001;Westphal *et al.*, 2003), a lipid transfer protein, Sola l 3 (Foetisch *et al.*, 2001) and a Bet v 1-related protein, Sola l 4 (Ballmer-Weber and Hoffmann-Sommergruber, 2011). Furthermore, the PR-proteins chitinase (Diaz-Perales *et al.*, 1999), glucanase (Palomares *et al.*, 2005), thaumatin-like protein (Kondo *et al.*, 2001), and peroxidase (Weangsripanaval *et al.*, 2003) are known tomato allergens; and also polygalacturonase and pectinesterase are two well-known and probably major tomato allergens (Foetisch *et al.*, 2001;Kondo *et al.*, 2001;Dölle *et al.*, 2011a). Recently, our group detected and described two seed storage proteins, a vicilin and a legumin, as putative tomato allergens (Bässler *et al.*, 2009). The last entry into the 'Allergome' database was a ribosomal protein, reacting with tomato-allergic subjects' sera from Spain (Lopez-Matas *et al.*, 2011b).

Some of these tomato allergens are glycoproteins containing cross-reactive carbohydrates (CCD) in complex N-glycan structures (Foetisch *et al.*, 2003;Altmann, 2007;Kaulfuerst-Soboll *et al.*, 2011). Based on N-glycosylated 1,3-α-fucose and 1,2-β-xylose, which do not occur in mammals some people develop specific anti-CCD-IgEs (Foetisch *et al.*, 2003;Altmann, 2007). These reactions to CCDs, however, might be clinically less significant (Foetisch *et al.*, 2003;Mari *et al.*, 2008). Glycoproteins of tomato are Sola l 2, polygalacturonase, and different pectinesterases, whereupon with Sola l 2 a glycosylation-dependent IgE reactivity has already been shown (Foetisch *et al.*, 2003;Westphal *et al.*, 2003;Kaulfuerst-Soboll *et al.*, 2011).

Transfer to this study

General mechanisms, prevalence, and diagnosis of food allergy are described since this is essential for understanding the significance and the statements of this study. Tomato is used in this study as a model organism, able to provoke clinically relevant allergies. As many other food allergies, tomato allergy becomes an increasing problem in industrialised countries and at once tomato is one of the most consumed and healthiest vegetables. Therefore, it is crucial to prove the safety of commercially cultivated tomato for tomato-allergic subjects.

1.6 Cyclophilin - A cross-reactive allergen

Another group of allergens are the cyclophilins, mainly known from allergies against certain fungi, like *Aspergillus* (Asp f 11), moulds (Mala s 6), mushrooms (Psi c 2), Candida (Cand a cyp), or baker's

yeast (Sac c cyp). However, they also can be found among pollen allergens, like birch (Bet v 7, Bet pu 7), grass (Lol p cyp), periwinkle (Cat r 1), oriental plant tree (Pla or cyp), and in vegetables and fruits, like carrot (Dau c cyp), pumpkin (Cac ma cyp), raspberry (Rub i cyp), or even in humans (Hom s cyp).

Tomato cyclophilin has been first described by Gasser and colleagues in 1990 and recently its cross-reactivity to a Bet v 7 antibody has been shown (Cadot et al 2006). Cyclophilins are well-known pan-allergens, meaning that they possess homologous regions leading to a high cross-reactivity in between cyclophilin allergens from various sources (Fluckiger *et al.*, 2002;Roy *et al.*, 2003).

Cyclophilin is a peptidyl-prolyl *cis-trans* isomerase that catalyses the isomerisation of the peptide bond from *trans*-form to *cis*-form on the amino side of proline residues and facilitates protein folding. It is also known to bind cyclosporine drugs, and therefore, plays an important role in immunosuppression (Handschumacher *et al.*, 1984;Fischer *et al.*, 1989;Takahashi *et al.*, 1989;Wang and Heitman, 2005). According to its function cyclophilin is a widely distributed and abundant protein in all described organisms, and therefore, might be of particular interest for further allergy research.

1.7 Allergen identification

The identification of new allergens in a respective food is often based on detected cross-reactivities to already known allergens from other sources, e.g. as described for cyclophilin in chapter 1.6. For diagnosis and therapy of allergies it is very important to more or less completely uncover the underlying elicitors of allergic symptoms and to identify and recombinantly produce as many allergens as possible (Chapman *et al.*, 2000).

In general, new putative allergens are initially detected on immunoblots with certain food stuff and allergic subjects' sera. For verification and confirmation further evaluation and characterisation is necessary. The respective putative new allergen has to be purified from total protein extract or cloned, sequenced, and recombinantly produced in another organism. Popular overexpression systems for recombinant allergen expression are bacteria, yeast, insect cells, or plants. Even if allergens seem to be routinely, recombinantly produced, methods have to be individually adapted and optimised for each protein (Schmidt and Hoffman, 2002).

The bacterial system *Escherichia coli* is the most commonly used overexpression system and possesses many advantages: *E. coli* is easy to cultivate and to transform, it is fast-growing and on average produces high yields of recombinant protein; it is a cost-effective system and many strains and expression vectors for many different protein characteristics are available. The major disadvantages, if expressing eukaryotic proteins in *E. coli,* are the different codon usage of prokaryotic bacteria in contrast to eukaryotes, the lack of proper protein folding, and the absence of post-translational protein modification mechanisms, like glycosylation or phosphorylation. Other systems, e.g. yeast, as an eukaryotic system is still very easy to cultivate and to transform and is additionally able to post-translationally modify the proteins, including disulphide bond formation, lipid- and carbohydrate addition, and general folding (Schmidt and Hoffman, 2002). Obviously, the recombinantly produced protein resembles the most to its naturally occurring representative if it is produced in the same species. For some plant food allergens the overexpression in tobacco plants was achieved with the A*grobacterium tumefaciens* mediated transformation system or certain virus-plant systems (Breiteneder *et al.*, 2001;Schmidt *et al.*, 2008). However, these plant overexpression

systems are more complex and cost intensive and are not easy to establish in an inexperienced laboratory. Considering the structure of the allergen and the experimental requirements the suitable overexpression system has to be carefully evaluated and often it is recommended to start with the well-understood and probably simplest overexpression system *E. coli*.
Subsequently, the purified or recombinant allergen can be independently tested in immunoblots with subjects' sera and in clinical allergy tests, like skin prick and basophil tests. For the inclusion into the allergen nomenclature database of IUIS (www.allergen.org) the putative allergen has to fulfil certain molecular and immunological requirements (e.g. the IgE binding of at least 5% of all tested subjects' sera, allergic to the respective allergen source).

Transfer to this study

It is important to uncover the underlying mechanisms of food allergy and to identify the putative allergens, provoking symptoms of allergy to improve allergy diagnosis and therapy. The identification of all clinically relevant putative allergens of an organism is also necessary to develop hypoallergenic cultivars. The determination of new allergens can be achieved through immunoblots or structural and sequence comparisons with known allergens to identify pan-allergens.

1.8 The VEGAL project

The initiative for this study was developed in the allergy project VEGAL (**Veg**etable **Al**lergies). Therein, the Leibniz-Institute of vegetable and ornamental crops in Großbeeren (IGZ; involved people: Philipp Franken, Dietmar Schwarz, Eckhard George and the current author) closely cooperates with the Charité in Berlin (involved people: Sabine Dölle and Margitta Worm) and the Proteome Factory AG in Berlin (involved people: Karola Lehmann and Christian Scheler). The operations for this study mainly took place at the IGZ, where the tomato plants were cultivated and the molecular analyses were carried out. The protein analyses were done in cooperation with the Proteome Factory AG and the clinical allergy tests have been conducted in the Charité. Other partners in this project were the University of Vienna (involved people: Wolfram Weckwerth and Luis Recuenco-Munoz) and the Fraunhofer-Institute for biomedical engineering in Potsdam (involved person: Eva Ehrentreich-Förster).

2. HYPOTHESES AND OBJECTIVES

This project was motivated on two previous studies of our working group VEGAL, showing that the selection of tomato cultivars and not the cultivation under different environmental conditions can have an impact on the allergenic potential (Dölle *et al.*, 2011a;Dölle *et al.*, 2011b). Commercially cultivated tomatoes are not only exposed to different environmental conditions but also to various biotic stressors. Therefore, it was necessary to investigate whether these biotic stressors might influence the allergenic potential of tomato fruits. Due to the activation of the plant's defence after biotic stress exposure, including the expression of defence- and PR-proteins, resembling putative allergens, we developed the hypothesis that symbiotically colonised or pathogen-infected tomato fruits hold a higher allergenic potential (figure 2.1). One objective was to determine the allergenic potential of tomato fruits colonised with the most important plant symbiont, the mycorrhizal fungus. Furthermore, it was aimed to unravel the impact of the *Pepino mosaic virus,* a major greenhouse tomato pathogen, on the whole tomato plant in an entire cultivation period; including its impact on the allergenic potential of tomato fruits.

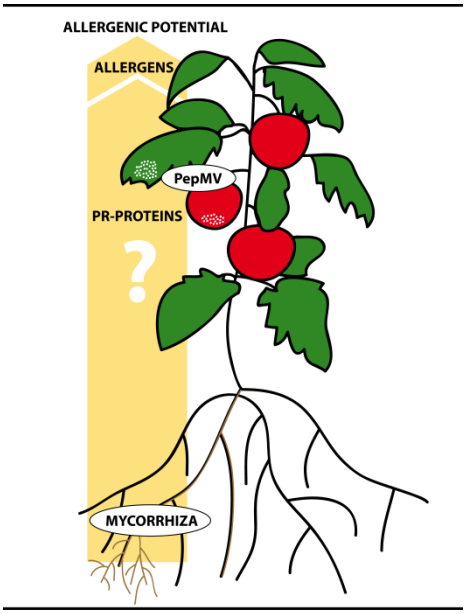

Figure 2.1: Illustration of the main hypotheses of this study: Due to the activation of the plant's defence, including expression of defence- and PR-proteins, resembling putative allergens, after mycorrhiza colonisation and *Pepino mosaic virus* (PepMV) infection, tomato fruits possess a higher allergenic potential.

Another objective was to identify new putative tomato allergens due to tomato cultivation under distinct conditions, and to additionally confirm new candidates through recombinant production and individual testing on tomato-allergic subjects. With single subjects' reactions in immunoblots with tomato protein extracts, deriving from differentially cultivated tomato plants, it was aimed to uncover the background and reason for the high individual differences of allergic subjects' reactions in clinical allergy tests.

3. MATERIALS AND METHODS

3.1 Software

- Adobe Photoshop CS4 and Illustrator CS2
- Bioedit (Ibis Bioscience, Carlsbad, CA, USA)
- FCS Express V3 software (De Novo Software, Los Angeles, CA, USA)
- DNAStar Lasergene 8 (DNASTAR Inc., Madison, WI, USA)
- LinReg (Amsterdam, The Netherlands, (Ramakers *et al.*, 2003))
- Mascot (Matrix Science, London, Great Britain)
- Microsoft Office 2010
- Protein Pilot 4.0 (AB Sciex, Framingham, MA, USA)
- Proteomweaver 3.1 (Definiens AG, Munich, Germany)
- qBase^{+} (Biogazelle, Zwijnaarde, Belgium, (Hellemans *et al.*, 2007))
- Sigma Plot (Systat Software GmbH, Erkrath, Germany)
- Statistica (version 9, Tulsa, OK, USA)

3.2 Accession numbers and primers

Based on tomato sequences deposited in NCBI (National Center for Biotechnology Information; Bethesda, USA) database, all listed primers were designed using the DNAStar Primer Select software (GATC Biotech, Konstanz, Germany) or are marked with a reference. All primers were designed for usage at 60°C annealing temperature in a polymerase chain reaction (PCR). Primers were obtained from Eurofins MWG Operon (PCR primer grade; MWG Biotech, Ebersberg, Germany).

Accessions and primers used for allergen-encoding genes, rubisco, and reference genes are listed in tables 3.1 and 3.2.

Accessions and primers used for cloning are listed in table 3.3. Those primers were designed according to the coding sequence (CDS) of the respective gene without start and stop codons. Specific restriction sites (bold) and three additional bases, for an efficient binding of restriction enzymes, were added (italic). Specific restriction enzymes were chosen after excluding their restriction sites in the respective gene with a freely available restriction mapper software (www.restrictionmapper.org). If these primers failed to work, the same primers were designed without restriction sites and primers with restriction sites were used with the PCR product, amplified before.

For size and sequence control of putative tomato allergens and the *Pepino mosaic virus* coat protein (PepMVCP) inserts in respective vectors, primer pairs flanking the insert region of the vectors were used (table 3.4).

The detection and quantification of the two *Pepino mosaic virus* (PepMV) isolates CH2 and EU with PCR could be achieved with isolate-specific and general PepMV primer pairs (table 3.5).

Table 3.1: Protein and gene accessions (NCBI) of known and putative tomato allergens and corresponding primer pairs used in quantitative real-time RT-PCR.

abbre-viation	name	protein accession	gene accession	primer sequence for (5'-3')	primer sequence rev (5'-3')	amp [bp]
Sola l 1	profilin	AAL29690	AY061819	TGGGCTCAATCTGCTAAT	AGTCATCGGCTCGTCATA	237
Sola l 2	β-fructo-furanosidase precursor	P29000	AF465612	CGTACCCCGCCAACTTATCTG	CAATGCCGGGTGGAGGAA	86
Sola l 3	non-specific lipid transfer protein	CAJ19705	AM051295	TGGCTCCTTGTCTCCCTTATCT	TTGCCCAAATTGAGTCCTGTA	163
Sola l 4	TSI 1 protein	CAA75803	Y15846	AACCACAATTTCCCCAACAAG	CCACCATCTCCCTCAATAGTCTC	117
GLU	1,3-β-glucanase	Q01413/ AAA03618	M80608	TCAAACATCATGGCTACCTCACAA	TCCCCATCATTCCATAACAAACAC	115
CHI	chitinase	Q05538	Z15140	ATGGGGTTACTGTTTCCTTAGAGA	TGGCCCATAGTTGTAGTTGTGT	139
NP24	PR-protein osmotin precursor, thaumatin-like	AAC64171	AF093743	TATATGGGGTCGTACTGGTTGC	GCTAGGGTGTTTGGGGGTTTG	117
HSC70	heat shock protein cognate 70	AAB42159	L41253	TTCTGTGATACTTTTGCTTTACTA	TGCTGCCATACACCTACAC	122
PER	suberization-associated anionic peroxidase	P15003	X15853	ATTAATTAAACCTGGCCGTATGAT	ATTAATTAAACCTGGCCGTATGAT	121
SOD	superoxide dismutase	AAA34194	M37150	TCGCCGTCCTTAACAGCAGTG	AAGCCATGAAGTCCAGGTTTTAGG	118
CYC	cyclophilin	P21568	M55019	TGGATGTGATTAAGAAGGCAGAGG	GACCCGACCAAAGCAGTAGAGATA	195
PG	polygalact-uronase 2a	P05177	A15981	CAGGTGATGATTGTATTTCAATTGTT	CCATGACCTGGACCACAAGTAA	104
PME1.9	pectinmethyl-esterase	P14280	U50986	CCCGCTAAAGCTATCCCGTTCA	GTTATGCTTGCTCTGCCCTGCTC	145
PME2.1	pectinmethyl-esterase	AAL02367	U50985	GGGCTGAGTGGCACGGAGATT	CCAGGCCACTTGACACGCTTACT	103
MAN	β-mannosidase	AAL37714	AF403444	GTTGGGCCGTGAAGTCGTGAA	CGTACCCCAAGGCAGCAACC	125
VIC	vicilin	CAP69670	AJ270964	GCCTCGCGCTCAACTCAG	TTCGGGACGTGCTTCAAA	114
EXP	expansin	AAC63088	U82123	TAACCCTCCTCGCCCTCACTTT	TATCCCCTGCACCTGCTACATTC	196
RUB	rubisco	AAA19771	L14403	TACTTGAACGCTACTGCAG	CTGCATGCATTGCACGGTG	190

amp [bp]: amplicon length in base pairs

Table 3.2: Primer pairs and gene accessions (NCBI, Sol Genomics Network) of reference genes used for the normalisation of quantitative real-time RT-PCR.

abbreviation	name	gene accession	primer sequence for (5'-3')	primer sequence rev (5'-3')	amp [bp]
18S rRNA[*1][*2]	17S rRNA gene	X51576	GTGCATGGCCGTTCTTAGTTGGTG	AAGAAGCTGGCCGCGAAGGGATAC	113
25S rRNA	25S rRNA gene	X13557	CCGAATCAACTAGCCCCGAAAATG	ACCGCCGCGCCCTCCTACTC	102
CAC[*3]	clathrin adaptor complex medium subunit	SGN-U314153	CCTCCGTTGTGATGTAACTGG	ATTGGTGGAAAGTAACATCATCG	173
SAND[*3]	SAND family protein	SGN-U316474	TTGCTTGGAGGAACAGACG	GCAAACAGAACCCCTGAATC	164
GAPDH[*1]	glyceraldehyde 3-phosphate dehydrogenase	U93208	ACCACAAATTGCCTTGCTCCCTTG	ATCAACGGTCTTCTGAGTGGCTGT	110
UBI[*1]	ubiquitin	X58253	TCGTAAGGAGTGCCCTAATGCTGA	CAATCGCCTCCAGCCTTGTTGTAA	119

amp [bp]: amplicon length in base pairs

*[1]: (Mascia *et al.*, 2010), *[2]: (Kitagawa *et al.*, 2006), *[3]: (Exposito-Rodriguez *et al.*, 2008)

Table 3.3: Primer pairs and gene accessions (NCBI) used for cloning of putative tomato allergens and the *Pepino mosaic virus* coat protein. The restriction sites are marked in bold and the three additional bases for an efficient binding of restriction enzymes in italic.

abbre-viation	name	gene accession	primer sequence for (5'-3')	primer sequence rev (5'-3')	restriction enzymes	an.temp. [°C]/ amp [bp]
PepMVCP	*Pepino mosaic virus* coat protein	DQ000985	*GCA* **GGATCC** GAAAACCAACCTACAGCTTCTAA CCCATC	*CAG* **AAGCTT** AAGTTCAGGAGGTGCATCAA TTGCGTAC	BamHI; HindIII	65/725
HSC70	70kDa heat shock protein cognate	L41253	*GCA* **GGATCC** GCCGGAAAAGGTGAAGGACCA GCG	*CAG* **CTGCAG** GTCCACTTCCTCAATCTTAGG GCCTG	BamHI; PstI	68/1966
MAN	β-mannosidase	AF403444	*GCA* **GGATCC** AAAGCAAATCCTCCATGTCTTAA TTTTGC	*CAG* **GTCGAC** ATGCTTCTGGCGCTTAAGCAA TTTC	BamHI; SalI	61/1556
NP24	PR-protein osmotin precursor, thaumatin-like	AF093743	*GCA* **GGATCC** GGCTACTTGACATC	*CAG* **AAGCTT** CTTGGCCACTTCATC	BamHI; HindIII	50/755

amp [bp]: amplicon length in base pairs, an.temp.: annealing temperature in °C

Table 3.4: Sequencing and colony PCR primer pairs of vectors used for overexpression of putative tomato allergens and the *Pepino mosaic virus* coat protein. The sequences for the primer pairs were obtained from respective vector cards.

vector	primer for (5'-3')	primer rev (5'-3')
pGemTEasy (Promega, Mannheim, Germany)	M13 for: TGTAAAACGACGGCCAGT	M13 rev: CAGGAAACAGCTATGACC
pCDF Duet (Novagen, Merck, Darmstadt, Germany)	ACYCDuetUP1: GGATCTCGACGCTCTCCCT	DuetDOWN1: GCTAGTTATTGCTCAGCGG
pET15b (Novagen, Merck, Darmstadt, Germany)	T7 promotor: TAATACGACTCACTATAGGG	T7 terminator: GCTAGTTATTGCTCAGCGG

Table 3.5: Primer pairs used for isolate-specific and general *Pepino mosaic virus* detection and quantification and the cDNA synthesis of the *Pepino mosaic virus* genome. The sequences for the primer pairs were obtained from Inge Hanssen (Scientia Terrae, Belgium).

name	gene accession	primer sequence for (5'-3')	primer sequence rev (5'-3')	amplicon length [bp]/ third primer
PepMV general detection/quantification	all isolates	GGAGCATTCATACCAAATGGG	CCTAGGTGAACCTATAACTAAG	344
primer, specific for EU isolate	JQ314461	AAATTGTGAGAACACACCAG	TAGAAAACCCCACTCTGA	996
primer, specific for CH2 isolate	DQ000985	ACAATTTAACTCAACTATGG	TAGAAAACCCCACTCTGA	762
for cDNA synthesis of PepMV genome	all isolates	TAGAAAACCCCACTCTGA	GGTTGAATCATTGCTTTCTC	CCTTTAACCTGTTTTGG

3.3 Tomato-allergic subjects

Tomato-allergic subjects were recruited in the Allergy-Center Charité (Berlin, Germany). All subjects had a positive history of adverse reaction to tomato and characteristics are listed in table 3.6. All studies conducted with these subjects were approved by the local ethic committee (Ethikkommission der Charité Berlin, EC-No. 1832/Si.258) and all subjects gave written informed consent prior to the investigations.

Table 3.6: Tomato-allergic subjects' characteristics and their participation in respective studies.

no	sex	age[1]	total IgE [kU/L]	sp. IgE tomato [kU/L]	sp. IgE birch [kU/L]	sp. IgE *A.f./A.A.* [kU/L]	sp. CCD	symp-toms tomato	serum pool	mycor-rhiza (2004)	PepMV (2010)	immun-oblot (2004)	over-exp. (2011 -12)
NA	f	28	nd	nd	nd	nd	nd	-	-	+	+	+	+
1	m	33	nd	4.69	3.68	nd	nd	OAS, GIT, D	+	-	+	-	+
2	m	28	72	1.04	nd	0.04/ 0.01	nd	mEr, Bli, GIT	+	-	+	-	+
3	f	38	154	0.37	nd	0.04/ 0.01	no	OAS, mEr, GIT	+	-	+	-	+
4	f	59	84	0.14	nd	nd	no	OAS, mEr	+	-	+	-	+
5	f	36	405/ 862	1.57/ 1.17	100	0.09/ 0.05	no	OAS, Er, GIT	+	+	+	+	+
6	f	57	204	2.55	nd	0.04/ 0.02	no	OAS, Er	+	-	+	-	+
7	m	31	>5000	9.57	11.6	2.68/ 0.93	yes	OAS, Er	+	-	+	-	+
8	f	46	425	0.47	14.38	0.06/ 0.03	yes	OAS, mEr	+	-	+	-	+
9	m	32	3250	42.9	nd	nd	nd	eAD, OAS	+	-	+	-	+
10	m	46	290	0.02	nd	0.05/ 0.01	nd	OAS	+	-	+	-	+
11	m	28	3588	7.42	nd	3.82/ 6.39	yes	OAS, syst	-	-	-	-	+
12	m	51	1229	1.74	nd	0.11/ 1.67	no	Er, OAS, skin	-	-	-	-	+
13	m	40	114	2.02	30.4	nd	nd	GIT	-	+	-	+	-
14	m	41	184	<0.35	29.3	nd	nd	OAS	-	+	-	+	-
15	m	36	197	0.86	3.35	nd	nd	OAS	-	+	-	+	-
16	m	43	51.1	<0.35	24.3	nd	nd	OAS	-	+	-	+	-
17	m	38	155	<0.35	17.2	nd	nd	R	-	+	-	+	-
18	f	18	383	0.82	37.1	nd	nd	OAS	-	+	-	+	-
19	f	18	234	nd	nd	nd	nd	OAS	-	+	-	+	-
20	f	36	255	1.17	2.97	nd	nd	OAS	-	+	-	+	-
21	f	18	476	0.65	86.1	nd	nd	OAS	-	+	-	-	-

[1]age at time of the first clinical allergy test, -: not participated in the respective study, +: participated in the respective study.
Abbreviations: sp.: specific; *A.f.: Aspergillus fumigatus*; *A.a.: Aspergillus alternaria*; overexp.: overexpression; NA: non-allergic subject; nd: not determined; m: male; f: female; Bli: blister of the oral mucosa; D: dyspnoea; Er: facial erythema; mEr: mucosal erythema; GIT: symptoms of the gastro-intestinal tract, can include diarrhoea, nausea; OAS: oral allergic symptoms, can include numbness in the mouth, burning tongue, pruritus, swelling lips; eAD: exacerbation of atopic dermatitis; R: rhinitis; syst: systemic.

3.4 Secondary anti-human IgE antibodies

- Goat anti-human IgE:POD (Sigma, Taufkirchen, Germany)
  - peroxidase-conjugated
  - polyclonal
- Rabbit anti-human IgE:AP (Sigma, Taufkirchen, Germany)
  - alkaline phosphatase-conjugated
  - monoclonal

- Mouse anti-human IgE:HRP (RayBiotech, Norcorss, Georgia)
  - horseradish peroxidase-conjugated
  - monoclonal

Different anti-human IgE antibodies were tested and either an alkaline phosphatase (AP)- or a peroxidase (POD/HRP)-conjugated antibody was used in the immunoblots of this study, depending on the objectives of the experiment. Peroxidase-conjugated antibodies induce a chemilumescent reaction, and therefore, are highly sensitive and were used e.g. for the detection of new putative allergens. AP-conjugated antibodies induce a colorimetric reaction and were useful when conducting many immunoblots in parallel.

3.5 *Pepino mosaic virus* isolates

- PepMV EU
  - *Pepino mosaic virus*, European genotype
  - mild isolate, PepMV-Sav (E397), (Schwarz *et al.*, 2010a)
  - isolated from tomatoes obtained from a German supermarket
- PepMV CH2
  - *Pepino mosaic virus*, Chilean genotype
  - aggressive isolate, strain PCH 06/104 (DQ000985)
  - obtained from Inge Hanssen (Scientia Terrae, Sint-Katelijne-Waver, Belgium)

3.6 Mycorrhiza isolate

- Mycorrhiza inoculum
  - *Funneliformis mosseae* (=*Glomus mosseae*, Nicol.&Gerd.) BEG 12 (Biorhize, Dijon, France)

3.7 Tomato cultivars

- '76R'
  - commercial tomato cultivar (Peto Seed Company, Santa Maria, CA, USA)
- 'RMC'
  - isogenic mutant of 76R, showing less mycorrhizal colonisation of the roots (Barker *et al.*, 1998)
- 'Reisetomate'
  - landrace (Genbank, Gatersleben, Germany)
- 'Matina'
  - cultivar selected for organic farming (Hild Samen GmbH (now Nunhems), Marbach, Germany)
- 'Counter'
  - commercial tomato cultivar (DeRuiter (now Monsanto), Bergschenhoek, The Netherlands)

3.8 Tomato plant cultivation

Tomato plants were cultivated in greenhouses at the Leibniz-Institute of vegetable and ornamental crops in Großbeeren (IGZ). They were germinated in coarse sand and seedlings were transferred to respective cultivation systems at 6 - 11 leaf stage. Plants were maintained near to commercial cultivation and routinely, manually pollinated with an electric toothbrush.

3.8.1 '76R' and 'RMC' cultivation, mycorrhiza inoculation and determination

These plants were cultivated from May until July 2004. In total 16 tomato plants (eight of '76R' and eight of 'RMC' genotype, figure 3.1) were cultivated in 10 L buckets filled with a sand/vermiculite mixture (1/1, v/v). Tomato plants were manually irrigated until drain started. Once a day pots were supplied with tap water and twice a week with nutrient solution in mM: 1 NH_4NO_3, 2.75 $Ca(NO_3)_2$ x 4 H_2O, 6.5 KNO_3, 1.25 KH_2PO_4, 1.5 K_2SO_4, 1 $Mg(NO_3)_2$, in µM: 25 FeEDTA, 10 $MnSO_4$, 20 H_3BO_4, 0.5 MoO_3, 0.75 $CuSO_4$, 4 $ZnSO_4$. Mean temperature, daily radiation, and humidity in the greenhouse were 23.1°C, 20.2 mol $m^{-2}d^{-1}$, and 61.3%.

Four plants of each genotype were inoculated with the mycorrhizal isolate *Funneliformis mosseae* BEG 12 (+mycorrhiza) and the other four plants were inoculated with filtered (589/3 blue ribbon paper filter; Schleicher & Schuell Bioscience GmbH, Dassel, Germany) and autoclaved (121°C, 20 min) drain of BEG12 (-mycorrhiza). The respective inoculum was uniformly mixed (5%, v/v) with the sand/vermiculite substrate in the pots before planting the seedlings.

For mycorrhizal colonisation determination, roots were cleared with 10% KOH, acidified with 2 N HCl, and stained with 0.05% trypan blue in lactic acid (Phillips and Hayman, 1970). Infection frequency, relative colonisation intensity, and arbuscular frequency were determined under a microscope and calculated out of 30 root segments each with 10 mm length (Trouvelot *et al.*, 1986).

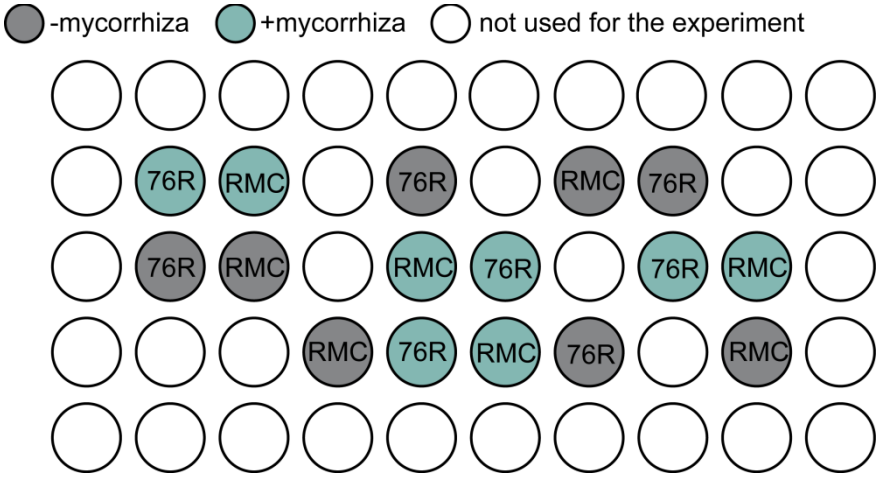

Figure 3.1: Plan of site for the mycorrhiza study: Tomato plants (genotype '76R' and 'RMC') grown in pots in the greenhouse. 50% of the plants were inoculated with mycorrhizal fungus *Funneliformis mosseae*.

3.8.2 'Matina' and 'Reisetomate' cultivation, *Pepino mosaic virus* inoculation and determination

Seeds were disinfected with 4% MennoFlorades (Menno Chemie Vertrieb GmbH, Norderstedt, Germany) for 30 min to exclude any pre-infection with PepMV. Plants were grown in hydroponic cultivation (figure 3.2, nutrient solution in mM: 12 NO_3, 4 K, 5 Ca, 0.1 NH_4, 0.5 P, 2.2 Mg and 3.4 SO_4, in µM: 50 B, 25 Fe, 5 Mn, 7 Zn, 0.7 Cu and 0.5 Mo). Nutrient solution was changed once a week and electric conductivity (EC) and pH were checked daily and adjusted to 3 dS m^{-1} for EC and to 5 - 6 for pH, if necessary.

Figure 3.2: Hydroponic cultivation of tomato plants in the greenhouse.

PepMV-infected as well as non-infected control plants were grown in the same greenhouse, separated with a net to prevent interchanging of insects which might be possible virus transmitters.
PepMV inoculation was conducted on the second fully developed leaf by rubbing with an extract of PepMV-infested tomato leaves. The extract was prepared by grinding infested leaf material in distilled water. For cultivating the plants protective clothing and equipment as well as routinely disinfection of material was necessary to prevent spreading the PepMV through the greenhouse and the whole complex.
Two weeks after PepMV inoculation (WPI), all plants were tested for a systemic PepMV infestation using an enzyme-linked immunosorbent assay (ELISA) kit (Agdia, Elkhart, Indiana, USA) according to manufacturer's instructions. All non-infected plants were controlled weekly following this procedure and always responded negative.

3.8.2.1 *Pepino mosaic virus* assessment study

The tomato plants were cultivated from January until April 2010. In total 96 (48 of cultivar 'Matina' and 48 of cultivar 'Reisetomate') were grown in the greenhouse (figure 3.3). PepMV inoculation was conducted six weeks after sawing. 24 plants were inoculated with PepMV isolate EU, 24 with isolate CH2 and 24 with both isolates. 24 plants served as controls without any PepMV infection. In addition to ELISA, all double-inoculated plants were tested with PCR and specific primers for EU and CH2 isolates (table 3.5), respectively, to confirm infection with both isolates. Unfortunately, infection with another pathogen, probably originating from the genus *Pythium* (Oomycota), was detected in every hydroponic channel on the roots of nearly all plants shortly before the harvest of fruits.

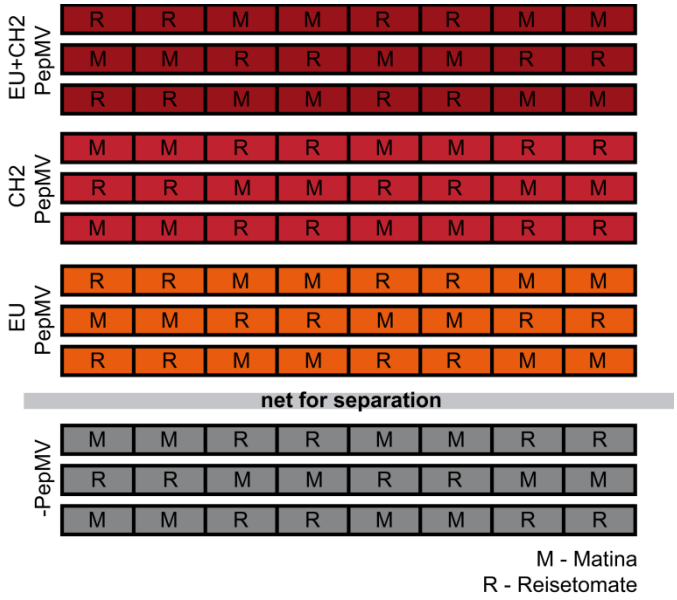

Figure 3.3: Plan of site for the *Pepino mosaic virus* assessment study: Tomato plants (cultivar 'Matina' and 'Reisetomate') grown in hydroponic cultivation in the greenhouse. Plants were infected with PepMV isolates EU or CH2 or with both.

3.8.2.2 *Pepino mosaic virus* study

The tomato plants were cultivated from May until November 2010. Mean temperature, daily radiation, and humidity in the greenhouse were 21.1°C, 28.9 mol $m^{-2}d^{-1}$, and 71.6%. In total 176 plants of the cultivar 'Matina' were grown (figure 3.4). PepMV inoculation was conducted ten weeks after sawing, after the first fruit development.

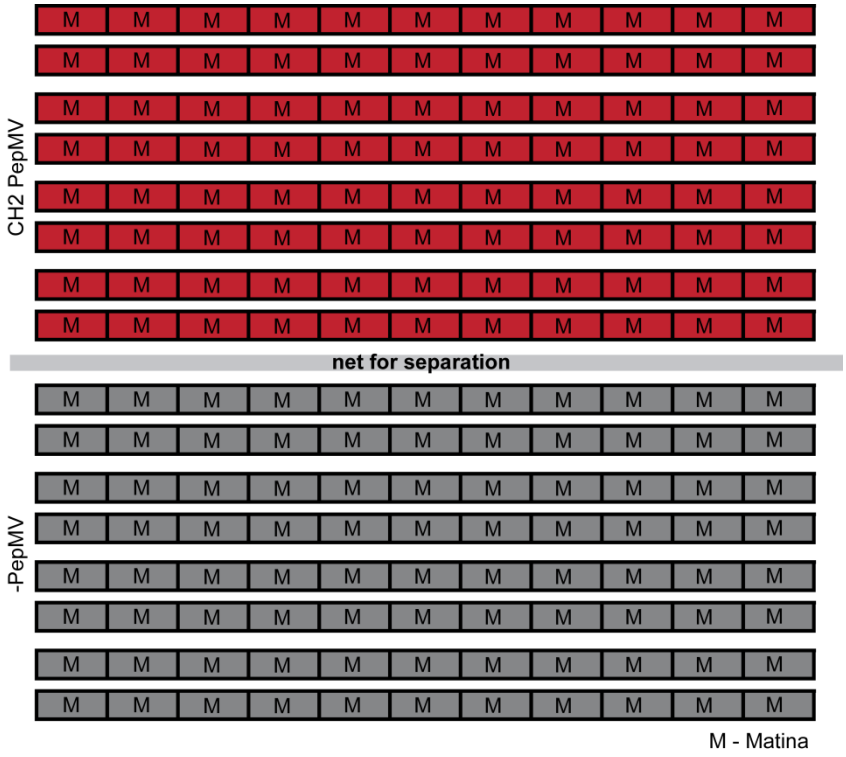

Figure 3.4: Plan of site for the *Pepino mosaic virus* study: Tomato plants, cultivar 'Matina', grown in hydroponic cultivation in the greenhouse. 50% of the plants were infected with PepMV isolate CH2.

3.8.3 'Counter' cultivation with different nitrogen conditions

These plants were cultivated from May to July 2004. In total 16 plants were cultivated in 10 L buckets (figure 3.5), filled with a commercial horticultural substrate (Terreau Professionel GePAC, Einheitserde Typ Null; Einheitserde Werkverband, Sinntal-Jossa, Germany). Tomato plants were irrigated manually until drain started.

Eight pots each were supplied either with organic nitrogen (Horngries, org N) or nitrate (NO_3) in deficit or excess, defined by the demand of a regular plant. The supplied nutrient solution consisted of (deficit/excess, in g per pot): 10/34 $Ca(NO_3)_2$ x 4 H_2O or 14/44 Horngries, 7 KH_2PO_4, 30 K_2SO_4, 25 K_2O/MgO, 30/30 $CaSO_4$ with Horngries, 15/0 $CaSO_4$ with NO_3 and (in mg per pot): 1.2 FeEDTA, 43 $MnSO_4$, 150 H_3BO_4, 6 MoO_3, 9.4 $CuSO_4$, 200 $ZnSO_4$. Mean temperature, daily radiation, and humidity in the greenhouse were 22.5°C, 20.1 mol $m^{-2}d^{-1}$, and 61%.

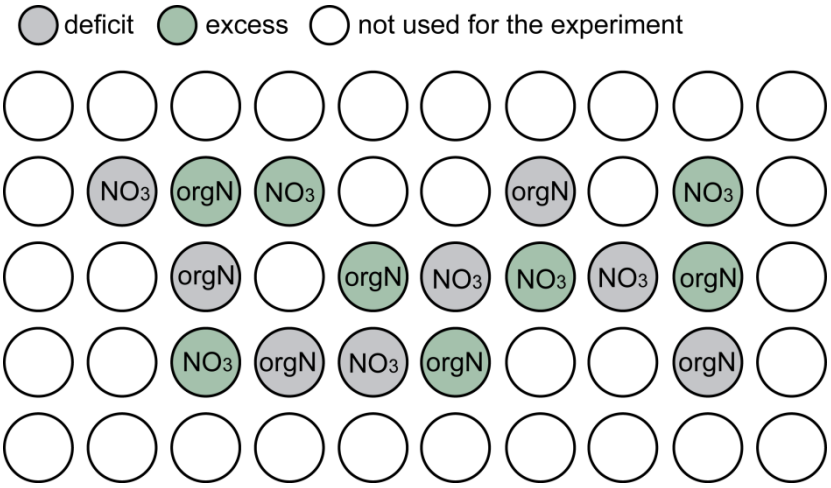

Figure 3.5: Plan of site of tomato plants cultivar 'Counter' grown in pots in the greenhouse. Plants were supplied with different forms (nitrate (NO_3) or organic nitrogen (org N)) and amounts (deficit or excess) of nitrogen.

3.9 Harvest and preparation of plant material and biological replications

Tomato fruits were harvested at comparable red-ripe stage (stage 10 - 11 of the colour screening scale for tomato, International standardisation of fruit and vegetables; figure 3.6) or at different ripening stages, according to the respective investigation. Tomato leaves and fruits, fruits after dividing them into four parts, were immediately frozen in liquid nitrogen and stored at -80°C until further processing.

Figure 3.6: Colour screening scale for tomato: International standardisation of fruits and vegetables.

3.9.1 Mycorrhiza study: Tomato roots and fruits

Roots from each plant were sampled. Per treatment four biological replicates were taken (one plant = one replicate) and further analysed. Root samples were taken with an auger (200 mm length, 225 mL) and remaining substrate was rinsed.

For RNA accumulation analyses, one tomato fruit of each plant was harvested, immediately frozen and ground with mortar and pistil in liquid nitrogen. Per treatment four biological replicates were taken (one plant = one replicate) and further analysed.

For the skin prick tests, fruits were harvested at the IGZ Großbeeren and brought to the Charité Berlin. Fresh fruits were used for skin prick tests.

3.9.2 *Pepino mosaic virus* studies: Tomato leaves and fruits

PepMV infection was determined by ELISA (Agdia, Elkhart, Indiana, USA). 300 mg of fresh leaf material were taken from each PepMV-inoculated plant. For weekly control of non-inoculated control plants always three to four plants were taken together as one sample of 300 mg. Leaf material was directly ground in ELISA kit containing buffer and further treated according to manufacturer's instructions.

For the RNA accumulation analyses four fruits or leaves were randomly taken at 11 -12 WPI (PepMV assessment study) at 3 and 10 WPI (PepMV study) from four different tomato plants, resulting in one mixed sample (four plants = one replicate). Per time point and treatment three biological replicates were analysed. After harvesting, fruits and leaves were immediately frozen in liquid nitrogen, freeze-dried, and pulverised with a mill (MM400; Retsch, Haan, Germany): Fine powder could be achieved by adding small sterilised metal balls (4 and 8 mm balls; ASK Kugellagerfabrik, Korntal-Münchingen, Germany) to the tubes containing the tomato material. This powder was directly used for RNA extraction.

For the RNA accumulation analyses of different fruit tissue in the PepMV assessment study, tomatoes were peeled and seeds as well as the florescence were separated from the pulp. Tissues were separately frozen in liquid nitrogen, freeze-dried, and ground to fine powder.

Figure 3.7 shows the sampling schedule for the respective analyses of the PepMV study. Tomato fruits were harvested at most 15 h before the clinical allergy tests between 3 and 13 WPI. Fruits were harvested at the IGZ Großbeeren and brought to the Charité Berlin, where a mush of ten tomatoes was prepared in a commercial food processor. This mush was directly used for skin prick and provocation tests or frozen in liquid nitrogen for basophil activation tests, skin prick tests, and RNA and protein, as well as immunoblot analyses.

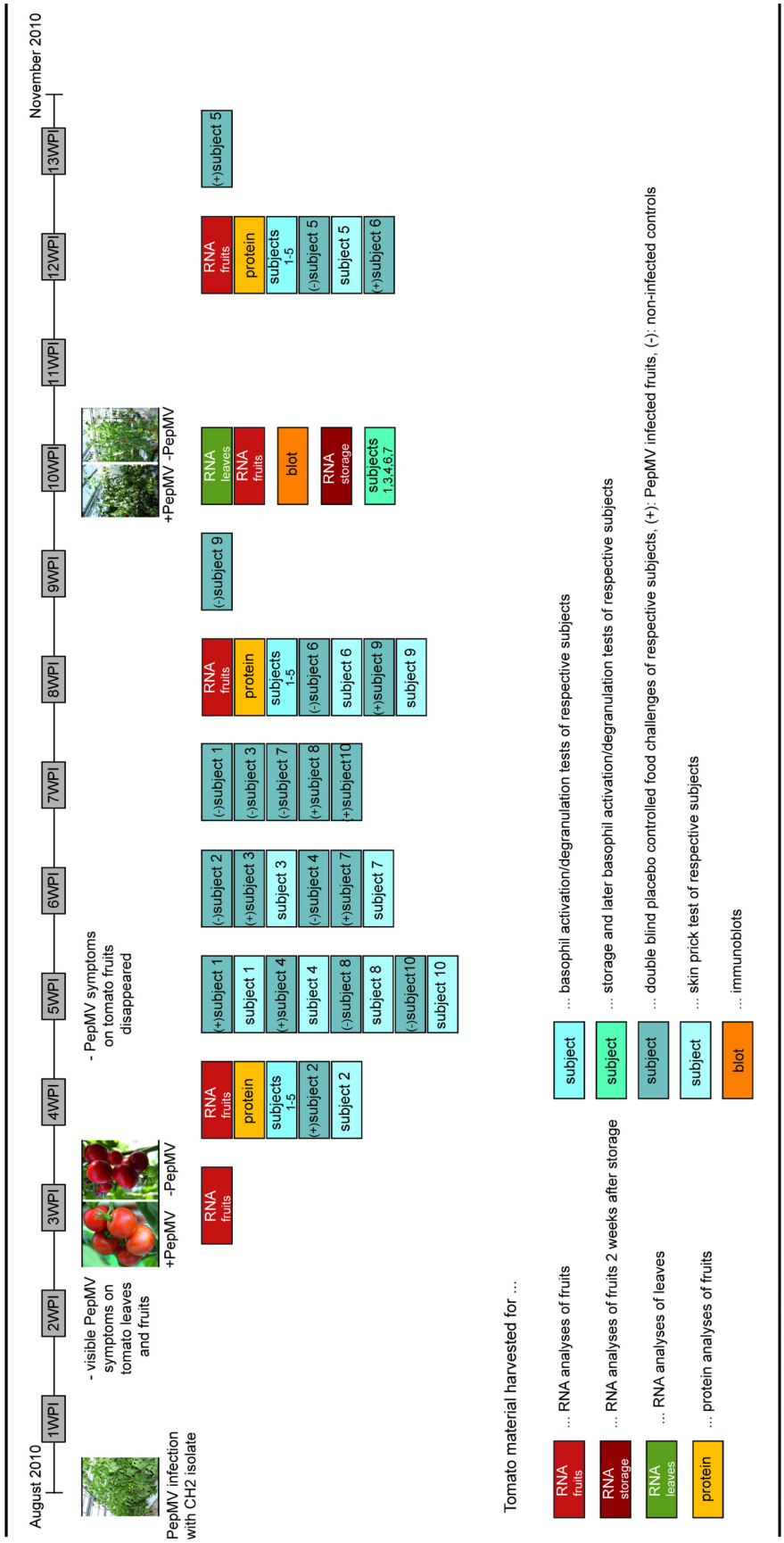

Figure 3.7: Overview of the tomato samples harvested for RNA and protein analyses as well as for clinical allergy tests and immunoblots over the time of the PepMV study in 2010. The small boxes tied to each other indicate that samples were taken from the same tomato mush or in case of leaves and fruits from the same tomato plant. WPI: weeks post *Pepino mosaic virus* (PepMV) inoculation.

3.9.3 Immunoblot study: Tomato fruits

For 2D protein gels and immunoblots ten tomato fruits of the genotypes '76R', 'RMC', and the cultivar 'Counter' were ground in a commercial blender and directly used for protein extraction.

3.10 Molecular analyses

The molecular analyses were carried out at the laboratories of the IGZ.

3.10.1 RNA extraction

RNA extraction was carried out with RNase free material and diethylpyrocarbonate (DEPC, Roth, Karlsruhe, Germany) or RNase free water (Qiagen, Hilden, Germany).

For the mycorrhiza study 100 mg of the frozen ground tomato fruit powder was used to extract RNA with the column based plant RNA extraction kit (Qiagen, Hilden, Germany) according to manufacturer's instructions.

For freeze-dried tomato fruits from the PepMV studies three different RNA extraction methods were evaluated. 10 mg of the freeze-dried ground tomato material was used.

RNA concentration and purity was determined photometrically with a NanoDrop at 260 nm (ND-100 spectrophotometer; Peqlab, Erlangen, Germany). The absorbance ratios 260/280 (to determine protein and phenol contamination) and 260/230 (to determine carbohydrate and phenol contamination) should be > 1.8 and > 2 for pure and non-contaminated RNA, respectively (NanoDrop technical support bulletin T009). Only values in that range were accepted. Additionally, RNA was checked after electrophoretic separation on an agarose gel for sharp ribosomal RNA (rRNA) bands.

Centrifugation was always performed in the centrifuge 5415R (Eppendorf, Wesseling-Berzdorf, Germany).

3.10.1.1 Plant RNA extraction kit

RNA was extracted with plant RNA extraction kit (Qiagen, Hilden, Germany) according to manufacturer's instructions on spin columns. Shortly, the samples were mixed with kit containing buffer and β-mercaptoethanol and put on the column. Samples were washed on column with ethanol and diluted from column with RNase free dH_2O. RNA was subjected to DNase digestion.

3.10.1.2 Phenol/chloroform RNA extraction

BUFFERS, SOLUTIONS, CHEMICALS, AND OTHER MATERIALS

- Extraction buffer
  - 0.1 M Natriumacetate (NaOAc)
  - 0.001 M Ethylenediaminetetraacetic acid (EDTA)
  - 4% Sodium dodecyl sulphate (SDS)
- PCIA
  - 50% Phenol
  - 48% Chloroform
  - 2% Isoamylalcohol
- CIA
  - 96% Chloroform
  - 4% Isoamylalcohol
- 8 M LiCl
- 70% Ethanol

All centrifugation steps were performed at 10,000 g.

The whole RNA extraction was performed on ice. Samples were mixed by vortexing with 500 µL extraction buffer and 500 µL PCIA and centrifuged for 5 min. Upper aqueous phase was used for further steps and extraction was repeated with remaining material. Aqueous phases were combined and an equal volume of PCIA was added, vortexed and centrifuged for 5 min. Upper aqueous phase was used and CIA was added, vortexed and centrifuged for 5 min. Upper aqueous phase was used, 0.5 volume LiCl was added, and RNA was precipitated at -20°C for 1 h. Tubes were centrifuged for 20 min and resulting pellet was washed with ethanol. After air drying the pellet was dissolved in RNase free dH_2O. RNA was subjected to DNase digestion.

3.10.1.3 TRIzol Reagent RNA extraction

BUFFERS, SOLUTIONS, CHEMICALS, AND OTHER MATERIALS

- Chloroform
- Isopropanol
- 75% Ethanol

All centrifugation steps were performed at 4°C and incubation steps at room temperature.

RNA was extracted with TRIzol Reagent (Invitrogen, Darmstadt, Germany) according to manufacturer's instructions. Shortly, the samples were mixed with 1 mL of TRIzol Reagent and incubated for 5 min. After centrifugation at 12,000 g for 10 min pellet was discarded and supernatant was further used. 200 µL chloroform were added to the supernatant, properly shaken, and incubated for 3 min. After centrifugation at 12,000 g for 15 min the upper aqueous phase was used and 500 µL isopropanol were added, incubated for 10 min and centrifuged again at 12,000 g for 10 min. The resulting pellet was washed with ethanol. After air drying pellet was dissolved in RNase free dH_2O. RNA was subjected to DNase digestion.

3.10.2 DNase digestion

BUFFERS, SOLUTIONS, CHEMICALS, AND OTHER MATERIALS

- 8 M LiCl
- 75% Ethanol

After RNA was extracted from tomato plant material, potentially remaining genomic DNA was digested with RNase free DNase set (Qiagen, Hilden, Germany). DNase digestion was performed in solution in accordance with the following protocol:
Dissolved RNA was incubated with DNase solution (7 units DNase enzyme solved in set containing RDD buffer) at room temperature for 30 min. DNase enzyme was inactivated at 70°C for 5 min. This procedure was repeated once. Afterwards RNA was precipitated with LiCl overnight at 4°C. After centrifugation (5415R; Eppendorf, Wesseling-Berzdorf, Germany) the pellet was washed with 75% Ethanol and resuspended in 50 µL RNase free water.

3.10.3. Control of genomic DNA contamination in RNA samples

Genomic DNA contamination was excluded by using extracted RNA as a template in a PCR reaction (chapter 3.10.5) and respective primer pairs for genomic DNA. The absence of a band in the agarose gel confirmed the absence of genomic DNA in the RNA solution and RNA quantity was photometrically determined and stored with 1 µL RNasin (Promega, Mannheim, Germany) at -80°C until further use.

3.10.4 cDNA synthesis

RNA was reverse transcribed with an M-MLV (*Moloney murine leukemia virus*) reverse transcriptase system (Promega, Mannheim, Germany) using oligo-dT primers for tomato mRNA or three PepMV genome-specific primers for PepMV RNA (table 3.5) according to manufacturer's instructions. Shortly, 1 µL oligo-dT (10 µM) primer was added to 1 µg RNA, RNase free dH_2O was added to a total volume of 15 µL and mixture was incubated at 70°C for 5 min. After cooling down on ice the following components were added:

- 5 µL M-MLV specific buffer
- 1.25 µL 10 mM dNTP mix
- 0.7 µL RNasin
- 1 µL M-MLV reverse transcriptase
- 2.05 µL RNase free dH_2O

The cDNA was synthesised at 42°C for 1 h and controlled on a 1.5% agarose gel.

3.10.5 Polymerase chain reaction

Polymerase chain reactions (PCR) were performed in a Primus thermocycler (MWG Biotech, Ebersberg, Germany). If not indicated differentially PCR reactions were prepared with respective primer pairs according to the protocol in table 3.7. PCR was conducted in a 20 µL reaction with a Taq (*Thermus aquaticus*)-polymerase, polymerase high specificity buffer (Peqlab, Erlangen, Germany) and a dNTP mix (ATP, TTP, GTP, CTP; Promega, Mannheim, Germany). The temperature program is shown

in table 3.8. Annealing temperature and elongation times were adapted according to the respective primer pairs and fragment lengths. Approximately 1 min elongation time was calculated for 1,000 bp.

Table 3.7: General PCR mix.

	volume [µL]	concentration
DEPC H_2O	12.9	
5x buffer (including $MgCl_2$)	2.0	1x
dNTP mix (2 mM)	2.0	0.2 mM
primer forward (10 µM)	1.0	0.5 µM
primer reverse (10 µM)	1.0	0.5 µM
Taq-polymerase	0.1	0.5 U
template (RNA/cDNA)	1.0	

Table 3.8: PCR temperature program of 35 repeating cycles.

		temperature [°C]	time [min]
initial denaturation		95	5.0
denaturation	**x35**	95	1.0
annealing	**x35**	depending on primer pair (50 – 65)	0.5
elongation	**x35**	72	depending on fragment length (1-2)
final elongation		72	10

For verifying respective fragment lengths of PCR products, 5 µL of PCR product was mixed with 1 µL loading dye and applied on an agarose gel with an appropriate DNA ladder.

3.10.6 Agarose gel electrophoresis

BUFFERS, SOLUTIONS, CHEMICALS, AND OTHER MATERIALS

- DNA ladders
  - 25 bp - 700 bp: low range DNA ladder (Fermentas, now Thermo Fisher Scientific, Rockford, USA)
  - 100 bp - 1,000 bp: peqGOLD (Peqlab, Erlangen, Germany)
  - 250 bp - 10,000 bp: peqGOLD (Peqlab, Erlangen, Germany)
- Agarose
- Ethidium bromide bath (1 µg/mL)
- TAE (Tris-acetate-EDTA) buffer
  - 40 mM Tris
  - 20 mM Acetic acid
  - 1 mM EDTA
- Loading dye
  - 250 mg Bromphenolblue
  - 34.5 mL Glycerol
  - → filled to 100 mL with dH_2O

Agarose gels were prepared with the respective percentage (1 - 3%) of agarose dissolved in TAE buffer by cooking in the microwave. Liquid agarose solution was polymerised in respective gel forms. The gel electrophoresis was performed in a Mupid-One apparatus (Nippon Genetics Europe, Düren, Germany) at 150 V for approximately 30 min.
Gels were stained in an ethidium bromide bath for 15 min and visualized and recorded under UV-light.

3.10.7 Quantitative real-time RT-PCR

For the design of a quantitative real-time reverse transcriptase PCR (qRT-PCR) experiment many things have to be considered and taken care of (Udvardi, 2008;Bustin *et al.*, 2009), and are described in detail in the previous and the following paragraphs.

In the real-time PCR reaction mix cDNA was used as a template in a 1:100 dilution of a master mix containing 50% Power SYBR Green (Applied Biosystems, Warrington, UK) and 200 nM of each primer (table 3.9). qRT-PCR was carried out using the 7,500 Fast Real-Time PCR System (Applied Biosystems, Warrington, UK) with a temperature program listed in table 3.10. Raw fluorescence data (Rn) or Ct (cycle threshold) values were used for further data processing (e.g. figure 3.8A). qRT-PCR reactions were always conducted in three technical replicates.

Table 3.9: Quantitative real-time RT-PCR mix.

	volume [µL]	concentration
Power SYBR Green	5	1x
primer forward (1 µM)	2	200 nM
primer reverse (1 µM)	2	200 nM
template (cDNA, diluted 1:10)	1	

Table 3.10: Quantitative real-time RT-PCR temperature program of 40 repeating cycles in Fast Real-Time PCR system.

		temperature [°C]	time
pre-heating		50	2 min
initial denaturation		95	10 min
denaturation	**x40**	95	15 sec
annealing	**x40**	60	1 min
melting curve analysis			

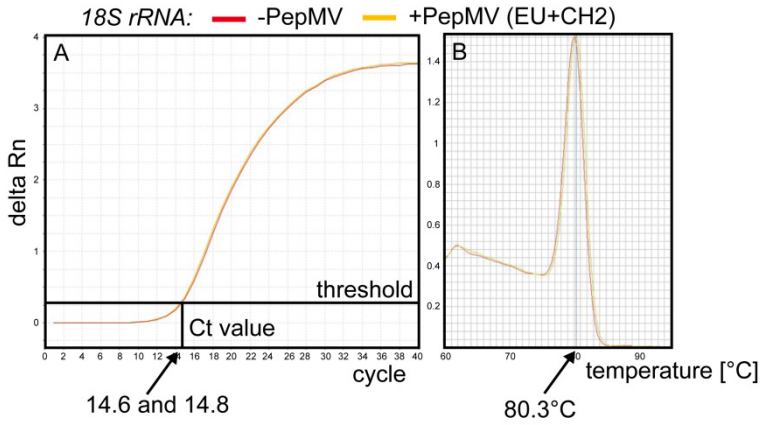

Figure 3.8: Amplification (A) and melt curve (B) of the reference gene *18S rRNA* of PepMV-infected (+PepMV, yellow) and non-infected control tomato fruits (-PepMV, red) in quantitative real-time RT-PCR. The amplification curve also shows the threshold, indicated by the black line and Ct values of 14.6 and 14.8 are marked. The melting temperature of both fragments is 80.3°C.

3.10.7.1 Primer design and validation

Primer pairs were designed with Primer Select software of DNAStar (GATC Biotech, Konstanz, Germany), avoiding if possible, mismatches between primers and targets, runs of identical nucleotides, T at 3'-end, and complementarities within and between primers. An amplicon length for qRT-PCR analyses of no more than 200 bp and a consistent annealing temperature of approximately 60°C was considered for all primer pairs to allow simultaneous analyses on one plate. All primer pairs had exponential increasing amplification curves starting between the 10^{th} and 30^{th} cycle. Melting of amplicons resulting from one primer pair always showed a single peaked melting curve (e.g. figure 3.8B). After curve analysis in the equipment software data were exported and primer pair efficiencies were calculated with LinReg software. All primer pairs showed efficiency values between 1.8 and 2.1 in all evaluated samples.

3.10.7.2 Calculation of quantitative real-time RT-PCR data

qRT-PCR results were calculated based on two relative quantification methods: the ΔCt method and the Biogazelle qBase$^+$ calculation. Ct values given by the Fast Real-Time PCR System were used for further evaluation. Reference genes were used for normalisation. In both methods and in case of no outlier the mean of the three technical replicates was used for further evaluation.

3.10.7.2.1 ∆Ct method

This classical relative quantification method was used in the beginning for the mycorrhiza study. The geometric mean of two reference genes was used for normalisation. Data were calculated based on the following formula:

ΔCt = Ct reference gene(s) – Ct target gene

$2^{\Delta Ct}$ was used for further calculation.

In this method PCR efficiency values are assumed to be optimal (2 = 100%).

3.10.7.2.2 Biogazelle qBase$^+$

Biogazelle qBase$^+$ software (qBase$^+$; Biogazelle, Zwijnaarde, Belgium) was used to evaluate qRT-PCR results (Hellemans *et al.*, 2007), based on given Ct values of the Fast Real-Time PCR system. With this software it is possible to take PCR efficiencies into account and to evaluate proper reference genes for respective samples. Additional calibration and normalisation factors are included in the calculation of relative RNA accumulation. The software calculates a CNRQ value due to the following formula, which is a modification from the classic ΔCt method:

ΔCt = geometric mean of Ct reference genes – Ct target gene

RQ = $\text{Efficiency}^{\Delta Ct}$

NRQ = RQ/Normalisation factor

CNRQ = NRQ/Calibration factor

3.10.7.2.3 Efficiency of quantitative real-time RT-PCR

PCR efficiency calculation was conducted with LinReg (Amsterdam, The Netherlands), a freely available software tool to calculate PCR efficiencies (Ramakers *et al.*, 2003). Efficiencies are calculated according to the raw fluorescence data exportable from the Fast Real-Time PCR System. For each experiment and primer pair a mean efficiency was calculated, recorded into the qBase$^+$ software, and further used for the calculation. Efficiency values between 1.8 and 2.1 were accepted.

3.10.7.2.4 Reference gene evaluation

Reference genes were chosen from literature or own experience because of their known stable expression (table 3.2). However, reference genes have to be newly validated for each sample set, depending on plant tissue and treatment to be compared. Therefore, reference gene stabilities were evaluated newly for every experiment with the qBase$^+$ software and an integrated software called geNorm (Vandesompele *et al.*, 2002). Both calculate a gene stability measure (M_{ES}), the mean pairwise variation of one reference gene with all other reference genes, and a coefficient of variance (CV). The smaller these values are the higher is the stability of the reference gene and the better for normalisation. In general it is recommended that M_{ES} should be < 0.5, and in very heterogeneous samples can rise until 1. CV should be < 0.2, and in very heterogeneous samples can rise until 0.5.

3.11 Biochemical analyses

The protein analyses with the tomato protein extracts were mainly carried out at the laboratories of the VEGAL project partner Proteome Factory AG. The protein analyses with the recombinant cyclophilin analyses were conducted at the IGZ.
Centrifugation was always performed in the centrifuge 5810R (Eppendorf, Wesseling-Berzdorf, Germany).

3.11.1 Protein extraction of tomato fruits for *Pepino mosaic virus* experiment

BUFFERS, SOLUTIONS, CHEMICALS, AND OTHER MATERIALS

- Extraction buffer
  - 25 mM Tris-HCl, pH 7.4
  - 50 mM KCl
  - 1.5 mM EDTA
  - 2.9 mM Benzamidine
  - 2.1 µM Leupeptine
  - 1 mM Phenylmethanesulfonylfluoride
  - 1 µM Pepsatin A
- Guanidinehydrochloride
- Urea
- Thiourea
- Carrier ampholytes (pH 2 - 4; Serva, Heidelberg, Germany)
- Dithiothreitol (DTT)

The tomato mush of ten tomatoes of cultivar 'Matina' was mixed with extraction buffer containing 7 M guanidinehydrochloride, centrifuged (10,000 g, 4°C, 30 min), and protein was precipitated from supernatant with five volumes ethanol at -20°C overnight. The pellet was resuspended in extraction buffer additionally containing 7 M urea, 2 M thiourea, 2% carrier ampholytes (pH 2 - 4), and 70 mM DTT. After concentration measurement protein extracts were stored at -20°C until further use.

3.11.2 Protein extraction of tomato fruits for immunoblot study

BUFFERS, SOLUTIONS, CHEMICALS, AND OTHER MATERIALS

- Extraction buffer
  - 1.4 M Sucrose
  - 0.2 M KCl
  - 100 mM Tris-HCl, pH 7.5
  - 50 mM EDTA
  - 2 mM Phenylmethanesulfonylfluoride
  - 4% Mercaptoethanol
  - 2% Polyvinylpolypyrrolidone
- Urea buffer
  - 7 M Urea
  - 2 M Thiourea
  - 2% Carrier ampholytes (pH 2 - 4; Serva, Heidelberg, Germany)

Tomato mush of either cultivar '76R', 'RMC', or 'Counter' was mixed with extraction buffer. After 30 min incubation on ice samples were mixed with phenol, followed by a centrifugation step (10,000 g, 4°C, 30 min). Supernatants were precipitated with five volumes methanol at -20°C overnight and resulting pellets were solved in urea buffer.

3.11.3 Protein concentration determination

Protein concentrations were determined by Bradford assay (Bradford, 1976), according to manufacturer's instructions (Proteome Factory AG, Berlin, Germany). Bradford determinations were carried out in three technical replicates and measured photometrically at 620 nm. Protein amount was determined according to a standard curve of bovine serum albumin (BSA), accompanying every measurement.

3.11.4 Separation of proteins: SDS-PAGE

Proteins were separated with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Depending on the demand proteins were separated with 1 dimensional (1D) or 2 dimensional (2D) SDS-PAGE.

3.11.4.1 1D gel electrophoresis

BUFFERS, SOLUTIONS, CHEMICALS, AND OTHER MATERIALS

- Gel composition
  - 15% (separation gel) or 4% (stacking gel) Acrylamide
  - 0.2% Bisacrylamide
  - 375 mM Tris-HCl, pH 6.8 (stacking gel) and 8.8 (separation gel)
  - 0.1% Sodium dodecyl sulphate (SDS)
  - 0.03% Tetramethylethylenediamine (TEMED)
  - 0.08% Ammonium persulphate

- Running buffer
  - 14.4 g Glycine
  - 3 g Tris
  - 1 g SDS
  → in 1 L dH_2O
- 375 mM Tris buffer, pH 7 - 8
- Leammli loading buffer (Roti-Load, Roth, Karlsruhe, Germany)
- Protein weight marker (Roti-Mark, Roth, Karlsruhe, Germany)

Separation and stacking gels were prepared according to the above recipe polymerised and ran in vertical gel electrophoresis systems (Mini-PROTEAN Tetra Cell; Bio-Rad, Munich, Germany). Laemmli loading buffer was added to the protein extracts and samples were cooked at 100°C for 10 min, applied to the gel and ran in running buffer at 150 V for 70 min. 10 µg total tomato protein extract and 1 – 2 µg of a single protein were normally used for separation on 1D gels. Full bacterial extract samples were diluted with 20 µL Tris buffer per OD600 ~1, before loading on the gel.

3.11.4.2 2D gel electrophoresis

BUFFERS, SOLUTIONS, CHEMICALS, AND OTHER MATERIALS

- First dimension gel composition
  - 9 M Urea
  - 4% Acrylamide
  - 0.3% Piperazine diacrylamide
  - 5% Glycerine,
  - 2% Carrier ampholyte (pH 2 - 11; Serva, Heidelberg, Germany)
  - 0.06% TEMED
  - 0.08% Ammonium persulphate
- Second dimension gel composition
  - 15% Acrylamide
  - 0.2% Bisacrylamide
  - 375 mM Tris-HCl (pH 8.8)
  - 0.1% SDS
  - 0.03% TEMED
  - 0.08% Ammonium persulphate

Two dimensional electrophoresis (2DE) was conducted following the protocol by Klose and Kobalz (Klose and Kobalz, 1995). Isoelectric focussing (first dimension) was performed in vertical rod gels. 60 µg or 120 µg of protein extract were focussed at 1,841 V. SDS–PAGE (second dimension) was performed in small (0.1 cm x 7 cm x 8 cm) or big gels (0.5 cm x 20 cm x 30 cm). 2D separations were performed in duplicate. One gel was stained with FireSilver (Proteome Factory, Berlin, Germany) for preparative applications; the other gel was used for immunoblotting.

3.11.4.3 Staining of SDS gels: Coomassie and silver

The visualisation of proteins was either done with colloidal coomassie or silver staining.
Coomassie staining of protein gels was performed with the Roti-Blue kit (Roth, Karlsruhe, Germany) according to manufacturer's instructions.
Silver staining of protein gels was performed with the FireSilver staining kit (Proteome Factory AG, Berlin, Germany) according to manufacturer's instructions.

3.11.5 Protein identification via mass spectrometry

Protein identification was performed by one of the following mass spectrometers:

- QTOF nanoLC-ESI-MS/MS: The MS system consisted of an Agilent 1100 nanoLC system (Agilent, Waldbronn, Germany), PicoTip emitter (New Objective, Woburn, USA) and a Qtof Ultima mass spectrometer (Micromass/Waters, Manchester, UK).
- LTQ FT nanoLC-ESI-MS/MS: The MS system consisted of an Agilent 1100 nanoLC system (Agilent, Waldbronn, Germany), NanoMate 100 (Advion, Ithaca, USA) and a Finnigan LTQ-FT mass spectrometer (ThermoFisher, Bremen, Germany).
- Q Star nanoLC-ESI-MS/MS: The HPLC system was coupled to an MS detection was performed on a Qstar XL mass spectrometer (ABI, Foster City, CA, USA).

Protein spots were in-gel digested with trypsin (Promega, Mannheim, Germany) according to manufacturer's instructions and applied to nanoLC-ESI-MSMS. Peptides were trapped and desalted on an enrichment column (Zorbax SB C18, 0.3 x 5 mm; Agilent, Böblingen, Germany) using 1% acetonitrile/0.1% formic acid solution for 5 min and subsequently separated on a Zorbax 300 SB C18, 75 µm x 150 mm column (Agilent, Böblingen, Germany) using an acetonitrile/0.1% formic acid gradient from 5% to 40% acetonitrile within 40 min. MS spectra were automatically taken by Esquire 3,000 plus according to manufacturer's instrument settings for nanoLC-ESI-MS/MS analyses. Proteins were identified using MS/MS ion search of Mascot search engine (Matrix Science, London, Great Britain) and a protein database (NCBI; Bethesda, MD, USA).

Ion charge in search parameters for ions from ESI-MS/MS data acquisition were set to "1+, 2+ or 3+", according to the instruments' and methods' common charge state distribution. Mascot expresses the probability that peptides match at random to a given protein by a probability score. A score larger than 57 indicates identity or extensive homology (p=0.05).

3.11.6 Protein quantification via 2D gels

Tomato protein extracts of PepMV-infected and non-infected tomato fruits of 4, 8, and 12 WPI were separated on big 2D protein gels (0.5 cm x 20 cm x 30 cm) to quantify representative putative tomato allergens. Identification and quantification was achieved by comparison with immunoblots. The 2D gels were digitised at a resolution of 150 dpi using a PowerLook 2100XL scanner with transparency adapter. 2D image analysis and protein spot quantification was performed using the Proteomweaver software 3.1 (Definiens AG, Munich, Germany). The quantification is based on the size and density of the respective protein spot and the software displays values of single spots for further calculation.

3.11.7 Protein quantification via iTRAQ

A commercially available iTRAQ (isobaric tag for relative and absolute quantification) 4plex kit (AB Sciex, Framingham, MA, USA) was used to quantify putative tomato allergens. iTRAQ reagents (isotopes: 114 - 117) are multiplexed , amine-specific stable isotope labelling reagents that can label all peptides in up to four different biological replicates.

25 µg tomato protein extract of PepMV-infected and non-infected tomato fruits of 4, 8, and 12 WPI were labelled according to manufacturer's instructions. The relative quantification was achieved by using a standard, consisting of a mixture of the six samples together. Four samples, differentially labelled, were mixed to one sample (sample 1 and sample 2, table 3.11), ran on a 1D SDS-PAGE and

stained with coomassie. Protein bands were excised, trypsin digested, and analysed and identified by mass spectrometry. For protein quantification the Protein Pilot 4.0 software (AB Sciex, Framingham, MA, USA) was used.

Table 3.11: Samples for iTRAQ quantification.

sample	components
sample 1	standard (iTRAQ 114) -PepMV 4 WPI (iTRAQ 115) +PepMV 8 WPI (iTRAQ 116) -PepMV 12 WPI (iTRAQ 117)
sample 2	standard (iTRAQ 114) +PepMV 4 WPI (iTRAQ 115) -PepMV 8 WPI (iTRAQ 116) +PepMV 12 WPI (iTRAQ 117)

3.12 Allergen detection: Immunoblots

The immunoblot analyses with the tomato protein extracts were mainly carried out at the laboratories of the VEGAL project partner Proteome Factory AG. In contrast to the immunoblot analyses with the recombinant cyclophilin analyses which were conducted at the IGZ.

3.12.1 Dot blot

For dot blot analyses respective protein extracts were dropped on a methanol-activated Immobilon-P membrane (PVDF, pore size 0.45 mm; Millipore, Bedford, USA) and then immune stained with respective tomato-allergic subjects' sera.

3.12.2 Western blot

1D and 2D gels were blotted on a methanol-activated Immobilon-P membrane (PVDF, pore size 0.45 mm; Millipore, Bedford, USA) using a Trans-Blot SD semi-dry transfer cell (Bio-Rad, Munich, Germany) at a constant current (5 V) at 4°C overnight.

3.12.3 Immunostaining

BUFFERS, SOLUTIONS, CHEMICALS, AND OTHER MATERIALS

- TBS (Tris buffered saline) Tween
  - 20 mM Tris-HCl
  - 154 mM NaCl
  - 0.1% Tween 20
  → pH 7.3
- Blocking buffer
  - TBS Tween
  - 3% Bovine serum albumin (BSA)
- Antibody buffer
  - TBS Tween
  - 1% BSA
- Alkaline phosphatase buffer
  - 200 mM Tris-HCl
  - 2 mM $MgCl_2$
  → pH 8.1

Immunoblots were conducted with tomato-allergic subjects' sera. Individual sera or a serum pool was used to detect putative new tomato allergens. All sera were used in a 1:10 dilution in antibody buffer, unless indicated differentially. After washing and blocking, membranes were incubated with sera dilutions overnight. Then, the membrane was incubated with one of the above described anti-human IgE secondary antibodies (chapter 3.4) in a respective dilution in antibody buffer for 2 h. Between all incubation steps the membrane was washed with TBS Tween (5 x 10 min). Immunoblots were developed with Pierce ECL (enhanced chemilumescent) Western blotting substrate (Thermo Fisher Scientific, Rockford, USA) according to manufacturer's instructions in the case of POD-conjugated secondary antibody. Immunoblots were exposed to films (Kodak scientific imaging film; Eastman Kodak Company, Rochester, NY, USA) until positive reacting spots were visible, respective times are indicated with the immunoblot figures. In the case of AP-conjugated secondary antibody the membrane was incubated with alkaline phosphatase buffer for 5 min after the last washing step and then developed with Fast Red TR/Naphtol AS/MX alkaline phosphatase tablets (Sigma-Aldrich, Saint Louis, USA) according to manufacturer's instructions. Respective incubation times are indicated with the immunoblot figures.

3.12.4 Immunoblot inhibition

Immunoblots of the recombinant cyclophilin were carried out as described above; with the exception that tomato-allergic subjects' sera were incubated with denatured recombinant cyclophilin at 4°C overnight, before the addition to the membrane. Immunoblots were then developed with the POD-conjugated secondary antibody.

3.13 Clinical allergy tests

The clinical allergy tests were conducted in the Allergy-Center Charité in Berlin by the group of Prof. Dr. Worm.

3.13.1 Determination of total and specific IgE

The total and specific IgE to tomato were determined with the ImmunoCAP System (Thermo Fisher Scientific, Uppsala, Sweden) according to manufacturer's instructions. Measurement of the specific IgE to tomato ensures that subjects are sensitised towards tomato and might reflect subjects' reaction, presupposed it matches their clinical history.

3.13.2 Skin prick test

Skin prick tests were performed according to the recommendations of GA²LEN (Heinzerling *et al.*, 2005) and performed as prick-to-prick. Prick-to-prick tests were performed on the surface of the interior forearm with standardised prick needles. Histamine dichloride (10 mg/mL; ALK-Abelló, Wedel, Germany) and saline solution (pH 7.4; ALK-Abelló) served as positive and negative controls. Allergenicity of the different tomatoes was analysed by measuring the wheal diameters. The skin reactions were considered positive when the wheal diameter was ≥ 3 mm after 15 min, in the absence of a reaction towards the negative control.

Skin prick tests for the mycorrhiza and immunoblot study were done by pricking into one fresh tomato fruit and then on the forearm of the tomato-allergic subject.
In the PepMV study skin prick tests were conducted with freshly prepared or frozen (stored at -80°C until use) tomato mush of ten tomatoes.
Recombinant cyclophilin (rCYC) was also tested on the forearms of tomato-allergic subjects. rCYC solution had a concentration of 312 µg/mL and was first tested as a 1:10 dilution, in the case of an absent reaction in a 1:2 dilution, and finally undiluted.

3.13.3 Basophil activation and degranulation test

BUFFERS, SOLUTIONS, CHEMICALS, AND OTHER MATERIALS

- PBS (phosphate buffered saline)
  - 137 mM NaCl
  - 10 mM Na_2HPO_4
  - 2.7 mM KCl
  - 2 mM KH_2PO_4,
  → pH 7.4
- PBS NP40
  - PBS
  - 5% NP40
- Beriglobin (Aventis Behring, Marburg, Germany)
- anti-CD63 FITC (BD, Franklin Lakes, NJ, USA)
- anti-CD203c-PE (Aventis Behring, Marburg, Germany)

For basophil activation tests tomato proteins were extracted with PBS NP40, centrifuged, and protein was precipitated from supernatant with acetone at -20°C overnight. The pellet was resuspended in PBS with a protease inhibitor mix (complete mini-EDTA free tablets; Roche, Indianapolis, USA).
Blood of tomato-allergic subjects was investigated in a basophil activation and degranulation test. Tomato protein extracts in different concentrations (0.005, 0.05, 0.5, 5, 50, 500 µg/mL) as well as a positive (5 µg/mL human anti-IgE; Biozol HP6061, Eching, Germany) and a negative (medium/ 10% foetal calf serum) control served for stimulation of the cells at 37°C for 15 min. Cells were stained for 45 min with anti-CD203c-PE in the case of basophil activation measurements and with anti-CD63 FITC in the case of basophil degranulation measurements with 2 µL Beriglobin, respectively. Activation and degranulation were determined by flow cytometry using MACS Quant Analyser (Miltenyi Biotec, Bergisch Gladbach, Germany) and data were analysed using FCS Express V3 software (De Novo Software, Los Angeles, CA, USA). CD3 negative and CCR3 positive cells were considered as basophils. Raw data were normalised relative to the positive control. Activation was determined by $\%CD203c^+$, whereas degranulation was determined by $\%CD63^+$ basophils.

3.13.4 Double blind placebo controlled food challenge

Tomato-allergic subjects from the PepMV study were tested for clinical relevant tomato allergy and the severity of reaction in double blind placebo controlled food challenges (DBPCFC). These provocation tests were performed according to the guideline of the European Academy of Allergy and Clinical Immunology (Bindslev-Jensen *et al.*, 2004).
Every subject was provoked on different days with a placebo and a tomato mush containing challenge meal, prepared shortly before the test. Challenge meals consisted of 150 g or no tomato

mush blinded with hypoallergenic infant formula (Nestlé Nutrition, Frankfurt, Germany), 250 mL beet root juice (Alnatura, Germany), and a trace of aroma (orange and black currant; SHS, Gesellschaft für klinische Ernährung, Heilbronn, Germany).

3.14 Cloning of putative allergen-encoding genes

Newly identified and putative allergens were tried to clone and overexpress in *Escherichia coli*.

3.14.1 Primer design

The whole coding sequence (CDS) of the respective protein-encoding gene was used for primer design. Specific restriction sites, not present in the gene sequence (tested in: www.restrictionmapper.org), were introduced on the 3'-end and ATG initiation was removed. In front of each restriction site three bases (CGA-for; CAG-rev) were added for facilitating the binding of the restriction enzyme (table 3.3). If these primers failed to work, primer pairs without restriction sites were designed, afterwards primers with restriction sites were used on the amplified PCR product.

3.14.2 Coding sequence amplification and purification

For amplifying the coding sequence (CDS) of respective genes with PCR proof reading polymerases (Pfu) instead of a Taq-polymerase (Promega, Mannheim, Germany) was used to increase the probability for the exact sequence. The PCR product was then purified with the Qiaquick PCR purification kit (Qiagen, Hilden, Germany). Amplified products were checked for their right size, before and after purification, on an agarose gel. Purified products were stored at -20°C until further use.

3.14.3 Restriction digestion and purification

Restriction digestion with fast digest restriction enzymes was carried out according to manufacturer's instructions (Fermentas, now Thermo Fisher Scientific, Rockford, USA). All restriction enzymes could be used in one single reaction and the following reaction mix was prepared:

- 15 µL nuclease-free dH_2O
- 2 µL 2x Fast digest restriction buffer
- 1 µL (~0.5 - 1 µg) Vector
- 1.5 µL of each restriction enzyme

The reaction mix was incubated in a water bath at enzyme specific temperature for 30 min.
Empty restricted vectors were additionally treated with 1 µL of SAP (shrimp alkaline phosphatase; Fermentas) to avoid re-ligation after restriction. Restriction enzymes and SAP were inactivated according to the respective enzyme and manufacturer's instructions at 80°C for 20 min.
Restriction digestion was only performed with vectors (empty or including PCR fragments), not on single PCR fragments, to assure high efficiency and proper restriction. Proper digestion was verified by separating restriction products on an agarose gel and checking fragment and vector size.

Resulting CDS fragments were purified from agarose gel with a gel extraction kit (Qiagen, Hilden, Germany). Restricted vectors were purified on columns with PCR purification kit (Qiagen). Purified restriction products were stored at -20°C until further use.

3.14.4 Cloning into pGemTEasy vector

The purified PCR fragment was first cloned into pGemTEasy vector (Promega, Mannheim, Germany). This vector allows a colour screening of *E. coli* colonies after transformation because of harbouring a β-galactosidase which can be insertionally inactivated. The pGemTEasy vector is delivered as an open vector with single 3' T-overhangs simplifying PCR product insertion. Pfu-polymerases generate blunt-end fragments, therefore, a poly-A tailing with Taq-polymerase was necessary just before ligation into this vector. This was achieved according to the following protocol:

- 3 µL purified PCR product
- 0.1 µL Taq Polymerase (Promega)
- 0.1 µL 2 mM dATPs (Promega)

The reaction mix was incubated at 70°C for 10 min.

Directly afterwards ligation was conducted with adjusted ligation time and temperature for the respective inserts and vectors. The general ligation was conducted due to the following protocol at 4°C overnight:

- 5 µL 2x Rapid ligase buffer (Promega, Mannheim, Germany)
- 3 µL purified and tailed PCR product
- 1 µL pGemTEasy vector
- 1 µL T4 Ligase (Promega, Mannheim, Germany)

3.14.5 Cloning into pCDFDuet and pET15b vector

Restricted CDS fragments were either cloned into pCDFDuet (figure 3.9) or pET15b (figure 3.10) vectors (Novagen, Merck, Darmstadt, Germany). The molar ratio of vector to insert in the ligation reaction should be approximately 1:3 to achieve effective ligation. Therefore, volumes of vectors and insert were adjusted in the ligation reaction mix:

- 5 µL 2x Rapid ligase Buffer (Promega, Mannheim, Germany)
- ~1 µL restricted CDS fragment
- ~1 µL restricted empty vector
- 1 µL T4 Ligase (Promega, Mannheim, Germany)
- → adjusted to 10 µL with nuclease-free dH_2O

The ligation was carried out at 16°C overnight.

Properly ligated new vectors were stored at -20°C until further use.

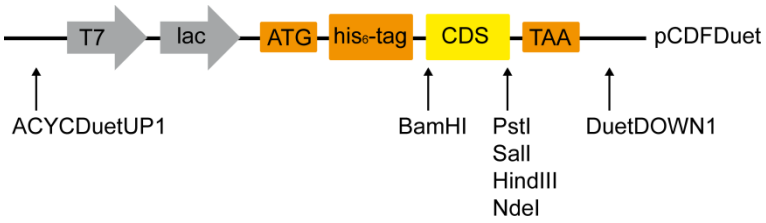

Figure 3.9: pCDFDuet expression vector construct containing CDS of respective putative allergen inserts for overexpression in *E. coli*. ACYCDuetUP1: vector specific primer forward; DuetDOWN1: vector specific primer reverse; T7: T7 promoter; lac: lac operator; ATG: start codon; TAA: stop codon; BamHI, PstI, SalI, HindIII, NdeI: respective restriction enzymes; his_6-tag: 6 x histidine; CDS: coding sequence for respective putative allergen.

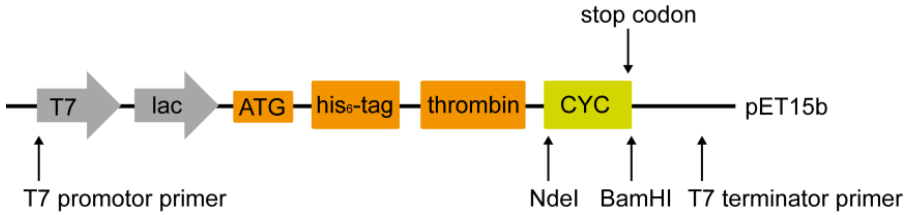

Figure 3.10: pET15b expression vector construct containing CYC insert for overexpression in *E. coli*. T7 promoter primer: vector specific primer forward; T7 terminator primer: vector specific primer reverse; T7: T7 promotor; lac: lac operator; ATG: start codon; NdeI¸ BamHI: respective restriction enzymes; his_6-tag: 6 x histidine; thrombin: thrombin cleavage site; CYC: coding sequence for tomato cyclophilin.

3.15 Transformation of *Escherichia coli* cells

BUFFERS, SOLUTIONS, CHEMICALS, AND OTHER MATERIALS

- LB (lysogeny broth) medium
  - 5 g/L Yeast extract
  - 10 g/L Tryptone
  - 10 g/L NaCl
  → pH 7
- Ampicillin (100 µg/mL; Roth, Karlsruhe, Germany)
- Spectinomycine (50 µg/mL; Calbiochem, EMD Bioscience, Merck, Darmstadt, Germany)
- Isopropyl-β-D-1-thiogalactopyranoside (IPTG; Roth, Karlsruhe, Germany)
- X-β-Gal (Roth, Karlsruhe, Germany)
- LB plates
  - LB medium
  - 15 g/L Agar
- SOC (super optimal broth) medium
  - 2% Tryptone
  - 0.5% Yeast extract
  - 10 mM NaCl
  - 2.5 mM KCl
  - 10 mM $MgCl_2$
  - 10 mM $MgSO_4$
  - 20 mM Glucose

COMPETENT *ESCHERICHIA COLI* CELLS

- JM109 (Promega, Mannheim, Germany)
  - produces high yield and quality of plasmid DNA through endonuclease A mutation
- BL21-DE3 (Merck, Darmstadt, Germany)
  - carries the T7 RNA polymerase gene
  - transformed plasmids containing T7 promoter driven expression are repressed until IPTG induction of T7 RNA polymerase from a lac promoter.
- Rosetta (Merck, Darmstadt, Germany)
  - BL21-DE3 derivates, designed to enhance the expression of eukaryotic proteins that contain codons rarely used in *E. coli*
  - these strains supply tRNAs for the codons AUA, AGG, AGA, CUA, CCC, GGA on a compatible chloramphenicol resistant plasmid
- Origami (Merck, Darmstadt, Germany)
  - K-12 derivatives that have mutations in both the thioredoxin reductase (trxB) and glutathione reductase (gor) genes, which greatly enhances disulphide bond formation in the cytoplasm.
  - kanamycin and tetracycline resistant

JM109 competent *E. coli* cells used for plasmid propagation and BL21 (DE3), Rosetta, and Origami used for overexpression were transformed according to the following protocol:

- 5 µL ligation reaction
- 20 µL competent cells

Ligation reaction mix was incubated with competent cells on ice for 30 min. A heat shock was conducted in a water bath at 42°C and the reaction mix then was cooled down on ice for 2 min. After the addition of 300 µL SOC medium cells were shaken at 37°C for 1.5 h.
Agar plates with respective antibiotics were prepared and, in the case of JM109 cells harbouring the pGemTEasy, additional 10 µL IPTG and 40 µL X-β-Gal were added to the plates for later blue-white screening of *E. coli* colonies. 50 - 150 µL of the transformation reaction mix were plated. After incubation at 37°C overnight or two to three days at room temperature, plates were screened for (white) colonies and further checked for the right insert with colony PCR.

3.15.1 Colony PCR

A PCR directly on the bacterial colony can be used to check for the right insert size in the *E. coli* propagated vector. Colonies were picked from agar plates and diluted in 50 µL LB medium, containing ampicillin. Colony PCRs were carried out with the KAPA robust PCR kit (Peqlab, Erlangen, Germany) and the appropriate protocol is shown in table 3.12. PCR temperature program was adapted according to the vector specific primer pairs and resulting fragment length (table 3.4 and 3.8). PCR product size was checked on an agarose gel.

Table 3.12: Colony PCR mix with vector-specific primers (table 3.4) and KAPA robust PCR kit.

	volume [µL]	concentration
DEPC H_2O	10.90	
5x buffer B (including $MgCl_2$)	5.00	1x
enhancer	5.00	
dNTP mix (10 mM)	0.50	0.2 mM
primer forward (10 µM)	1.25	0.5 µM
primer reverse (10 µM)	1.25	0.5 µM
polymerase	0.10	0.5 U
template (colony)	1.00	

3.15.2 Plasmid propagation and isolation

BUFFERS, SOLUTIONS, CHEMICALS AND OTHER MATERIAL

- Solution 1
  - 25 mM Tris-HCl, pH 8
  - 10 mM EDTA
  - 50 mM Glucose
  - 5 mg/mL Lysozyme
- Solution 2
  - 0.2 M NaOH
  - 1% SDS
- Solution 3
  - 3 M Potassium acetate, pH 4.8 (with acetic acid)
- 96% and 70% Ethanol
- 3 M Natrium acetate, pH 4.8 (with acetic acid)

Centrifugation was always performed in the centrifuge 5415R (Eppendorf, Wesseling-Berzdorf, Germany).

Transformed *E. coli* cells, including respective plasmids can be propagated in a shaking culture at 30 or 37°C overnight in 5 - 10 mL LB including the respective antibiotic.
2 mL of *E. coli* LB was centrifuged at 8,000 g for 5 min and supernatant was discarded. The resulting pellet was suspended in 100 µL solution 1, including 5 µL RNase (10 mg/mL; Qiagen, Hilden, Germany), and incubated for 5 min at room temperature. 200 µL of solution 2 were added, incubated for 5 min on ice, and then 200 µL of solution 3 were added. After 5 min incubation on ice solution was centrifuged at 13,000 g for 5 min. Plasmid DNA was participated with 800 µL ethanol

(96%) and 40 µL 3 M Natrium acetate at -20°C for 30 min. After 5 min centrifugation at 13,000 g the supernatant was discarded and the resulting DNA pellet was washed with 70% ethanol. Air dried pellet then was dissolved in 30 µL nuclease-free dH_2O. Plasmid DNA was stored at -20°C until further use.

3.15.3 Sequencing

CDS fragments in respective vectors were sent for sequencing with the vector-specific primer pairs to MWG Eurofins (MWG Biotech, Ebersberg, Germany). Obtained sequences were controlled with the freely available software Bioedit (Ibis Bioscience, Carlsbad, CA, USA) by comparison with the original CDSs.

3.16 Protein overexpression in *Escherichia coli* and purification of recombinant protein

BUFFERS, SOLUTIONS, CHEMICALS, AND OTHER MATERIALS

- LB (lysogeny broth) medium
  - 5 g/L Yeast extract
  - 10 g/L Tryptone
  - 10 g/L NaCl
  - → pH 7
- Ampicillin (100 µg/mL; Roth, Karlsruhe, Germany)
- Spectinomycine (50 µg/mL; Calbiochem, EMD Bioscience, Merck, Darmstadt, Germany)
- Isopropyl-β-D-1-thiogalactopyranoside (IPTG; Roth, Karlsruhe, Germany)
- LB plates
  - LB medium
  - 15 g/L Agar
- SOC (super optimal broth) medium
  - 2% Tryptone
  - 0.5% Yeast extract
  - 10 mM NaCl
  - 2.5 mM KCl
  - 10 mM $MgCl_2$
  - 10 mM $MgSO_4$
  - 20 mM Glucose

Centrifugation was always performed in the centrifuge 5810R (Eppendorf, Wesseling-Berzdorf, Germany).

100 mL LB medium, containing respective antibiotics, were inoculated with one colony of transformed *E. coli* strains BL21, Rosetta, or Origami and grown at 30 °C overnight. 1.5 L LB, containing respective antibiotics, were inoculated with 100 mL overnight bacterial culture and incubated at 37°C until OD600 was about 0.6. Overexpression of respective recombinant protein was induced with 0.5 or 1 mM IPTG and further grown at 37°C for another 3 - 4 h. Other overexpression temperatures and times were also tested and protein- and strain-specific conditions are indicated with the results. Cells were harvested by centrifugation at 6,000 g und 4°C for 20 min and resulting pellet was stored at -20°C until further use.

The bacterial cell pellet was resuspended in approximately 10 mL lysis (for native his-tag purification, chapter 3.16.2) or Gu-HCl (for denatured his-tag purification, chapter 3.16.1) buffer and one complete EDTA-free proteinase inhibitor tablet (Roche, Basel, Switzerland) was added to prevent protein degradation. This mix was shaken on ice for 30 - 60 min. Bacterial cells were disrupted by sonicating (Sonicating bar, Hieltscher, Teltow, Germany) on ice 2 x for 2 min. After centrifugation at

18,000 g at 10°C for 40 min the supernatant containing the recombinant protein was taken and subjected to native or denatured his-tag purification.

3.16.1 Denatured his-tag purification of recombinant protein

BUFFERS, SOLUTIONS, CHEMICALS AND OTHER MATERIAL

- 5 M NaCl
- 2 M Imidazole
- 1 M Tris-HCl, pH 8
- PBS
  - 137 mM NaCl
  - 2.7 mM KCl
  - 10 mM Na_2HPO_4
  - 2 mM KH_2PO_4
  - → pH 7.4
- Gu-HCl buffer
  - 6 M Guanidine-HCl
  - 0.5 M NaCl
  - 20 mM Tris-HCl
  - 5 mM Imidazole
  - → pH 7-8
- Washing buffer I
  - 6 M Urea
  - 5 mM Imidazole
  - → pH 7-8
- Washing buffer II
  - 6 M Urea
  - 10 mM Imidazole
  - → pH 7-8
- Elution buffer
  - PBS
  - 300 mM Imidazole
  - → pH 7-8

1.5 mL Ni-NTA agarose beads (Qiagen, Hilden, Germany) were washed with dH_2O and equilibrated on a column (Flex-Column with Flow Adapter; Kimble chase, Rockwood, TN, USA) with Gu-HCl buffer. Bacterial protein lysate of a 1.5 L culture was slowly applied on the column. Beads were washed with approximately 50 mL washing buffer I, 50 mL washing buffer II and finally with 50 mL PBS. Recombinant his-tag protein was eluted from agarose beads with 5 mL elution buffer with a decreased flow rate. This recombinant protein containing solution was concentrated on centrifugal filter units (Millipore, Merck, Darmstadt, Germany) with centrifugation at 4,000 g and 4°C for 20 min. Protein concentration was determined with a BCA assay (Roti-Quant Universal; Roth, Karlsruhe, Germany) according to manufacturer's instructions. An SDS-PAGE was carried out with the flow through of the different purification steps and the purified, concentrated, recombinant protein.

3.16.2 Native his-tag purification of recombinant protein

BUFFERS, SOLUTIONS, CHEMICALS AND OTHER MATERIAL

- Lysis buffer
  - 50 mM NaH_2PO_4
  - 300 mM NaCl
  - 5 mM Imidazole
  → pH 8
- Washing buffer I
  - 50 mM NaH_2PO_4
  - 300 mM NaCl
  - 10 mM Imidazole
  → pH 8
- Washing buffer II
  - 50 mM NaH_2PO_4
  - 300 mM NaCl
  - 15 mM Imidazole
  → pH 8
- Elution buffer
  - 50 mM NaH_2PO_4
  - 300 mM NaCl
  - 250 mM Imidazole
  → pH 8

1.5 mL Ni-NTA agarose beads (Qiagen, Hilden, Germany) were washed with lysis buffer and incubated with bacterial protein lysate of a 1.5 mL culture and shaken on ice for 30 - 60 min. This mix was applied on the column (Flex-Column with Flow Adapter; Kimble chase, Rockwood, TN, USA) and washed with approximately 50 mL lysis buffer. The second washing step was performed with approximately 70 mL washing buffer I and the third one with approximately 30 mL washing buffer II. Recombinant his-tag protein was eluted from agarose beads with 5 mL elution buffer with a decreased flow rate. This recombinant protein containing solution was concentrated on centrifugal filter units (Millipore, Merck, Darmstadt, Germany) with centrifugation at 4,000 g and 4°C for 20 min. Protein concentration was determined with a BCA assay (Roti-Quant universal; Roth, Karlsruhe, Germany) according to manufacturer's instructions. An SDS-PAGE was carried out with the flow through of the different purification steps and the purified, concentrated, recombinant protein.

3.16.3 Thrombin cleavage of his-tag

After the his-tag purification of recombinant cyclophilin (rCYC), the his-tag was cleaved with thrombin (Thrombin cleavage kit; Novagen, Merck, Darmstadt, Germany) according to manufacturer's instructions with optimised concentrations. Shortly, thrombin enzyme was diluted 1:50 in kit containing thrombin dilution/storage buffer and the following mix was prepared:

- 100 µL 10x thrombin cleavage/capture buffer
- 200 µg purified rCYC with his-tag
- 20 µL diluted thrombin
→ adjust to 1mL with dH_2O

The mixture was incubated at room temperature overnight.

For thrombin removal 25 µL streptavidin agarose were applied to the reaction mix and incubated at room temperature with gentle mixing for 30 min. The reaction mix was transferred to the sample cup of a spin filter and centrifuged at 500 g for 5 min. The filtrate in the collection tube was free of biotinylated thrombin. The thrombin cleave site is between the amino acids arginine and glycine (LeuValProArg ↓ GlySer), therefore, at least two additional amino acids remain on the purified and thrombin-cleaved recombinant cyclophilin.

3.17 Enzyme-linked immunosorbent assay with recombinant cyclophilin

BUFFERS, SOLUTIONS, CHEMICALS AND OTHER MATERIAL

- PBS
  - 137 mM NaCl
  - 2.7 mM KCl
  - 10 mM Na_2HPO_4
  - 2 mM KH_2PO_4
  - → pH 8
- PBS Tween
  - PBS
  - 0.05% Tween 20
- Blocking buffer
  - PBS Tween
  - 3% BSA
- Antibody buffer:
  - PBS Tween
  - 0.5% BSA
- 2.5 M sulphuric acid

ELISAs were conducted on MaxiSorp 96 well plates (Nunc, Wiesbaden, Germany). The plates were coated with 100 µL rCYC diluted in PBS with a final concentration of approximately 15 µg/mL and shaken at 4°C overnight.
The next day plates were washed with 200 µL PBS Tween 5 x for 5 min. Then plates were blocked with 200 µL blocking buffer at room temperature for 2 h and again washed with 200 µL PBS Tween 2 x 1 min. 100 µL serum, diluted in antibody buffer (1:50), was applied and the plates were shaken at room temperature overnight. The next day plates were washed with 200 µL PBS Tween 5 x for 5 min. 100 µL of POD-conjugated anti-human IgE secondary antibody (chapter 3.4), diluted in antibody buffer (1:1,000), were added and the plates were shaken at room temperature for 2 h, afterwards again washed with 200 µL PBS Tween 5 x for 5 min.
The TMB substrate kit (Thermo Fisher Scientific, Rockford, USA) was used according to manufacturer's instructions and immediately before its use, equal volumes of TMB solution and peroxidase solution were mixed. 100 µL of TMB substrate were applied to each well and incubated at room temperature for 15 - 30 min until desired blue colour developed. The reaction was stopped with 100 µL 2.5 M sulphuric acid and the solution turned yellow. The absorbance was measured in a plate reader (Anthos, Zenyth 200rt; Mikrosysteme GmbH, Krefeld, Germany) at 450 nm.

3.17.1 ELISA inhibition

ELISA inhibition with rCYC was carried out as the above described ELISAs; with the exception that tomato-allergic subjects' sera were incubated with different concentrations (0.00002 - 200 µg/mL) of native recombinant cyclophilin or, as a negative control, with the same amounts of BSA at room temperature for 2 h, before addition to the plates. ELISAs were then developed with the POD-conjugated secondary antibody.

3.18 *Pepino mosaic virus* isolation and coat protein identification

BUFFERS, SOLUTIONS, CHEMICALS AND OTHER MATERIAL

- TCA buffer
  - 0.1 M Tris-citric acid
  → pH 9
- SDT buffer
  - 0.1 M Tris-citric acid
  - 0.01 M Sodium thioglycolat
  → pH 9
- PEG solution
  - 5% Polyethylene glycol (PEG8000)
  - 2% NaCl
- Diethyl ether
- Carbon tetrachloride

Centrifugation was always performed in the centrifuge 5810R (Eppendorf, Wesseling-Berzdorf, Germany).

20 g freeze dried fruit or leave material was mixed in a commercial blender with 300 mL SDT buffer and 75 mL diethyl ether and carbon tetrachloride, respectively. The mush was filtered through a 20 µm gauze and centrifuged at 7,600 g for 10 min. PEG solution was added to the supernatant and stirred at 4°C for 1 h. Solution was centrifuged at 9,000 g for 15 min. The resulting pellet was dissolved in 100 mL SDT buffer and stirred at 4°C overnight. Solution was centrifuged at 7,600 g for 10 min and supernatant was again centrifuged at 50,000 g for 70 min. The virus-containing pellet was dissolved in 500 µL TCA buffer.
An SDS-PAGE was carried out with the different purification steps and the PepMV coat protein could be identified via mass spectrometry.

3.19 Statistics

Statistical analyses were carried out using Statistica (version 9, Tulsa, OK, USA). RNA and protein expression data were subjected either to one-way or to factorial analysis of variance (ANOVA) procedures (p=0.05), always indicated with the data sets. Clinical allergy tests were evaluated with non-parametric tests and medians were separated by the Mann-Whitney U test procedure (p=0.05). Correlation analyses were also carried out using Statistica.

4. RESULTS

Cultivation of tomato plants and molecular biological experiments were conducted at the Leibniz-Institute of Vegetable and Ornamental Crops in Großbeeren (IGZ), 2D gel electrophoresis and immunoblots at the Proteome Factory AG in Berlin. Mass spectrometric measurements were carried out by Ulf Bergmann (Proteome Factory AG). All clinical analyses were conducted in the Allergy-Center Charité in Berlin by the group of Prof. Dr. Worm.

First of all, for a better understanding of the following paragraphs, confirmed, known, and newly identified putative allergens are listed in table 4.1. Reference genes used for normalisation in quantitative real-time RT-PCR analyses (qRT-PCR) are shown in table 4.2.

Table 4.1: Confirmed and putative tomato allergens. Listed allergens can either be found in the allergen database (www.allergome.org) or are identified as putative tomato allergens in the following experiments. Additional information and accession numbers can be found in table 3.1, 4.9, and 4.10.

abbreviation[1]	name	allergen family[2]	function	major physiological role
Sola l 1	profilin	profilin family	regulation of actin polymerisation	cell movement, cytokinesis, signalling
Sola l 2	acid β-fructofuranosidase	glycoside hydrolase family 32	invertase: hydrolysis of sucrose to fructose and glucose	ripening
Sola l 3	lipid transfer protein	prolamin super family	transfer of phospholipids	plant defence
Sola l 4	TSI 1	Bet v 1-related protein family	hydrolysis of glucans	PR-10, plant defence
GLU	1,3-β-glucanase	glycoside hydrolase family 17	hydrolysis of glucans	PR-2, plant defence
CHI	chitinase, basic endochitinase	class I chitinase family (glycoside hydrolase family 19)	hydrolysis of 1,4-β-acetyl-D-glucosamine in chitin polymers	PR-3, PR-4, PR-11, plant defence
NP24	PR23, thaumatin-like protein, osmotin precursor	thaumatin family	induced by osmotic stress	PR-5, plant defence, ripening
PG	polygalacturonase	glycoside hydrolase family 28	pectinase: hydrolysis of 1,4-α-D-galactosiduronic pectate/galacturonans	cell wall metabolism, ripening
PME1.9, PME2.1	pectinmethylesterase	pectin-methylesterase family	de-esterification of pectin in pectate and methanol	cell wall metabolism, ripening
MAN*	β-mannosidase	cellulase family A (glycoside hydrolase family 5)	degradation of cellulose and xylans	ripening
VIC	vicilin	cupin super family	seed storage globulin	storage
PER	anionic peroxidase	peroxidase family	oxidation of toxic reductans	stress response
CYC*	cyclophilin, peptidyl-prolyl cis-trans isomerase	cyclophilin family	catalyses peptidyl-prolyl isomerisation	chaperone, cell signalling
EXP	expansin	expansin family	catalyses cell wall loosening	cell growth, ripening
PEIU1*	pectinesterase inhibitor U1	-	inhibitor of pectinesterases	cell wall modification, ripening
11SG	11S globulin	cupin super family	seed storage globulin	storage
AP*	aspartyl protease family protein	-	proteolytic activity, degradation of denatured proteins	stress response

continued →

→ continued

abbreviation[1]	name	allergen family[2]	function	major physiological role
PAE*	pectinacetylesterase precursor	-	hydrolyses acetyl-ester in homogalacturonan regions of pectin	cell wall modification, plant pathogen interaction, ripening
HSC70*	heat shock protein 70 kDa, heat shock cognate	heat shock protein family	assists protein folding	chaperone
ACO*	1-amino-cyclopropane-1-carboxylate oxidase homolog	-	catalyses 1-aminocyclopropane-1-carboxylate	ethylene biosynthesis
FBA*	fructose-1,6-bisphosphate aldolase	fructose bisphosphate aldolase class II	condensation of fructose-1,6-bisphosphate	glycolysis
KIN*	nucleoside diphosphate kinase	-	catalyses exchange of phosphate groups between nucleoside diphosphates	signalling
EN*	enolase	enolase family	catalyses the interconversion of 2-phosphoglycerate and phosphoenolpyrovat	glycolysis
PGI*	polygalacturonase inhibitor protein	-	inhibitor of polygalacturonase	plant defence
ASR1*	abscisic stress-ripening protein	-	unknown	ripening, leaf water deficit
SOD*	superoxide dismutase [Cu-Zn], chloroplastic	Cu/Zn superoxide dismutase family	catalyses dismutation of superoxide into molecular oxygen and hydrogen peroxide	anti-oxidative response

*in this study newly identified as putative tomato allergen.
[1]: Allergens abbreviated as Sola l are confirmed by the International Union of Immunological Societies (IUIS, database: www.allergen.org). Other abbreviations indicate putative allergens.
[2]: Allergen families according to database AllFam (www.meduniwien.ac.at/allergens/allfam).

Table 4.2: Reference genes used for normalisation of quantitative real-time RT-PCR. Accession numbers can be found in table 3.2.

abbreviation	gene name	major physiological role	reference
18S rRNA	17S rRNA	ribosomal RNA, protein synthesis	(Kitagawa *et al.*, 2006;Mascia *et al.*, 2010)
25S rRNA	25S rRNA	ribosomal RNA, protein synthesis	-
CAC	clathrin adaptor complexes medium subunit	intracellular protein transport, endocytic pathway	(Exposito-Rodriguez *et al.*, 2008)
SAND	SAND family protein	transport	(Exposito-Rodriguez *et al.*, 2008)
GAPDH	glyceraldehyde 3-phosphate dehydrogenase	glycolysis, gluconeogenesis	(Mascia *et al.*, 2010)
UBI	ubiquitin 3	signal transduction, protein degradation	(Mascia *et al.*, 2010)

4.1 Mycorrhiza study: Impact of arbuscular mycorrhizal fungi on the allergenic potential of tomato

modified from Schwarz et al., 2010, Mycorrhiza

The preliminary work of this experiment (planting, harvesting and determination of plant and mycorrhiza parameters, and clinical allergy tests) has been conducted in 2004 before the start of the experiments for the current PhD thesis. Within this PhD thesis in 2009 additional molecular analyses were carried out for completion of this work.

The study was conducted in order to test the hypothesis that mycorrhization of roots has an impact on the expression of allergen-encoding genes in fruits. This hypothesis was tested with the tomato genotypes '76R' and the mutant 'RMC' which is not able to form a mycorrhizal symbiosis. Both genotypes were inoculated or not with the arbuscular mycorrhizal (AM) fungus *Funneliformis mosseae.* Since plants were cultivated under normal phosphate fertiliser conditions, inhibiting mycorrhization, colonisation parameters were low in the wild type '76R'. In the 'RMC' mutant fungal structures were attached, but the fungus was unable to colonise the roots (table 4.3).

Table 4.3: Mycorrhization characteristics of wild type '76R' and mycorrhizal mutant 'RMC' tomato plants. Data are recorded nine weeks after inoculation with the arbuscular mycorrhizal fungus *Funneliformis mosseae.* Asterisks represent statistical significant differences between '76R' and 'RMC' (one-way ANOVA, p=0.05; n=4). *Parameters were assessed by Kerstin Fischer (IGZ)*. Table is modified from Schwarz *et al.,* 2010, *Mycorrhiza*.

mycorrhization	'76R'	'RMC'	effect
colonisation frequency [%]	29.20	4.17	*
relative colonisation intensity [%]	4.20	0.75	*
relative arbuscular frequency [%]	1.89	0.00	*

4.1.1 RNA accumulation of tomato allergens

To analyse the impact of AM on the allergenic potential of tomato fruits, RNA accumulation of three confirmed and five putative allergen-encoding genes was determined by quantitative real-time RT-PCR (qRT-PCR). A genotype-independent up-regulation of six allergen-encoding genes was observed, when plants were inoculated with the AM fungus (figure 4.1). This up-regulation was independent of the genotype and no significant difference was observed between the wild type '76R' and the mutant 'RMC'.

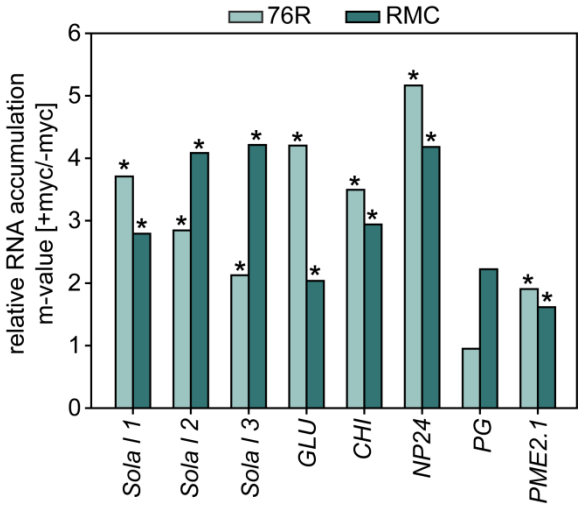

Figure 4.1: Relative RNA accumulation of allergens in mycorrhized versus non-mycorrhized tomato fruits. Wild type '76R' and mycorrhizal mutant 'RMC' tomato plants were inoculated or not with an arbuscular mycorrhizal fungus (+/- myc). Allergen expression was determined with qRT-PCR. Data were normalised with the geometric mean of two reference genes (*CAC* and *SAND*) and are given in m-values ($\log_2$ (+myc $2^{\Delta Ct}$/-myc $2^{\Delta Ct}$)). Interactions between the factors genotype and inoculation and influences of the factor genotype were not detected, except for Sola l 1. Significant differences between inoculated and non-inoculated plants are indicated by asterisks (factorial ANOVA, p=0.05; n=4). Figure is modified from Schwarz *et al.,* 2010, *Mycorrhiza*.

4.1.2. Skin prick test with tomato-allergic subjects

Skin prick tests were carried out by the group of Prof. Dr. Worm in the Charité Berlin, statistical analysis by the current author.

Skin prick tests were conducted as standardised allergy tests on ten tomato-allergic subjects. Skin reactions were positive (wheal diameter ≥ 3 mm) in six (-myc) or seven (+myc) out of ten subjects to wild type '76R'. The mutant 'RMC' provoked a higher number of positive skin reactions, namely eight (-myc) or nine (+myc) out of ten subjects. No significant differences could be observed neither between reactions to fruits from mycorrhizal and non-mycorrhizal plants, nor between the two genotypes (figure 4.2).

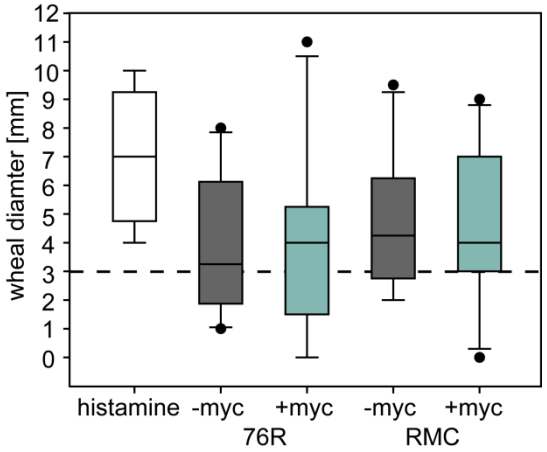

Figure 4.2: Skin prick test of ten tomato-allergic subjects (5, 13 - 21). Tomato fruits of the two tomato genotypes '76R' and 'RMC', inoculated or not with an arbuscular mycorrhizal fungus (+/-myc), were pricked. The median is depicted as black line. The dotted line represents a wheal diameter of ≥ 3 mm, in clinical practice considered as positive. No significant differences could be observed (Mann-Whitney U test, p=0.05; n=10). Histamine dichloride (10 mg/mL) was used as positive control. Figure is modified from Schwarz *et al.,* 2010, *Mycorrhiza*.

Summary and connection

These results showed that the symbiotic interaction of mycorrhizal fungi with tomato plant roots systemically induces RNA accumulation of allergen-encoding genes in fruits. It is known that pathogens induce the expression of defence-related proteins among which allergens could be identified. This induction is probably much stronger than the induction of respective genes after symbiotic colonisations. Based on these findings and considerations the hypothesis was developed that infection of tomato plants with a pathogen has a higher impact on the expression of allergen-encoding genes and in consequence also on the allergenicity of tomato fruits. To test this hypothesis the commercially important tomato pathogen *Pepino mosaic virus* (PepMV) was selected.

4.2. *Pepino mosaic virus* assessment study: 'Reisetomate' and 'Matina' infected with two virus isolates

This first assessment study was conducted to test two virus isolates in combination with two tomato cultivars and to work out the best approach for plant cultivation with PepMV, harvesting and subsequent analyses. The two tomato cultivars 'Matina' (used for organic farming, figure 4.3A) and 'Reisetomate' (old landrace, figure 4.3B) and the mild European (EU) and aggressive Chilean (CH2)

PepMV isolates were used. RNA accumulation analyses of allergen-encoding genes were conducted to get an idea about the molecular background before starting with the clinical analyses.
Unfortunately, this trial got additionally infected with another pathogen probably from the genus *Pythium* (Oomycota). Therefore, it was difficult to differentiate between the defence response of the plant to the intentional infection with PepMV and the other inadvertent pathogen regarding the RNA accumulation of allergen-encoding defence-related genes. Nevertheless, this experiment was used to evaluate different RNA extraction methods for freeze-dried tomato fruits, to test primer pairs for certain allergen-encoding genes, and to get used to different analysing methods for qRT-PCR results. The analyses were carried out with the cultivars 'Reisetomate' and 'Matina', double-infected with both isolates of PepMV (CH2 + EU).
Two weeks after PepMV inoculation leaves showed first typical PepMV disease symptoms (figure 4.3C). On the contrary, symptoms were absent on fruits during the whole study. Tomato fruits for RNA accumulation analyses were harvested between 11 and 12 weeks after PepMV inoculation.

Figure 4.3: Tomato fruits of cultivars 'Matina' (A) and 'Reisetomate' (B) and a tomato plant with typical PepMV symptoms on leaves: nettle head and leaf bubbling (C).

4.2.1 RNA extraction of tomato fruits

Tomato fruits were harvested, divided into four parts and immediately frozen in liquid nitrogen. Freeze-drying was used to handle mixed samples of up to ten tomato fruits later on. Freeze-dried mixed samples were ground and resulting powder was used for RNA extraction. Three RNA extraction methods for freeze-dried tomato fruits were compared to evaluate the most efficient one. Extractions with the commercially available Qiagen plant RNA extraction kit resulted in low RNA concentrations and 260/230 absorbance ratios of less than 1, probably resulting from high sugar content in freeze-dried tomato fruits. The two phenol based methods (chloroform/phenol and TRIzol) resulted in acceptable purity of the RNA but higher concentrations were achieved with the TRIzol reagent (table 4.4). For better comparison TRIzol reagent was used for further RNA extractions from all plant tissues and organs.

Table 4.4: RNA extraction methods for freeze-dried tomato fruits. Three different methods: The Qiagen plant RNA extraction kit, a phenol/chloroform method, and the TRIzol reagent, were compared. RNA concentration and purity parameter (optical density ratios at 260 nm/280 nm and 260 nm/230 nm) of three tomato samples (T1, T2, T3) are shown.

	Qiagen kit			phenol/chloroform			TRIzol		
	RNA conc. [ng/µL]	ratio 260/280	ratio 260/230	RNA conc. [ng/µL]	ratio 260/280	ratio 260/230	RNA conc. [ng/µL]	ratio 260/280	ratio 260/230
T1	54.40	2.09	0.47	123.87	2.14	2.08	526.17	2.11	2.09
T2	198.48	2.15	0.94	110.51	2.08	2.21	501.88	2.10	2.07
T3	23.25	1.87	0.23	104.90	2.15	2.30	477.96	2.10	2.11

4.2.2 Quantitative real-time RT-PCR

In order to analyse RNA accumulation in tomato fruits it was necessary to adapt the qRT-PCR method. The qBase$^+$ software (Hellemans *et al.*, 2007) was used for all qRT-PCR analyses to separately evaluate the different reference genes for every experiment. Efficiencies of PCR reactions were calculated with the LinReg software (Ramakers *et al.*, 2003) and loaded into qBase$^+$. Efficiency values were always between 1.8 and 2.1.

4.2.3 RNA accumulation of tomato allergens in different fruit tissue

Primers for putative tomato allergen-encoding genes were continuously designed based on information of new candidates in allergen databases (e.g. allergome.org) and on new identifications of immunoblot analyses (table 3.1 and 4.1). The spatial expression patterns of these genes in different tomato fruit tissues were analysed in the cultivar 'Matina' (figure 4.4). Since it was very difficult to find a set of optimal reference genes, constitutively expressed in florescence, pulp, peel, and seed, only one gene with acceptable stability values was finally used for normalisation (*18S rRNA*; M_{ES} = 0.503, CV = 0.231). The putative tomato allergen-encoding genes showed varying expression pattern according to their functions in respective fruit tissues (table 4.5).

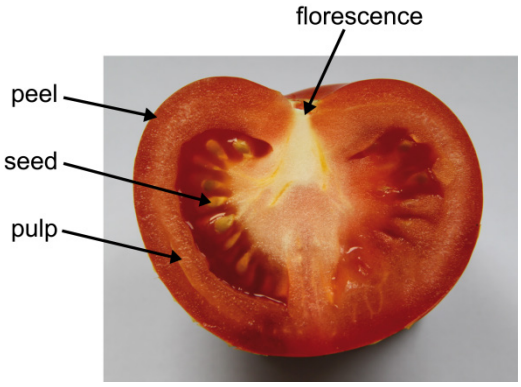

Figure 4.4: Tomato fruit tissue division.

Table 4.5: Allergen-encoding gene expression in different tomato fruit tissue. Pie charts are based on RNA accumulation analyses (CNRQ values, qBase^{+}) of six biological replicates. *18S rRNA* was used as reference gene.

tomato allergen name	major physical role	RNA accumualtion in tomato fruit pulp florescence seed peel
Sola l 1	cell movement	
Sola l 2	ripening	
Sola l 3	plant defence	
Sola l 4	plant defence	
GLU	plant defence	
CHI	plant defence	
NP24	plant defence, ripening	
PG	ripening	
PME2.1	ripening	
MAN	ripening	
VIC	storage	

4.2.4 RNA accumulation of allergens in tomato fruits

Samples from both tomato cultivars, 'Reisetomate' and 'Matina' infected or not with both PepMV isolates CH2 and EU, were used to do a first assessment of gene expression patterns in fruits. Stability values for the reference genes, encoding *18S* and *25S rRNA*, were acceptable (table 4.6).

Table 4.6: Stability values of the reference genes *18S* and *25S rRNA* (qBase$^+$). Analysed samples were taken from three biological replicates of tomato fruits from cultivars 'Reisetomate' and 'Matina' infected or not with two *Pepino mosaic virus* isolates (CH2 and EU). Stability values M_{ES} (expression stability) and CV (coefficient of variance) are shown.

	M_{ES}	CV
18S rRNA	0.198	0.069
25S rRNA	0.198	0.068

Relative viral load was determined with qRT-PCR in tomato cultivars 'Matina' and 'Reisetomate' (figure 4.5). A general detection primer pair for PepMV was used for the quantification of both PepMV isolates together. This shows that tomato fruits from non-infected plants were virus-free. 'Matina' showed highly varying amounts of virus particles and differences between the cultivars were therefore not significant.

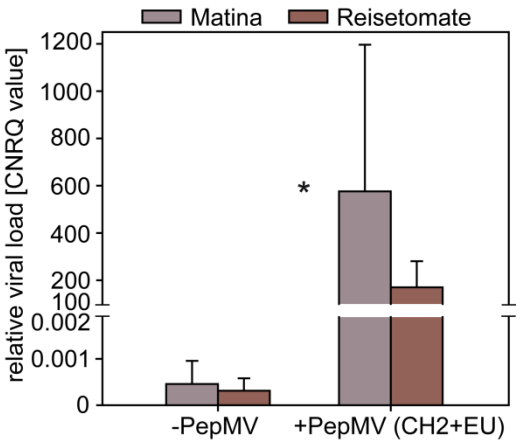

Figure 4.5: Relative viral load in tomato fruits. Double *Pepino mosaic virus* (isolates CH2 + EU)-infected and corresponding non-infected control fruits from cultivars 'Matina' and 'Reisetomate' were analysed. qRT-PCR results are shown and target gene was normalised with the geometric mean of two reference genes (*18S rRNA* and *25S rRNA*). Data are given in CNRQ values (qBase$^+$). Significant differences were detected between infected and non-infected plants. Differences between cultivars were not significant (one-way ANOVA, p=0.05; n=3).

RNA accumulation of selected genes, encoding allergens and ribulose-1,5-bisphosphat-carboxylase-oxygenase (rubisco, *RUB*) was analysed with qRT-PCR (figure 4.6). Rubisco is known to be negatively affected by PepMV infection (Hanssen *et al.*, 2011) and the only significantly down-regulated gene in 'Reisetomate'. Although a tendency was observed that most of the allergen-encoding genes were also down-regulated in PepMV-infected tomato fruits, no significant differences could be detected. One reason might be the high standard deviations in between the biological replicates (data not shown).

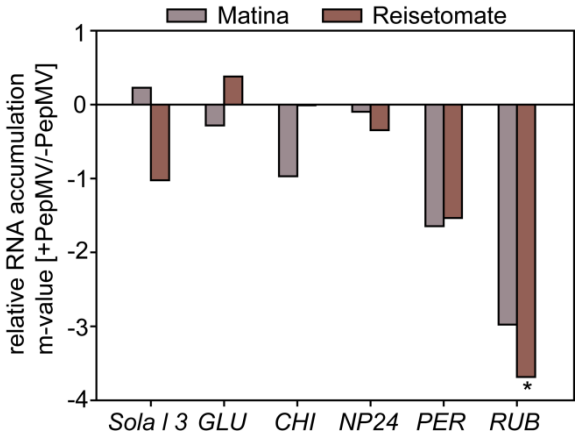

Figure 4.6: Relative RNA accumulation of putative allergens in *Pepino mosaic virus*-infected versus non-infected control tomato fruits. Cultivars 'Matina' and 'Reisetomate' were inoculated or not with two PepMV isolates (CH2 and EU). Allergen and rubisco (RUB) expression was determined by qRT-PCR. Data were analysed with qBase^{+}. Target genes were normalised with the geometric mean of two reference genes (*18S rRNA* and *25S rRNA*). Data are given in m-values ($\log_2$ (+PepMV CNRQ/-PepMV CNRQ)). Significant differences between PepMV-infected plants and non-infected controls are indicated by asterisks (one-way ANOVA, p=0.05; n=3).

4.2.5 RNA accumulation of expansin and Sola l 2 in different ripening stages of tomato fruit

Red-ripe tomato fruits were harvested by colour at stage 10 - 11 of the colour screening scale for tomato (figure 3.6). To exclude different developmental stages of the tomato fruits as the reason for the high standard deviations between the biological replicates, RNA accumulation of expansin (*EXP*)- and β-fructofuranosidase (*Sola l 2*)-encoding genes was analysed at different developmental stages. Expansin is known as a developmentally regulated protein with increasing expression during colour development of the tomato fruit (Anjanasree and Bansal, 2003;Payasi *et al.*, 2009). Additionally, the expression of the Sola l 2-encoding gene was analysed, as being also developmentally regulated during tomato ripening (Elliott *et al.*, 1993). The two potential reference genes, encoding a clathrin adaptor complex medium subunit (CAC) and a SAND family protein, recommended for RNA accumulation analyses in different developmental stages of tomato fruits (Exposito-Rodriguez *et al.*, 2008), were tested for their stability and showed acceptable values (table 4.7). Five ripening stages (3 - 12 on the colour screening scale) were analysed for both cultivars ('Matina' and 'Reisetomate'), infected or not with PepMV. No constant increase of expansin from green to red-ripe fruits could be observed and differences in biological replicates in one single ripening stage were similarly high as in previous experiments. As an example RNA accumulation of expansin and β-fructofuranosidase of non-infected control tomato fruits from the cultivar 'Matina' is shown in figure 4.7.

Table 4.7: Stability values of the two reference genes *CAC* and *SAND* (qBase$^+$). Analysed samples were taken from three biological replicates of non-infected tomato fruits from cultivar 'Matina' harvested at five ripening stages. Stability values M_{ES} (expression stability) and CV (coefficient of variance) are shown.

	M_{ES}	CV
CAC	0.430	0.148
SAND	0.430	0.149

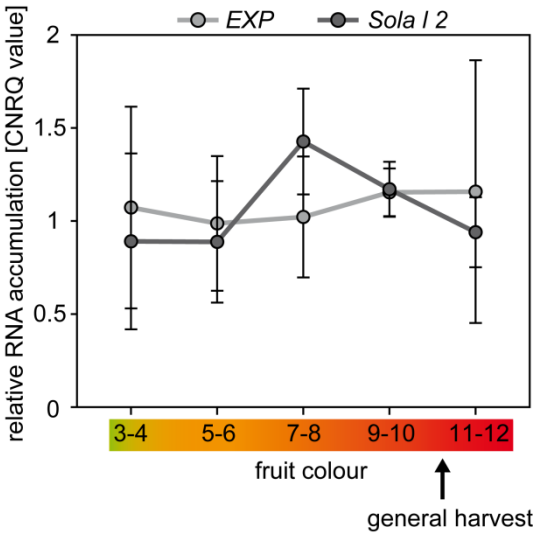

Figure 4.7: Relative RNA accumulation of expansin (*EXP*) and β-fructofuranosidase (*Sola I 2*) in tomato fruits. Fruits from cultivar 'Matina' were harvested at five ripening stages (colour 3-12 on the colour screening scale, figure 3.6). Gene expression was determined by qRT-PCR. General harvest colour of tomato fruits for all other experiments is indicated with an arrow. Target genes were normalised with the geometric mean of two reference genes (*CAC* and *SAND*). Data are given in CNRQ values (qBase$^+$). Significant differences between ripening stages could not be observed (one-way ANOVA, p=0.05; n=3).

Summary and connection

After having established cultivation and all relevant molecular methods for PepMV-infected tomato plants, the major PepMV trial was carried out in summer 2010. Due to the assessment study it was decided to use 'Matina', a commercially grown tomato cultivar used for organic farming. 'Matina' was easier in cultivation and handling due to its growth parameters and more consistent fruit colour development than the cultivar 'Reisetomate'. Additionally, 'Reisetomate' is not a hybrid cultivar and plants might be genetically not uniform, leading to variations in different parameters. As pathogen challenge, the aggressive CH2 PepMV isolate was used, because higher plant defence responses could be expected than from the mild one. Inoculating the plants with both PepMV isolates was excluded because equal distribution of both in every plant could not be guaranteed, and therefore, could be a source for unpredictable variations.

4.3 *Pepino mosaic virus* study: Impact of *Pepino mosaic virus* infection on the allergenic potential of tomato

modified from Welter et al. 2013, PLOS One

The main focus of this study was to determine differences between PepMV-infected and non-infected control tomato fruits in allergen expression on RNA accumulation and protein level, in immunoblots and in clinical allergy tests. Due to interesting observations during the time course of the experiment, investigations were extended and analyses of tomato leaves and different storage conditions of tomato fruits were included (a harvest schedule can be found in figure 3.7).

Tomato plants were not inoculated with PepMV until the first fruits were developed. Therefore, and in contrast to the PepMV assessment study, not only tomato leaves but also fruits showed typical PepMV symptoms (figure 4.9).

4.3.1 *Pepino mosaic virus* detection via ELISA

Plants were tested for systemic PepMV infestation with a commercial enzyme-linked immunosorbent assay (ELISA) kit two weeks after inoculation (WPI) and all investigated plants showed positive reactions. All non-infected plants were controlled weekly following this procedure and always responded negative.

4.3.2 Reference gene evaluation

The reference genes, encoding *CAC, SAND*, and the *25S rRNA*, used in the two previous experiments showed no stable expression within fruit and leaf samples of this experiment. Therefore, *18S rRNA* and two additional reference genes, encoding *UBI* and *GAPDH,* were evaluated separately for leaf and fruit samples. The reference calculation software geNorm and qBase$^+$ gave acceptable stability values (table 4.8). For leaves it was recommended to use the reference genes *18S rRNA* and *GAPDH*. In fruits all evaluated reference genes were less stable, but acceptable values could be achieved. Further analyses were conducted with all three reference genes (*18S rRNA, GAPDH,* and *UBI*). Unless indicated differently these reference genes for fruits and leaves were used during all further RNA accumulation analyses.

Table 4.8: Stability values of the reference genes *18S rRNA*, *GAPDH,* and *UBI* (geNorm, qBase$^+$). Analysed samples were taken from three biological replicates of PepMV-infected and non-infected tomato fruits and leaves. Stability values M_{ES} (expression stability) and CV (coefficient of variance) are shown.

	leaves		**fruits**	
	geNorm [M_{ES}]	qBase$^+$ [M_{ES}/CV]	geNorm [M_{ES}]	qBase$^+$ [M_{ES}/CV]
18S rRNA	0.262	0.217/0.007	0.538	0.623/0.259
GAPDH	0.288	0.217/0.074	0.471	0.471/0.165
UBI	0.312	-/-	0.496	0.519/0.209

4.3.3 *Pepino mosaic virus* quantification

Relative viral load was determined by qRT-PCR in fruits and leaves. For the comparison of viral content of fruits and leaves a single reference gene was used (*18S rRNA*). Virus titres revealed higher PepMV accumulation in fruits than in leaves at 10 WPI. Interestingly, the appearance of marbling symptoms on fruits at 3 WPI were accompanied by higher virus titres at this time point compared to 10 WPI where no symptom was left on the fruits (figures 4.8 and 4.9). Two weeks storage of tomato fruits had no influence on the virus content.

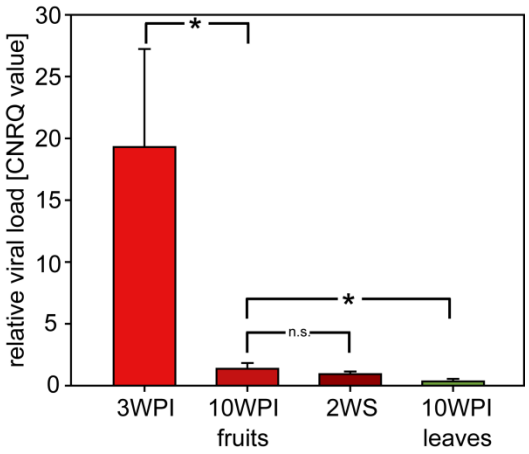

Figure 4.8: Relative viral load in tomato fruits and leaves. *Pepino mosaic virus*-infected tomato plants were analysed and values determined by qRT-PCR. Target gene was normalised with the reference gene, encoding *18S rRNA*. Data are given in CNRQ values (qBase^{+}). Viral loads of fruits 3 and 10 weeks post-inoculation (WPI), as well as fruits and leaves at 10 WPI and those, stored at 4°C for 2 weeks (2 WS), were compared. Significant differences are marked with asterisks (one-way ANOVA, p=0.05; n=4). n.s.: not significant. Figure is modified from Welter *et al.,* 2013, *PLOS One*.

Figure 4.9: *Pepino mosaic virus*-infected tomato fruits at 3 and 10 weeks post-inoculation (WPI). Fruits showed typical PepMV marbling symptoms at 3 WPI in contrast to fruits at 10 WPI, where no symptoms could be observed. Figure is modified from Welter *et al.,* 2013, *PLOS One*.

4.3.4 RNA accumulation of allergens in tomato fruits 3 and 10 weeks after *Pepino mosaic virus* inoculation

Seventeen confirmed and putative tomato allergen-encoding genes, including those, encoding for defence-related proteins were selected for RNA accumulation analyses using qRT-PCR (table 3.1). The two time points (3 and 10 WPI) revealed higher differences in allergen-encoding gene expression than PepMV-infected and non-infected control tomato fruits. At 3 WPI four genes were significantly up-regulated and three down-regulated while at 10 WPI only one gene was induced by the virus (figure 4.10).

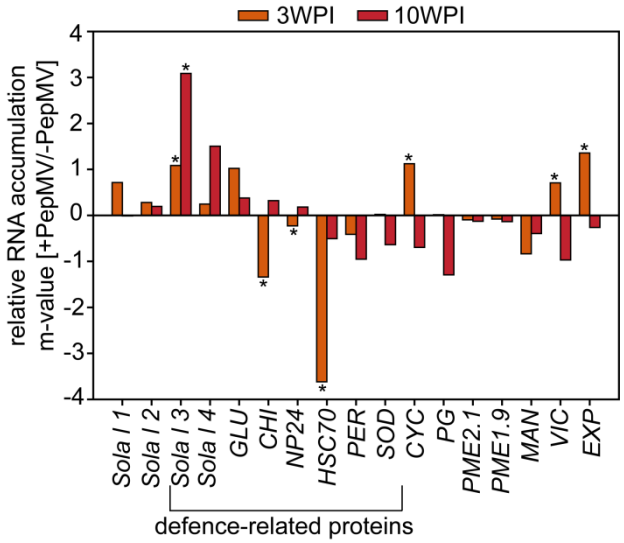

Figure 4.10: Relative RNA accumulation of putative allergens in *Pepino mosaic virus*-infected versus non-infected control tomato fruits at 3 and 10 weeks post-inoculation (WPI). Allergen expression was determined by qRT-PCR and data were analysed with qBase^{+}. Target genes were normalised with the geometric mean of three reference genes (*18S rRNA, GAPDH*, and *UBI*). Data are given in m-values ($\log_2$ (+PepMV CNRQ /-PepMV CNRQ)). Significant differences between PepMV-infected plants and non-infected controls are indicated by asterisks (one-way ANOVA, p=0.05; n=3). Figure is modified from Welter *et al.,* 2013, *PLOS One*.

4.3.5 RNA accumulation of allergens in tomato fruits after storage

It is known that allergen expression can be increased after storage of fruits (Matthes and Schmitz-Eiberger, 2009;Kiewning *et al.*, 2012). Since tomatoes will be stored at least for a few days before consumption, the impact on allergen expression of two weeks storage at 4°C was investigated. Significant differences could only be observed in non-infected control fruits, four of eleven genes were significantly down-regulated and Sola l 3-encoding gene was up-regulated after storage (figure 4.11).

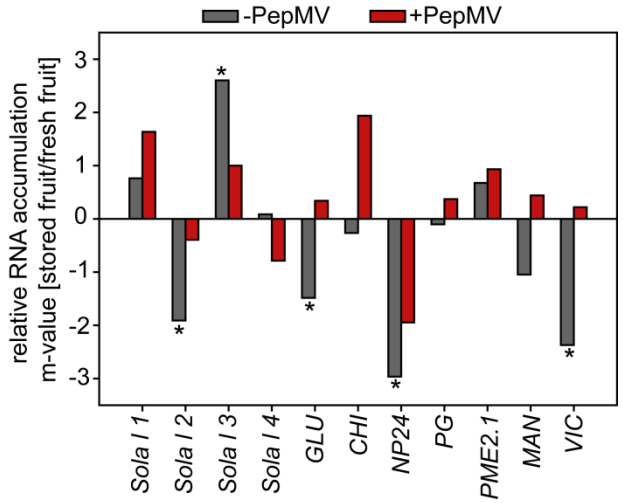

Figure 4.11: Relative RNA accumulation of putative allergens in fresh versus stored tomato fruits. *Pepino mosaic virus*-infected and non-infected control tomato fruits, harvested at 10 WPI, were used freshly or stored at 4°C for 2 weeks. Allergen expression was determined by qRT-PCR. Data were analysed with qBase^{+} and analysed with CNRQ values. Target genes were normalised with the geometric mean of three reference genes (*18S rRNA, GAPDH,* and *UBI*). Data are given in m-values ($\log_2$ (stored fruit CNRQ/fresh fruit CNRQ)). Significant differences between fresh and stored fruits are indicated by asterisks (one-way ANOVA, p=0.05; n=3).

4.3.6 RNA accumulation of allergens in tomato leaves, fresh, and stored fruits 10 weeks post *Pepino mosaic virus* inoculation

Contrary to expectations, tomato fruits showed no general up regulation of defence-related allergen-encoding genes after PepMV inoculation. In order to investigate if this is an organ (fruit)-specific phenomenon, leaf samples were taken from the same plants. The expression of the four confirmed allergens Sola l 1, Sola l 2, Sola l 3, Sola l 4, and of selected defence-related allergen-encoding genes was analysed on RNA accumulation level additionally in leaves. Interestingly, five of ten allergen-encoding genes showed a significantly enhanced expression of more than two fold in leaves of PepMV-infected plants (figure 4.12).

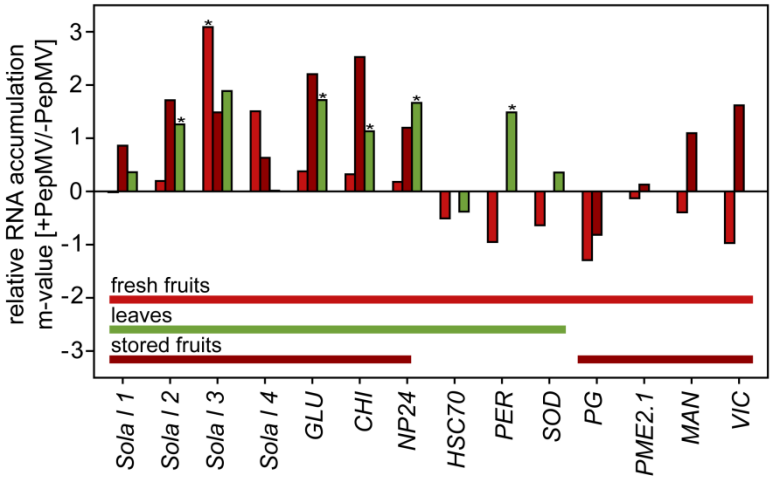

Figure 4.12: Relative RNA accumulation of putative allergens in *Pepino mosaic virus*-infected versus non-infected control tomato leaves and fresh and stored fruits. Fruits and leaves were harvested 10 WPI and used freshly or stored at 4°C for 2 weeks. Allergen expression was determined by qRT-PCR. Data were analysed with qBase^{+} and analysed with CNRQ values. Target genes were normalised with the geometric mean of two (leaves) or three (fruits) reference genes (*18S rRNA, GAPDH*, (and *UBI*)). Data are given in m-values ($\log_2$ (+PepMV CNRQ/-PepMV CNRQ)). Significant differences between PepMV-infected and non-infected control fruits are indicated by asterisks (one-way ANOVA, p=0.05; n=3).

4.3.7 Immunoblot analyses and identification of putative tomato allergens

After analysing tomato allergen expression on RNA accumulation level differences between PepMV-infected and non-infected control fruits were investigated also on protein level. Putative tomato allergens were identified on immunoblots with the sera from tomato-allergic subjects (table 3.6) and a POD-conjugated secondary antibody (chapter 3.4). Proteins were extracted from tomato fruits 10 WPI. Total protein content of PepMV-infected tomato fruits was higher (~0.6 mg/mL tomato mush) than those of non-infected control fruits (~0.2 mg/mL tomato mush), probably due to the high accumulation of viral proteins.

All investigated materials (protein extract, subject's serum, and anti-human IgE antibody) were tested in a dot blot. A serum pool of ten tomato-allergic subjects showed a stronger reaction to protein extract of PepMV-infected tomato fruits compared to non-infected controls (figure 4.13). The negative control bovine serum albumin (BSA) provoked no reaction at all.

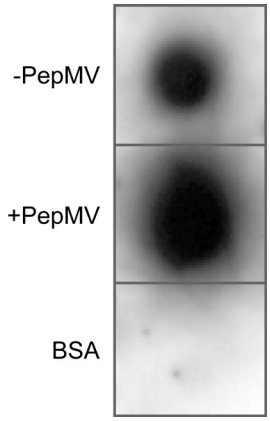

Figure 4.13: Dot blot of *Pepino mosaic virus* -infected and non-infected control tomato fruits with a serum pool of tomato-allergic subjects. 1 µg protein extracts of tomato fruits 10 WPI and BSA (negative control) were used. Serum pool of ten subjects was diluted 1:10. A peroxidase-conjugated anti-human IgE antibody (dilution: 1:5,000) was used for detection (exposure to film: 10 min).

Due to this result, potential protein candidates triggering these differences were aimed to be identified. Therefore, protein extracts were separated on 2D gels. 2D immunoblot analyses with a serum pool of ten tomato-allergic subjects were conducted in three technical replicates, in order to detect new putative allergens, which might be differentially expressed in tomato fruits infected or not with PepMV (figure 4.14). Among those proteins that reacted with the serum pool, 20 could be identified by mass spectrometry. Nine of the putative allergens occurred in protein extracts from both, while five or six only were present in extracts from infected or non-infected fruits, respectively (table 4.9). Confirmed allergens, like Sola l 1 and Sola l 2, known putative allergens, like polygalacturonase, peroxidase and glucanase and other particular defence- and pathogenesis-related (PR) proteins, but also newly identified proteins, for example heat shock proteins and a mannosidase (figure 4.14, table 4.9), could be identified. The 70 kDa heat shock protein was identified from *Solanum tuberosum* and a subsequent Blast search for tomato revealed identities with a heat shock cognate (HSC70). Interestingly, some extracted positive spots resulted in peptide sequences belonging to the PepMVCP (arrows in figure 4.14A) and further analyses are described in chapter 4.7.

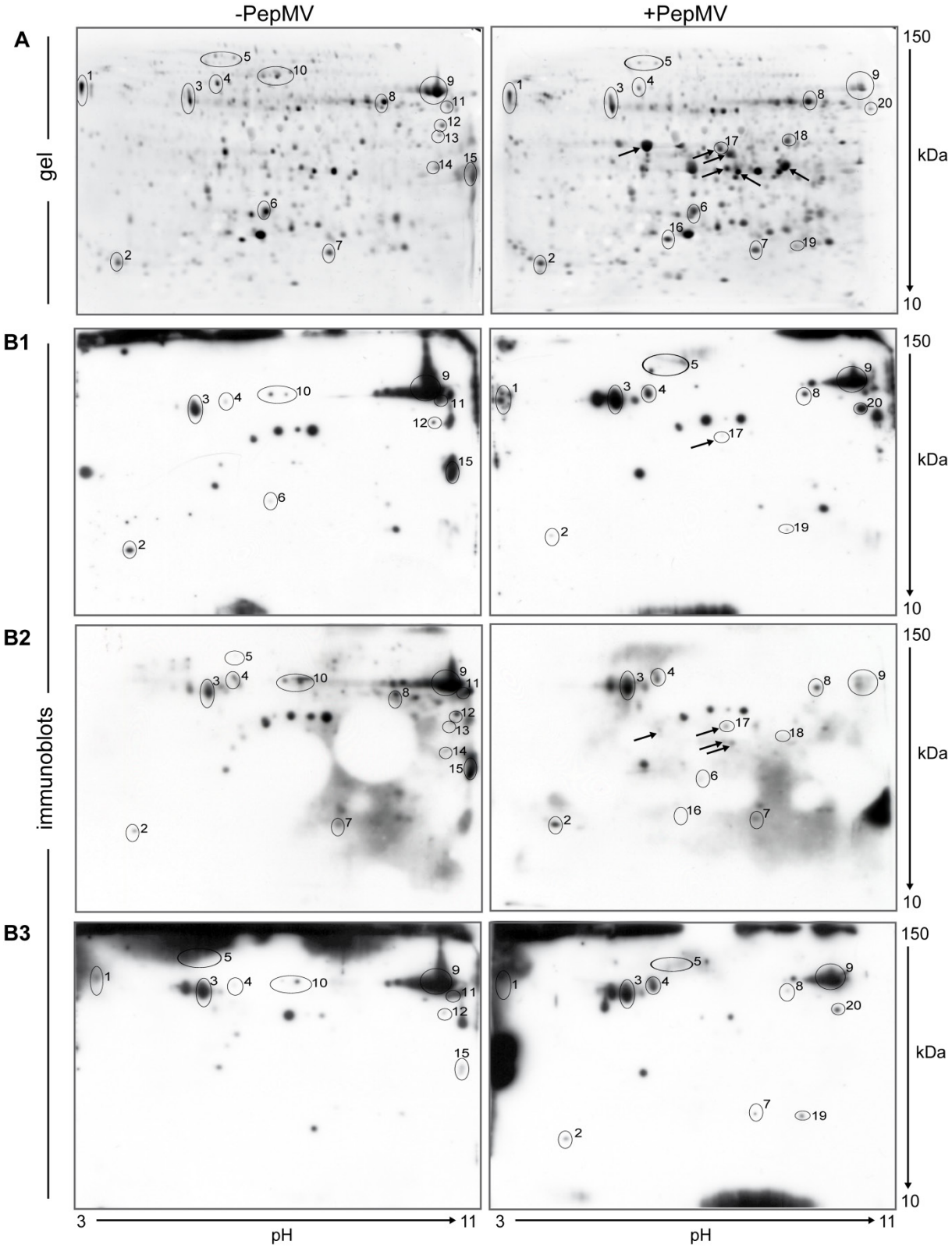

Figure 4.14: Small 2D protein gels and corresponding immunoblots of *Pepino mosaic virus* -infected and non-infected tomato fruits 10 WPI. A serum pool of ten tomato-allergic subjects (dilution: 1:10) was used. Proteins detected in immunoblots and identified with mass spectrometry are marked and numbers are listed in table 4.9. A peroxidase-conjugated anti-human IgE antibody (dilution: 1:5,000) was used for detection (exposure to film: 5 min). **A**: Representative 2D protein gels. **B1-3**: Immunoblots in three technical replicates. Figure is modified from Welter *et al.,* 2013, *PLOS One*.

Table 4.9: Putative tomato allergens identified in *Pepino mosaic virus* -infected and non-infected tomato fruits. Tomato fruit protein extracts were separated by 2D gel electrophoresis and analysed by immunoblotting with an IgE serum pool of ten tomato-allergic subjects. Positive spots were eluted from parallel gels and identified using mass spectrometry. Spots are indicated by numbers and shown in figure 4.14. Table is modified from Welter *et al.,* 2013, *PLOS One*.

spot no. in gel	name	accession number	molecular weight/ isoelectric point	score/sequence identity [%]	known as allergen in tomato	abbre-viation
putative allergens identified in PepMV-infected (+PepMV) and non-infected (-PepMV) fruits						
2	profilin	gi|17224229	14.1/4.7	-127/24 (+137/43)	yes	Sola l 1
4	acid β-fructofuranosidase	gi|124701	70.1/5.5	-1221/28 (+424/27)	yes	Sola l 2
9	polygalacturonase-2	gi|129939	50/6.4	-1707/52 (+669/41)	yes	PG
1	suberization-associated anionic peroxidase 1	gi|129807	38.7/4.9	-886/26 (+357/19)	yes	PER
3	1-aminocyclopropane-1-carboxylate oxidase homolog	gi|119640	41.1/5.6	-219/25 (+131/17)	no	-
8	fructose-1,6-bisphosphate aldolase	gi|14484932	36.5/8.7	-1333/55 (+283/74)	no	-
7	nucleoside diphosphate kinase	gi|575953	15.4/6.8	-422/40 (+347/40)	no	-
5	heat shock 70 kDa protein, mitochondrial (*Solanum tuberosum*) Blast search tomato: heat shock cognate	gi|585273 gi|762844	73/6.4	-606/24 (+nd)	no	HSC70
6	small heat shock protein	gi|4836469	17.7/5.8	-436/54 (+555/65)	no	-
putative allergens only identified in non-infected fruits (-PepMV)						
12	glucan endo-1,3-β-glucosidase B	gi|461979	39.7/7.9	333/37	yes	GLU
13	vicilin	gi|166053040	66.1/8.2	241/12	yes	VIC
14	pathogenesis-related protein PR P23	gi|19315	25.1/6.1	102/8	yes	PR23
15	NP24 protein precursor	gi|170467	25.7/8.3	180/27	yes	NP24
11	mannan endo-1,4-β-mannosidase 4	gi|125951563	45.3/8.9	331/24	no	MAN
10	enolase	gi|119354	47.8/5.7	1463/51	no	-
putative allergens only identified in PepMV-infected fruits (+PepMV)						
17	ascorbate peroxidase	gi|21039134	42.1/8.7	234/21	(yes)	-
18	basic 30 kDa endochitinase	gi|544011	34.3/6.2	381/48	yes	CHI
20	polygalacturonase inhibitor protein	gi|469457	36.5/8.7	305/29	no	-
19	abscisic stress-ripening protein 1	gi|584786	13.1/6.8	159/22	no	-
16	superoxide dismutase [Cu-Zn], chloroplastic	gi|134682	22.2/5.8	293/34	no	SOD

-: -PepMV, +: +PepMV; nd: not detectable, but visible in gel, Sola l 1 and Sola l 2 confirmed tomato allergens by IUIS. Mascot and Blast searches were performed in 08/2011.

4.3.8 Clinical allergy tests

All following clinical allergy tests were carried out by Sabine Dölle, Dennis Ernst, and Juliane Hiepe in the Charité Berlin. Data were analysed by the current author.

In order to evaluate the allergenic potential of PepMV-infected fruits in comparison to non-infected control tomato fruits, different clinical allergy tests were conducted. Skin prick tests, basophil activation/degranulation tests, as well as double blind placebo controlled food challenges were carried out with a tomato-allergic subject cohort (table 3.6).

4.3.8.1 Skin prick test

Skin prick tests were conducted during the whole study and freshly prepared mush of tomato fruits, harvested between 3 and 13 WPI, was used (figure 4.15A). Reactions to PepMV-infected as well as to non-infected control tomato fruits were positive (wheal diameter ≥ 3 mm) in eight out of ten subjects and no significant difference was observed between both. The ten tomato-allergic subjects (1 - 10), however, showed high individual variation in their reactions to these tomatoes, freshly harvested at the different time points. In chapter 4.3.4 it is shown that different harvest time points after PepMV infection affected RNA accumulation of allergen-encoding genes. Therefore, skin prick tests on nine subjects (1, 3, 4, 5, 7, 8, 10, 11, 12) were repeated with frozen tomato mush of one single time point (figure 4.15B, 3 WPI, PepMV-infected tomatoes showed typical symptoms). Reactions to PepMV-infected tomato fruits were positive in seven out of nine subjects. In contrast only six out of nine subjects showed a positive reaction to non-infected controls. Similarly high individual variability was, however, observed in this analysis and results failed to be significant different. The results of both skin prick tests were comparable, although subjects in skin prick tests with frozen tomato mush tended to higher reactions with PepMV-infected tomato fruits (figure 4.15C).

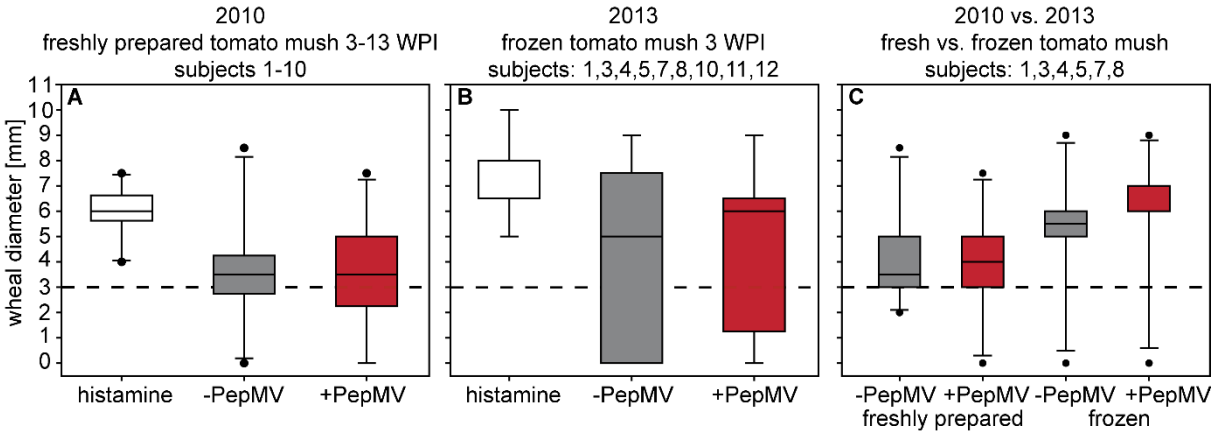

Figure 4.15: Skin prick test of tomato-allergic subjects. A: Subjects 1 - 10 pricked with freshly prepared tomato mush (n=10) of *Pepino mosaic virus*-infected and non-infected control tomato fruits harvested between 3 and 13 weeks post-inoculation (WPI). **B**: Subjects 1, 3, 4, 5, 7, 8, 10, 11, and 12 pricked with frozen tomato mush (n=10) of PepMV-infected and non-infected control fruits harvested at 3 WPI. **C**: Comparison of six subjects (1, 3, 4, 5, 7, 8) pricked with freshly prepared (3 - 13 WPI) or frozen (3 WPI) tomato mush. Histamine dichloride (10 mg/mL) was used as a positive control. The median is depicted in the black line. The dotted line represents a wheal diameter of ≥ 3 mm, in clinical practice considered as positive. No statistically significant differences could be observed (Mann-Whitney U test, p=0.05; n=10/9/6). Figure is modified from Welter *et al.,* 2013, *PLOS One*.

4.3.8.2 Double blind placebo controlled food challenge

In addition to skin prick tests, double blind placebo controlled food challenges (DBPCFC) were conducted with the ten tomato-allergic subjects. PepMV-infected and non-infected control tomato fruits were harvested between 3 and 13 WPI and tomato mush was freshly prepared for each subject. All subjects investigated showed very weak reactions in DBPCFC and nearly all subjects' reactions were below a certain limit (symptom score of 3, (Dölle *et al.*, 2011a)). However, higher variability in reactions could be observed with PepMV-infected tomato fruits (figure 4.16).

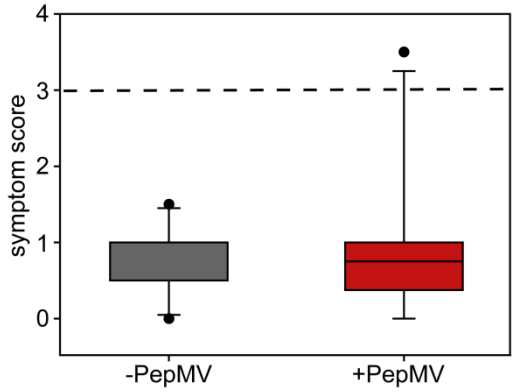

Figure 4.16: Double blind placebo controlled food challenges of ten tomato-allergic subjects. Tomato fruits of *Pepino mosaic virus*-infected and non-infected control fruits were harvested between 3 and 13 WPI and used for provocation tests. Symptoms were evaluated according to a symptom score (Dölle *et al.*, 2011a). The median is depicted in the black line. The dotted line represents a symptom score of ≥ 3 mm, in clinical practice considered as a positive reaction. No statistical significant differences could be observed (Mann-Whitney U test, p=0.05; n=10).

4.3.8.3 Basophil activation and degranulation test with fresh and stored tomato fruits

Basophil activation and degranulation tests were carried out on five tomato-allergic subjects (1, 3, 4, 6, 7) using tomato protein extracts of tomato fruits harvested at 10 WPI and fruits stored at 4°C for two weeks. Basophil activation and degranulation increased in a dose-dependent manner (figure 4.17). However, basophil activation and degranulation revealed neither a significant difference between the reaction to PepMV-infected and non-infected control fruits nor between the fruits of fresh and stored tomato fruits. Individual variation was similarly high as in the skin prick tests and DBPCFCs.

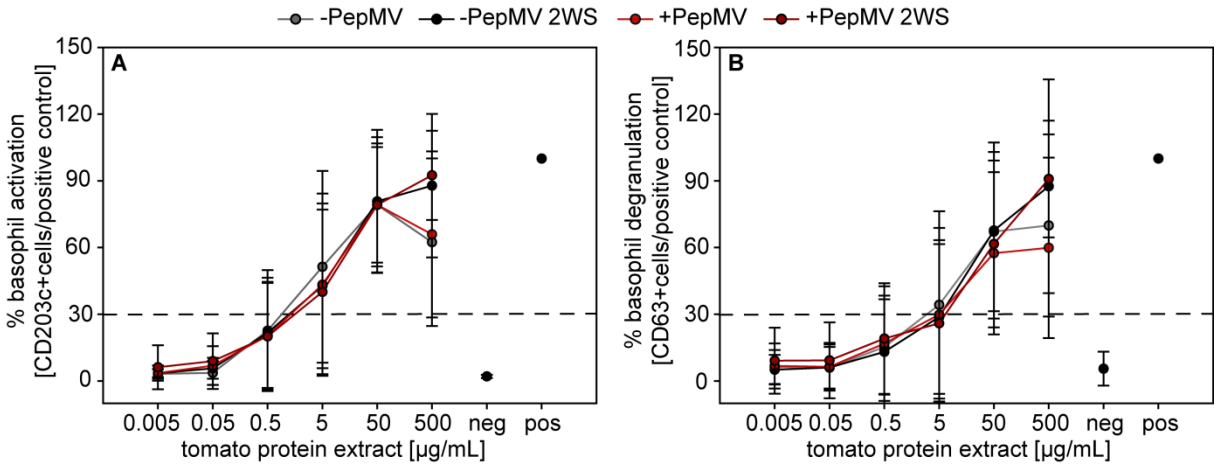

Figure 4.17: Basophil activation and degranulation tests of five tomato-allergic subjects (1, 3, 4, 6, 7). Basophil dose response curves with protein extracts of *Pepino mosaic virus*-infected and non-infected control tomato fruits 10 weeks post-inoculation (WPI) and corresponding fruits stored at 4°C for 2 weeks (2 WS) are shown. **A**: Basophil activation is shown in %CD203c^{+} cells normalised to positive control. **B**: Basophil degranulation is shown in %CD63^{+} cells normalised to positive control. The dotted line shows 30% of basophil activation or degranulation, in clinical practice considered as a positive reaction. Data points represent the mean of five subjects. No statistical significant differences could be observed (Mann-Whitney U test, p=0.05; n=5).

4.3.9 Comparison of different harvest time points after *Pepino mosaic virus* infection

In chapter 4.3.4 it was shown that the time point of harvest affected RNA accumulation of allergen-encoding genes. Since skin prick tests and DBPCFC showed high variability in reaction to tomato fruits harvested at different time points (chapters 4.3.8.1 and 4.3.8.2), allergen expression on RNA and protein level and were analysed in the same tomato fruits used for clinical studies. With additional basophil activation tests, results are shown in the following.

4.3.9.1 Tomato allergen expression on gene and protein level

To quantify allergen expression on protein level 2D protein gels with tomato fruit protein extracts of PepMV-infected and non-infected control fruits of 4, 8, and 12 WPI were analysed. The overall protein pattern looked very similar and only minor differences could be detected. Two representative gels are shown in figure 4.18. Nine putative allergens from tomato and the PepMVCP could be identified by comparison with previously analysed immunoblots and quantified due to their spot intensity by the 'proteome weaver' software. Most of these putative allergens have already been described in chapter 4.3.7 (figure 4.14, table 4.9). As expected the PepMVCP was not visible in gels of non-infected control fruits.

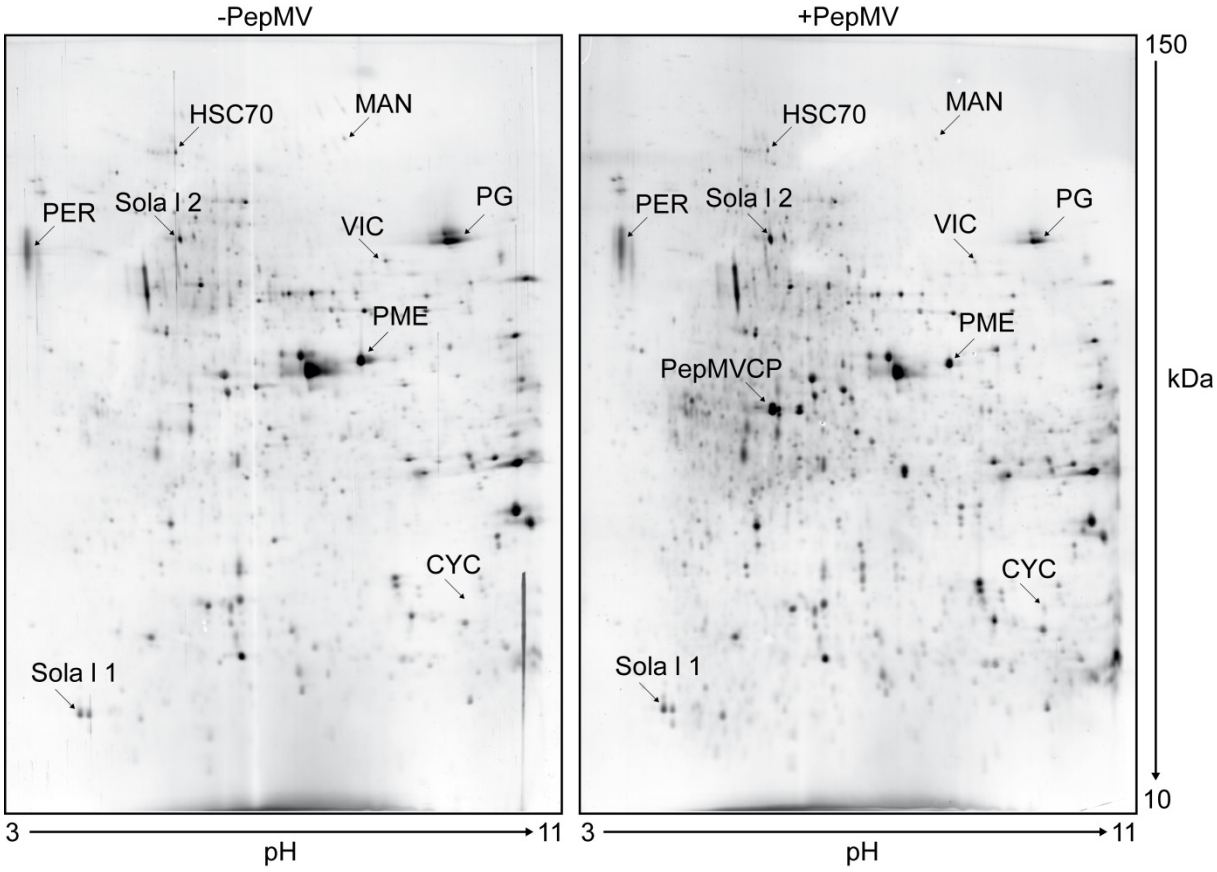

Figure 4.18: Big 2D protein gels of *Pepino mosaic virus*-infected (+PepMV) and non-infected control (-PepMV) tomato fruits. Gels with protein extracts of tomatoes from 4 WPI are shown. Quantified putative tomato allergens and the PepMV coat protein (PepMVCP) are indicated.

Putative tomato allergens of the same samples were additionally quantified on protein level with iTRAQ. *The iTRAQ analyses were carried out by Mario Köhler at the Proteome Factory AG and are integrated in his bachelor thesis.* Three putative tomato allergens and the PepMVCP could be identified and quantified with this method. The quantity of the PepMVCP was used to prove the comparability of the two protein quantification methods. Even when total values of both methods are not comparable, relative to each other they showed the same trend (figure 4.19).

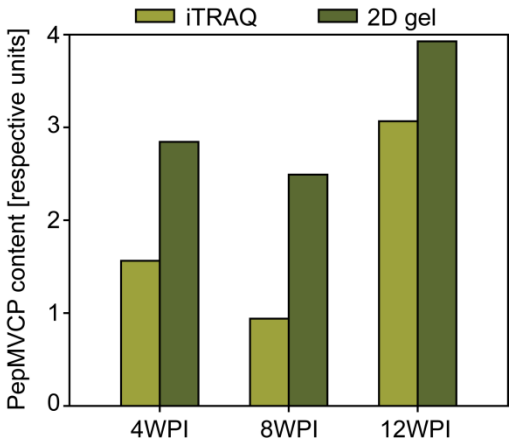

Figure 4.19: Quantification of *Pepino mosaic virus* coat protein. 2D gel quantification (spot intensity, quantified with Proteomweaver software) and iTRAQ measurements (intensity ratios of iTRAQ 115, 116, and 117 reagents towards the standard with iTRAQ reagent 114) of PepMV-infected tomato fruits 4, 8 and 12 weeks post-inoculation (WPI).

RNA of the same samples was extracted and allergen expression was also analysed on transcript level. A comparative heat map of expression levels quantified on 2D gels, by iTRAQ and by qRT-PCR is shown in figure 4.20 and revealed no correlation. Within one quantification method allergen expression in tomato fruits differed between the investigated harvest time points (4, 8, and 12 WPI). Neglecting the iTRAQ analyses, no general up-regulation of putative allergens in PepMV-infected tomato fruits could be observed. It has, however, to be considered that samples were taken during the clinical tests, therefore, each time point is presented by only one sample, a fruit mush of ten tomatoes.

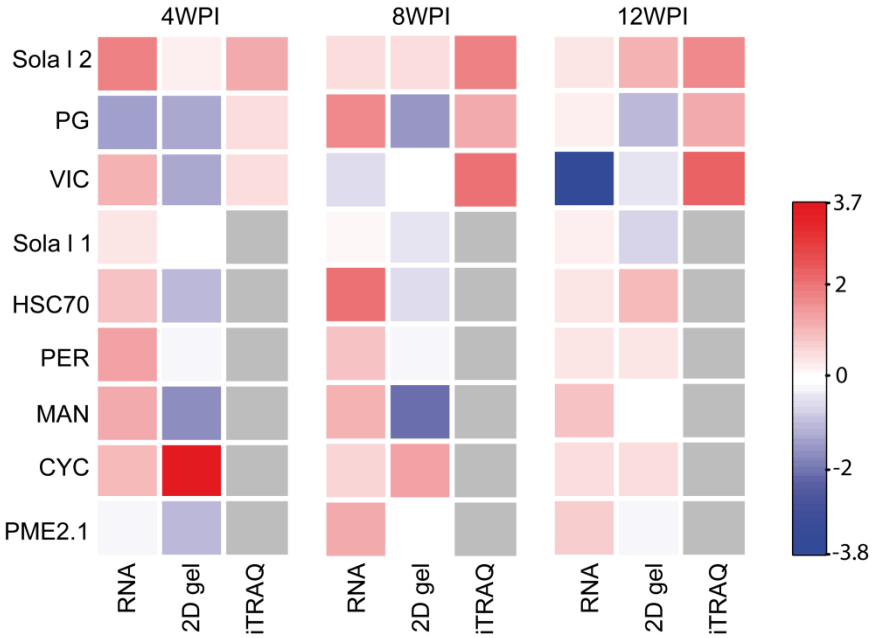

Figure 4.20: Expression of putative tomato allergens in tomato fruits 4, 8, and 12 weeks post-inoculation (WPI). Three different methods were used to analyse allergen expression. RNA: allergen-encoding gene expression measured by qRT-PCR, 2D gel: protein amounts were quantified based on their spot intensities on the gel by 'proteome weaver', iTRAQ: protein amounts were quantified by iTRAQ. Data were transformed to m-values (log_2 (+PepMV/-PepMV)) and in PepMV-infected fruits up-regulated allergens are reddish, in contrast to down-regulated allergens are bluish (scale). Grey fields indicate allergens which could not be analysed by iTRAQ.

4.3.9.2 1D immunoblot

In order to visualise potential differences in the IgE reactions to differentially expressed proteins in tomato fruits harvested at 4, 8, and 12 WPI, 1D immunoblot analyses with a serum pool of ten tomato-allergic subjects were carried out (figure 4.21). Differential expression of some putative allergens during this time (figure 4.20) seemed not to impact the general IgE reaction to protein extracts of tomatoes harvested at the different time points. Additionally, no obvious difference was observed in immunoblots with PepMV-infected and non-infected control fruits.

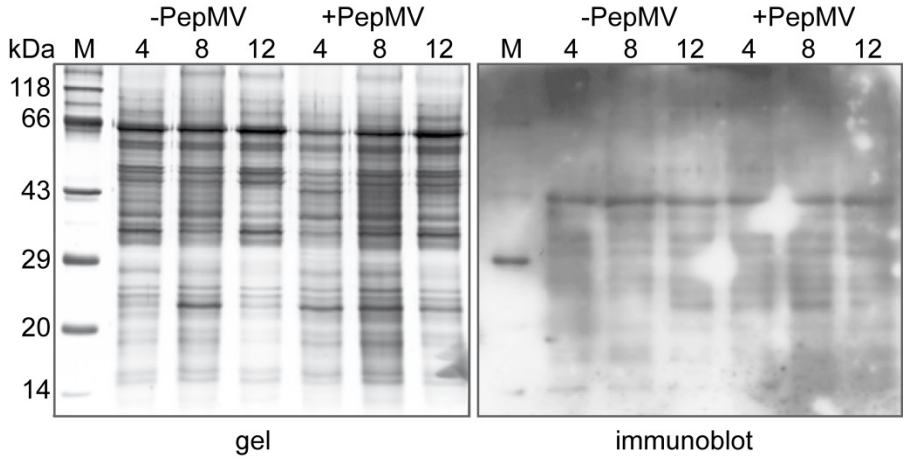

Figure 4.21: 1D protein gel and corresponding immunoblot of *Pepino mosaic virus*-infected and non-infected control tomato fruits from 4, 8, and 12 weeks post-inoculation (WPI). A serum pool of ten tomato-allergic subjects (dilution: 1:10) was used. A peroxidase-conjugated anti-human IgE antibody (dilution: 1:5,000) was used for detection (exposure to film: 1.5 min). M: protein weight marker.

4.3.9.3 Basophil activation and degranulation test

Basophil activation and degranulation tests were carried out by Dennis Ernst in the Charité Berlin. Data were analysed by the current author.

Clinical relevance was evaluated for tomato fruit protein extracts of 4, 8, and 12 WPI. Basophil degranulation and activation tests were carried out on five subjects (1 - 5). Basophil activation and degranulation increased in a dose-dependent manner. However, basophil activation and degranulation revealed neither a significant difference between the reaction to PepMV-infected and non-infected control extracts nor between the extracts from fruits harvested at different time points (figure 4.22). Subject showed again high inter-individual variation. Regarding basophil activation and degranulation gained with 5 µg/mL protein extract (figure 4.23) at 4 and 8 WPI a lower amount of tomato protein extract of non-infected tomatoes was necessary to activate (AC_{30} [µg/mL]: 4 WPI: -PepMV: 0.2, +PepMV: 1.4; 8 WPI: -PepMV: 0.2, +PepMV: 0.4) or degranulate (DC_{30} [µg/mL]: 4 WPI: -PepMV: 0.4, +PepMV: 3.5; 8 WPI: -PepMV: 1.3, +PepMV: 4.3) 30% of the basophils. At 12 WPI this was inverted and a lower amount of protein extract of PepMV-infected tomatoes was necessary (AC_{30} [µg/mL]: -PepMV: 1.3, +PepMV: 0.1; DC_{30} [µg/mL]: -PepMV: 3.7, +PepMV: 0.5).

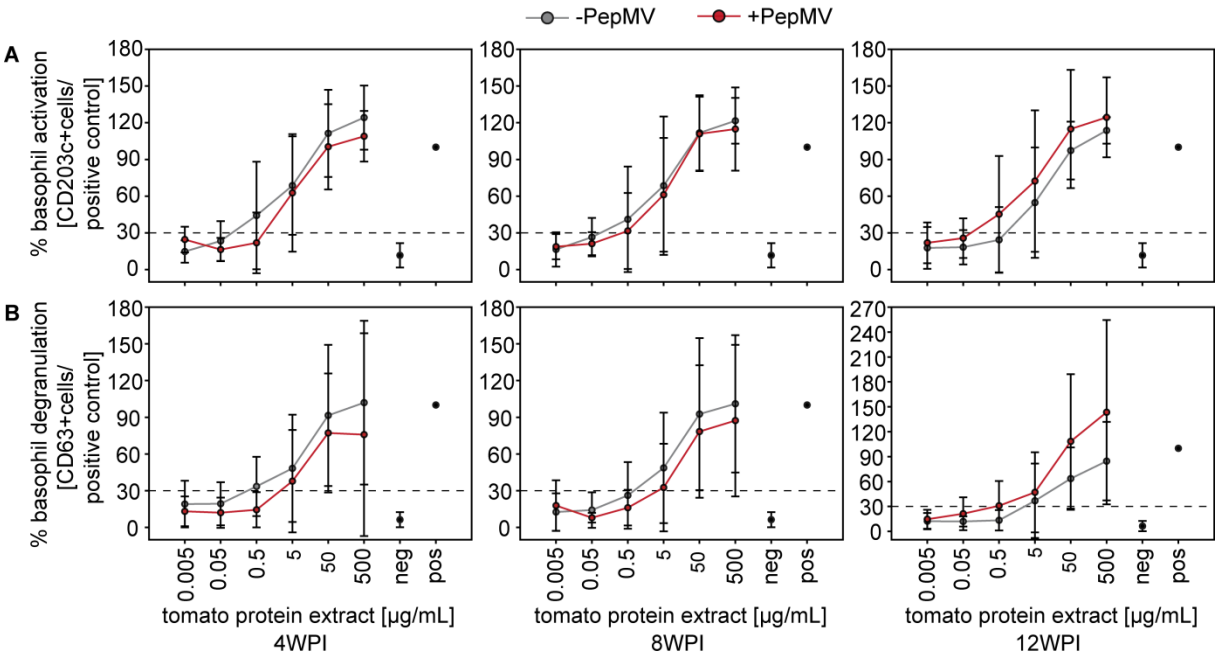

Figure 4.22: Basophil activation and degranulation tests of five tomato-allergic subjects (1-5). Basophil dose response curves with protein extracts of *Pepino mosaic virus*-infected and non-infected control tomato fruits 4, 8, and 12 weeks post-inoculation (WPI). **A**: Basophil activation is shown in %CD203c$^+$ cells normalised to positive control. **B**: Basophil degranulation is shown in %CD63$^+$ cells normalised to positive control. The dotted line shows 30% of basophil activation or degranulation, in clinical practice considered as a positive reaction. Data points represent the mean of five subjects. No statistical significant differences could be observed (Mann-Whitney U test, p=0.05; n=5). Figure is modified from Welter *et al.,* 2013, *PLOS One*.

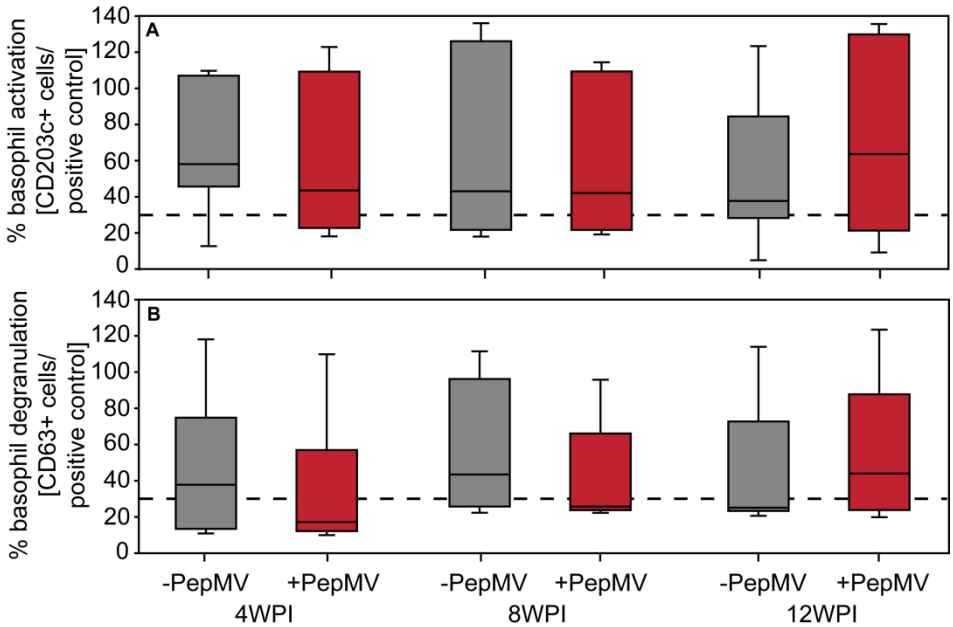

Figure 4.23: Basophil activation (A) and degranulation test (B) of five tomato-allergic subjects (1 - 5). 5 µg/mL of *Pepino mosaic virus*-infected and non-infected tomato fruit protein extract from 4, 8 and 12 weeks post-inoculation (WPI) was used. Basophil activation and degranulation is shown in %CD203c$^+$ and %CD63$^+$ cells normalised to positive control. The median is depicted in the black line. The dotted line shows 30% of basophil activation or degranulation, in clinical practice considered as a positive reaction. No significant differences could be observed (Mann-Whitney U test, p=0.05; n=5). Figure is modified from Welter *et al.,* 2013, *PLOS One*.

Summary and connection

Summarizing the results gained in the PepMV study, no general increase of putative tomato allergens and defence-related proteins in infected tomato fruits could be observed. This is the first study investigating the expression of those proteins tardy (weeks) after tomato inoculation with a virus and differences were shown between harvest time points and also between organs.
Against our hypothesis we could not observe any significant increase in the allergenic potential of PepMV-infected tomato fruits. Inter-individual variation in reaction of tomato-allergic subjects was high and analyses concerning the basis for this variation are described in the next chapter.

4.4 Immunoblot study: Identification of new putative tomato allergens and differential interaction with IgEs of tomato-allergic subjects

modified from Welter et al. 2013, in revision of Clinical and Experimental Allergy
The preliminary work of this experiment (planting, harvesting, and clinical allergy tests) was previously published (Schwarz *et al.*, 2010b;Dölle *et al.*, 2011b). *All immunoblots were conducted by Karola Lehman in the Proteome Factory AG. Data were analysed by the current author.*
In this study the basis of the observed high inter-individual differences between single tomato-allergic subjects in clinical allergy tests was aimed to clarify. It was hypothesised that single subjects react or do not react to particular allergens and that these allergens are differentially expressed in tomato plants with certain genetic background or grown under distinct cultivation methods. Therefore, single 2D immunoblots with individual sera from nine tomato-allergic subjects were carried out. Proteins for these immunoblots were extracted from tomato fruits of the two genotypes '76R' and 'RMC' (chapter 4.1) as well as of a third cultivar 'Counter', fertilised either with low (deficit) or high (excess) amounts of organic (org N) or mineral nitrogen (NO_3). Reactions of single subjects' IgEs to certain allergens could be detected and the corresponding proteins were identified by mass spectrometry. Simultaneous colorimetric development of single immunoblots to allow later comparison was achieved by using an AP-conjugated secondary antibody (chapter 3.4). The number of interactions between IgEs of particular subjects with allergens in the different fruits were counted, and compared to the results of the previously conducted skin prick tests. Additionally, known and new putative tomato allergens were identified and their importance as minor or major allergens suggested.

4.4.1 Identification of putative tomato allergens

Positive spots on immunoblots with tomato-allergic subjects were eluted from parallel 2D gels and analysed by mass spectrometry.
In total ten putative tomato allergens could be identified in the different tomato fruit protein extracts (figure 4.24, table 4.10). No reaction to any of the identified putative tomato allergens was observed using the serum of a non-allergic subject (data not shown). Among the identified proteins were five already known tomato allergens: two pectinmethylesterases (PME1.9 and PME2.1), polygalacturonase (PG), thaumatin-like protein (NP24), and a protein similar to 11S globulin (11SG). Four proteins were described in this study for the first time as putative tomato allergens: a pectinesterase inhibitor U1 (PEIU1), two proteins similar to a pectinacetylesterase (PAE), and an aspartyl protease family protein (AP), and a protein of unknown function. The mannosidase (MAN)

was already identified in the previous immunoblots of the PepMV study (figure 4.14), but to our knowledge, never described as a tomato allergen in literature. Reliable identifications could be confirmed through high sequence coverage and scores with the database entries obtained in Mascot or Blast searches (table 4.10), even when theoretical and experimental achieved molecular weights were often not in occurrence. Partly processed proteins might be one reason for these divergences. Except for the PEIU1 and the protein of unknown function, all other proteins were at least once recognised by IgEs of all tomato-allergic subjects (figure 4.25). Sola l 1 and Sola l 2, two well-known confirmed tomato allergens could not be detected with this subject cohort, but were present in the extracts of all genotypes and growth conditions (arrows in figure 4.24).

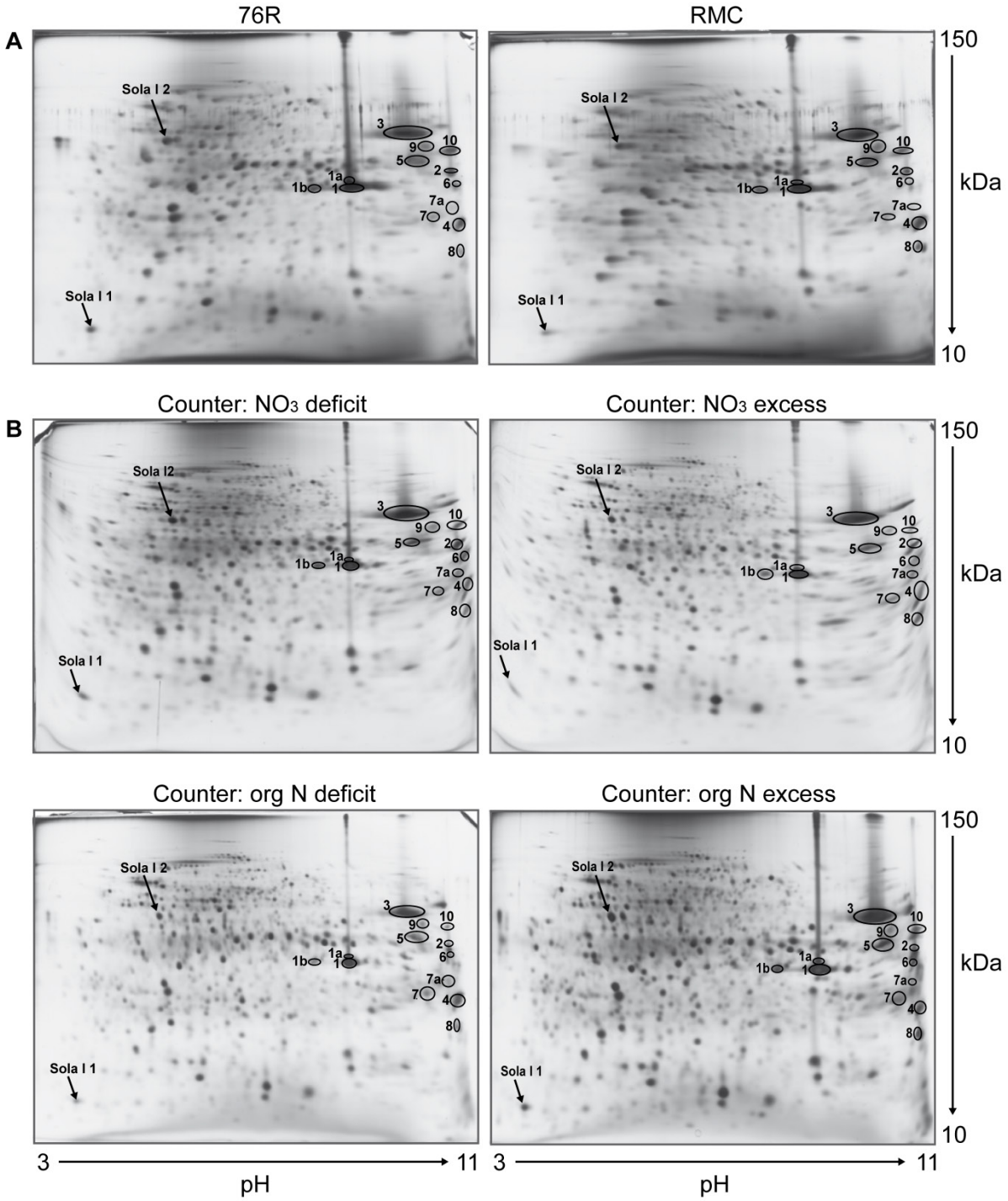

Figure 4.24: Small 2D protein gels with identified putative tomato allergens (circled) and the confirmed tomato allergens Sola l 1 and Sola l 2 (arrows). Proteins detected in immunoblots with the sera from nine tomato-allergic subjects and identified by mass spectrometry are marked with numbers and listed in table 4.10. **A**: Proteins were extracted from fruits of the non-mycorrhized tomato genotype '76R' and its isogenic mycorrhizal mutant 'RMC'. **B**: Proteins were extracted from fruits of the tomato cultivar 'Counter' cultivated with different nitrogen fertilisers (NO_3 or organic nitrogen in deficit or excess). Figure is modified from Welter *et al.,* 2013, in revision of *Clinical and Experimental Allergy*.

Table 4.10: Putative tomato allergens identified in tomato fruits of the genotypes '76R' and 'RMC' and the cultivar 'Counter' treated with different nitrogen fertilisers. Protein extracts were separated by 2D gel electrophoresis and analysed by immunoblotting with sera from nine tomato-allergic subjects. Positive spots were eluted from parallel gels and identified by mass spectrometry. Spots are indicated by numbers and shown in figure 4.24. Table is modified from Welter *et al.,* 2013, in revision of *Clinical and Experimental Allergy*.

spot no. in gel	name	accession number	molecular weight/ isoelectric point (theoretical)	molecular weight/ isoelectric point (in gel)	score/ sequence identity [%]	known as allergen in tomato	abbre-viation
1 (1a,1b)	pectinmethyl-esterase 1	gi|6174913	60/6.3	27/8.1	470/20	yes	PME1.9
2	pectinmethyl-esterase 2.1	gi|2507165	60.5/8.7	30/9.8	323/15	yes	PME2.1
3	polygalacturonase 2	gi|129939	50/6.4	37/9	758/30	yes	PG
4	np24	gi|170467	25.7/8.3	19/9.8	111/8	yes	NP24
5	endo-β-mannanase	gi|66360069	42.4/8.8	33/9	549/35	no	MAN
6	pectinesterase inhibitor U1	gi|6093740	64.1/9	29/10	330/16	no	PEIU1
7 (7a)	unknown	gi|924626	25.2/9.4	24/9.8	304/28	no	-
8	similar to 11S globulin isoform 4 (*Sesamum indicum*)	EST:BP891368 (TC241385)	18.3/10.1	18/9.8	282/43	yes	11S G
9	similar to aspartyl protease family protein (*Arabidopsis thaliana*)	EST:AI484598 (gi|15232503)	16.1/10.2	34/9.4	303/38	no	AP
10	similar to pectinacetylesterase precursor (*Vigna radiata*)	EST:BP891840 (gi|1431629)	17.8/8.2	34/10	156/40	no	PAE

Mascot and Blast searches were performed in 01/2012.

4.4.2 Distribution in minor and major putative tomato allergens

All subjects IgEs (100%) reacted with eight out of ten putative tomato allergens (figure 4.25). Interestingly, these reactions were dependent on genotype and growth condition of investigated tomato plants. The genotype '76R' provoked for example reaction of fewer subjects' IgEs than the genotype 'RMC'. Therefore, it is difficult to make a statement about minor and major tomato allergens. Anyway, the most prominent allergens, at least for this subject cohort and for the genotypes and growth conditions used for fruit production, were the known tomato allergens PME1.9 and NP24.

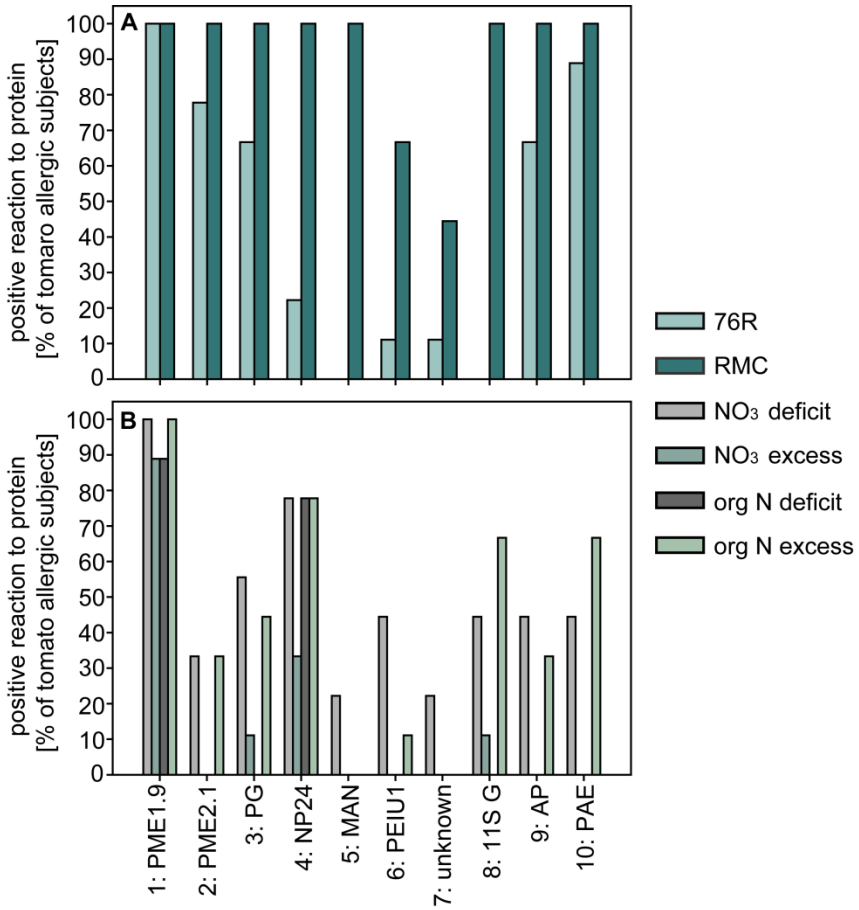

Figure 4.25: Percentage of nine tomato-allergic subjects showing positive reactions to putative tomato allergens. A: Reactions to the genotypes '76R' and its isogenic mycorrhizal mutant 'RMC'. **B**: Reactions to the cultivar 'Counter' fertilised with NO_3 or organic nitrogen (org N) in deficit or excess. Numbers indicate protein spots in figure 4.24 and table 4.10. Figure is modified from Welter *et al.,* 2013, in revision of *Clinical and Experimental Allergy*.

4.4.3 Correlation of skin prick tests versus immunoblots

The number of positive reacting spots on the immunoblots with the tomato-allergic subjects was correlated with wheal diameters of corresponding skin prick tests (figure 4.26). This analysis revealed no significant correlation neither within a particular tomato genotype or growth condition nor over all genotypes and growth conditions together (r=0.036).

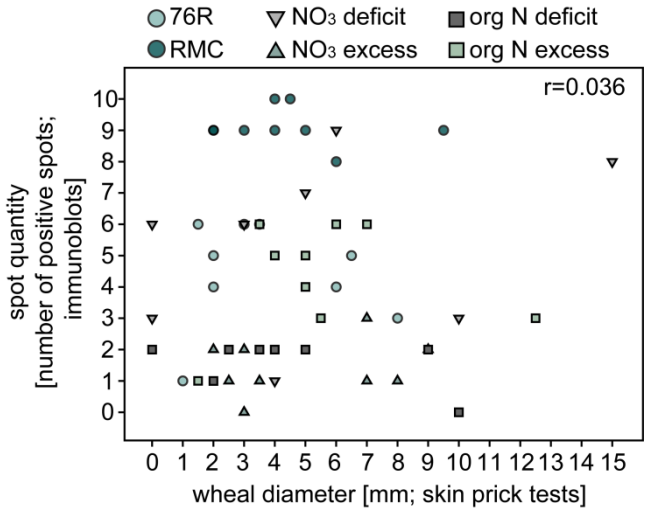

Figure 4.26: Correlation between wheal diameters (mm) of skin prick tests and spot quantity on immunoblots. Correlation analyses were carried out over nine tomato-allergic subjects with different tomato genotypes ('76R' or 'RMC') or with fruits from cultivar 'Counter' cultivated with different nitrogen fertilisers (NO_3 or organic nitrogen (org N) in deficit or excess). Wheal diameters [mm] of skin prick tests were correlated with the number of positive spots of the corresponding immunoblots. Figure is modified from Welter *et al.,* 2013, in revision of *Clinical and Experimental Allergy*.

Summary and connection

Within these two studies, including immunoblot analyses with tomato-allergic subjects, we could detect 14 new putative tomato allergen candidates - to our knowledge - never described before. Four of these candidates have been investigated in further studies (next chapter), including their expression as recombinant proteins, in order to confirm their allergenicity.

4.5 New putative tomato allergens: *Pepino mosaic virus* coat protein, heat shock protein, mannosidase and thaumatin-like protein

In the 2D immunoblots of the two previous chapters new putative tomato allergens were identified (table 4.9 and 4.10), among those a mannosidase (MAN), heat shock proteins (HSC70), and the PepMV coat protein (PepMVCP, figure 4.27). A thaumatin-like protein (NP24), already listed in the 'Allergome' database (www.allergome.org), but not yet confirmed as a tomato allergen by the International Union of Immunological Societies (IUIS; www.allergen.org), was also detected. Mass spectrometry and subsequent Mascot searches revealed the following accessions for MAN: AAL37714 and AAK56557, for NP24: AAA34175 (partial sequence) =AAC64171 (full sequence), for HSC70: AAB42159 (first identified as heat shock protein of *Solanum tuberosum*) and for PepMVCP: AAS79818 (US genotype) = AAY51623 (CH2 genotype). In order to confirm their allergenicity it was necessary to investigate the pure proteins in immunoblots and clinical allergy tests. For this purpose, the corresponding candidates had to be overexpressed.

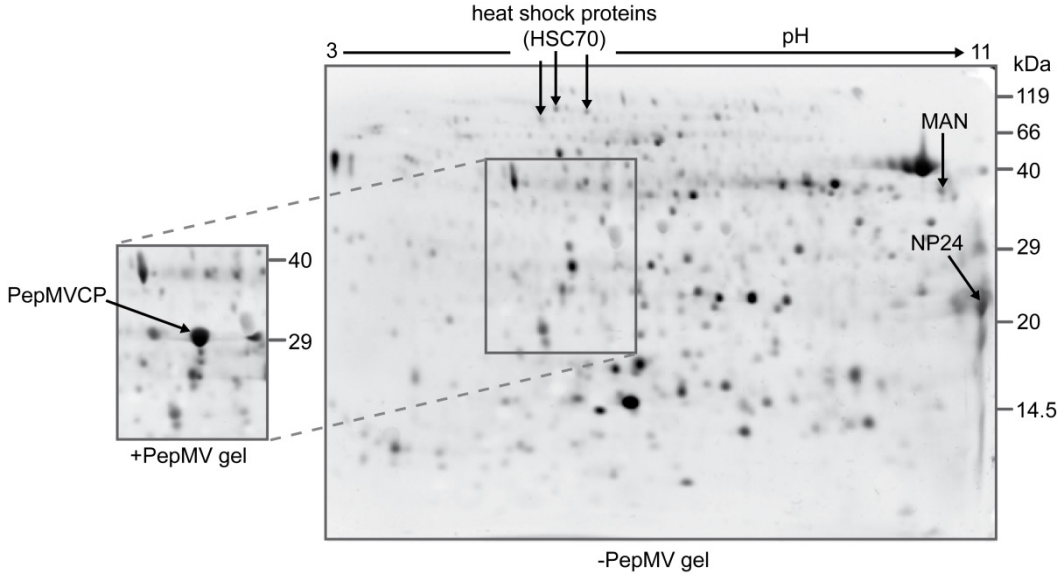

Figure 4.27: Small 2D protein gel of non-infected tomato fruit protein extract with a small section of the representative part of a gel of *Pepino mosaic virus*-infected tomato fruit protein extract. Selected proteins for overexpression analyses are indicated. These putative tomato allergens were detected in immunoblots with a serum pool of ten tomato-allergic subjects.

The full length coding sequences (CDSs) of the respective genes were available in the gene bank of the national centre for biotechnology (NCBI). To amplify the particular CDS-specific primer pairs with and without specific restriction sites were designed (table 3.3). PCR amplification products were cloned into pGemTEasy vector, excised with specific restriction enzymes and the CDSs were subsequently ligated into the pCDFDuet expression vector (figure 3.9, table 4.11). *PepMVCP* and *NP24* were successfully cloned and cloned CDSs were confirmed by sequencing.

Table 4.11: PCR amplification products of putative tomato allergen coding sequences. Fragments with and without introduced restriction sites and cloned into particular vectors are shown. Particular CDSs of *MAN* (AF403444), *NP24* (AF093743), *HSC70* (L41253), *PepMVCP* (DQ000985) and *CYC* (M55019) were amplified.

	HSC70 (BamHI, PstI)	***MAN*** (BamHI, SalI)	***NP24*** (BamHI, HindIII)	***PepMVCP*** (BamHI, HindIII)	***CYC*** (BamHI, NdeI)
primer with restriction sites[1]				725 bp	x
primer without restriction sites[2]	1954 bp	1544 bp	743 bp	x	x
primer with restriction sites[1]	1966 bp	1556 bp	755 bp	x	x
in pGemTEasy (M13 for/rev)[3]		1797 bp	996 bp	948 bp	x
in pCDFDuet (ACYCDuetUP1, DuetDOWN1)[3]	x		1006 bp	976 bp	x
in pCG19[3]	x	x	x	x	753 bp
in pET15b (T7 promotor, T7 terminator)[3]	x	x	x	x	976 bp

black box: no product; x: not done
[1]length: CDS + restriction sites + 3bp, without start and stop codon; [2]length: CDS, without start and stop codon; [3]length: amplified sequence with vector-specific primer (table 3.4).

Successful overexpression of PepMVCP and NP24 could not be achieved in any of the following *E. coli* strains: BL21-DE3, Rosetta, and Origami. Changing of expression temperature (25°C, 30°C, and 37°C), IPTG concentration (0.5 mM, 1 mM) and/or incubation time (1 h, 2 h, 3 h, 4 h, and overnight) provided no positive results. The general procedure was controlled by a positive control which could be always successfully overexpressed (data not shown). Personal contact to Yannis Livieratos (Crete, Greece) after a recent publication (Mathioudakis *et al.*, 2012) confirmed the unattainable overexpression in *E. coli* at least for PepMVCP and HSC70.

4.6 Cyclophilin - A tomato allergen candidate

Another putative tomato allergen, the cyclophilin (CYC, P21568), was previously detected (figure 4.18). CYC was already successfully overexpressed by the group of Prof. Dr. Charles Gasser from U.C. Davis (California, USA, (Gasser *et al.*, 1990)). He kindly provided us a vector plasmid (pCG19) with the *CYC* insert.

4.6.1 Overexpression of tomato cyclophilin in *Escherichia coli*

Overexpression of recombinant tomato cyclophilin (rCYC) was successful in *E. coli* BL21-DE3 at 37°C after 3 h and an IPTG induction concentration of 0.5 mM. To test the necessity for further purification, the gel with full bacterial protein extract including overexpressed rCYC was blotted. An

immunoblot with a serum pool of tomato-allergic subjects (n=10) showed a positive reaction to rCYC (figure 4.28).

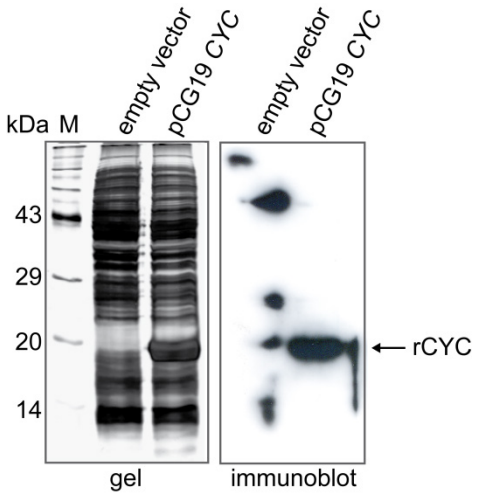

Figure 4.28: Silver stained protein gel and corresponding immunoblot with bacterial protein extract containing the recombinant cyclophilin. Overexpression of rCYC was induced with 0.5 mM IPTG and *E. coli* cells (BL21-DE3), carrying an empty vector (pET15b) or the pCG19 CYC vector construct, were grown for 3 h. A serum pool of ten tomato-allergic subjects (dilution: 1:10) was used for immunoblotting. A peroxidase-conjugated anti-human IgE antibody (dilution: 1:5,000) was used for detection (exposure to film: 30 min). M: protein weight marker.

For purification of the overexpressed rCYC from *E. coli* extract, the *CYC* insert of pCG19 vector was subcloned into a pET15b vector, including a his-tag (figure 3.10, table 4.11, (Cadot *et al.*, 2006)). Proper insertion was checked by sequencing. Overexpression was successfully achieved in *E. coli* isolates BL21-DE3 and Rosetta, after addition of 0.5 mM IPTG and incubation at 37°C for 3 h. The predicted molecular mass of rCYC, containing the his-tag and thrombin cleavage site, is 20.1 kDa (http://web.expasy.org/protparam) and was in good agreement with the observed protein band on the gel (figure 4.29).

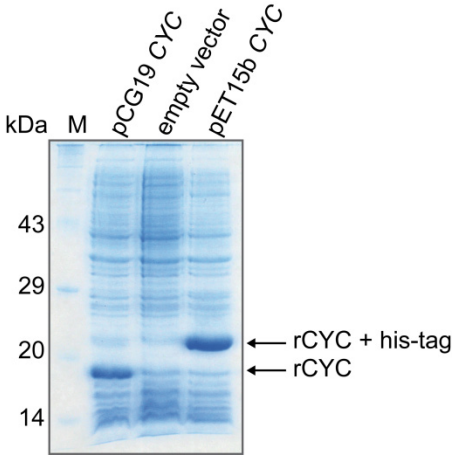

Figure 4.29: Coomassie stained protein gel of bacterial protein extract, containing the recombinant cyclophilin. Overexpression was induced in *E. coli* cells (BL21-DE3) with pCG19 CYC vector construct, empty vector (pET15b), and pET15b CYC vector construct with 0.5 mM IPTG and bacterial cells were grown for 3 h. Overexpressed rCYC is marked with an arrow. M: protein weight marker.

4.6.2 His-tag purification and thrombin cleavage of recombinant cyclophilin

His-tag purification of rCYC from bacterial extract with Ni-NTA agarose beads under denaturing conditions was not efficient and only low amounts of rCYC could be achieved. Therefore, protein concentration could not be exactly determined, but all immunoblots could be conducted with this rCYC. Subsequently the purification method could be optimised and rCYC was successfully purified under native conditions, yielding always a concentration around 3 mg/L bacterial cell culture. His-tag removal was achieved by thrombin cleavage. The predicted molecular masses of rCYC with and without the his-tag are 20.1 kDa and 18.2 kDa (http://web.expasy.org/protparam) and were in good agreement with the observed protein bands on the gel (figure 4.30).

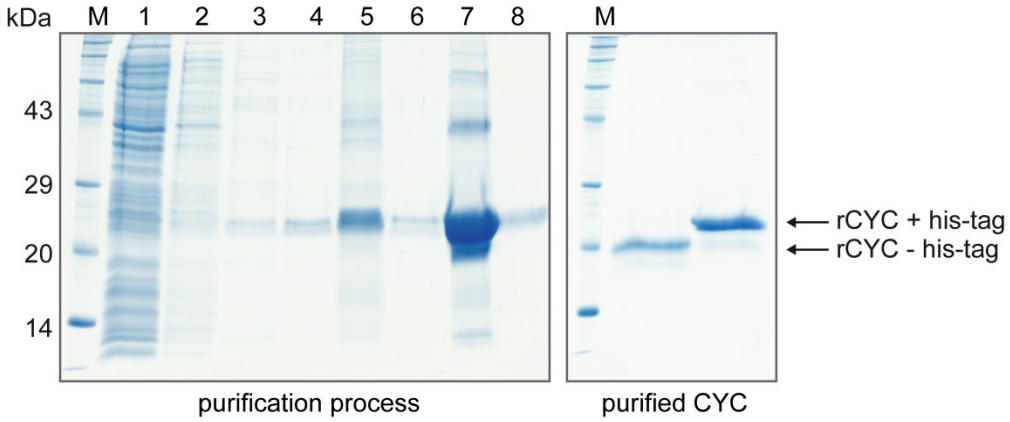

Figure 4.30: Coomassie stained protein gel of native his-tag purification process of recombinant cyclophilin. Purification was achieved with Ni-NTA agarose beads on column under native conditions. The purification process, flow through of, 1: sonicated whole bacterial extract, 2 - 5: different washing steps with increasing amount of imidazole (5 - 15 mM), 6 - 8: elution steps with 250 mM imidazole. Purified rCYC with and without the his-tag are marked with an arrow. M: protein weight marker.

The purified rCYC was trypsin digested and identified by mass spectrometry and Mascot search (figure 4.31). Cyclophilin from tomato could be verified with a Mascot score of 918 and 85% sequence coverage. The calculated nominal mass was 17.9 kDa and the pI 8.8. The differences in the molecular masses between this native tomato cyclophilin (P21568) and rCYC produced here resulted from three additional amino acids, still remaining after the thrombin cleavage.

```
  1 MANPKVFFDL TIGGAPAGRV VMELFADTTP KTAENFRALC TGEKGVGKMG
 51 KPLHYKGSTF HRVIPGFMCQ GGDFTAGNGT GGESIYGAKF NDENFVKKHT
101 GPGILSMANA GPGTNGSQFF ICTAKTEWLN GKHVVFGQVV EGMDVIKKAE
151 AVGSSSGRCS KPVVIADCGQ L
```

Figure 4.31: Recombinant cyclophilin identification via mass spectrometry. Purified protein (figure 4.30) was used. Matched peptides are indicated in red, sequence coverage with the native tomato cyclophilin (P21568) was 85% and the Mascot score was 918.

4.6.3 Immunoblots with recombinant cyclophilin

The purified rCYC was firstly tested for IgE reactivity on a dot blot with a serum pool from tomato-allergic subjects (n=10). This immune dot blot was positive in regard to rCYC, as well as to the positive control (tomato protein extract). A slight reaction could also be observed to the negative control BSA (data not shown). This indicated that tomato-allergic subjects reacted to rCYC, but further investigations were necessary to confirm the allergenicity of rCYC.

A 1D immunoblot was carried out with the same material (tomato protein extract as positive control, BSA as negative control, and the rCYC). As an additional negative control the immunoblot was developed without any sera to exclude reaction of the anti-human IgE antibody to the rCYC (figure 4.32). Except of some slight signals obtained with the tomato extract, the POD-conjugated secondary antibody alone did not show any reaction. The serum pool (n=10) reacted to the rCYC, as it could be expected from the dot blots, but again some slight reactions to BSA were visible.

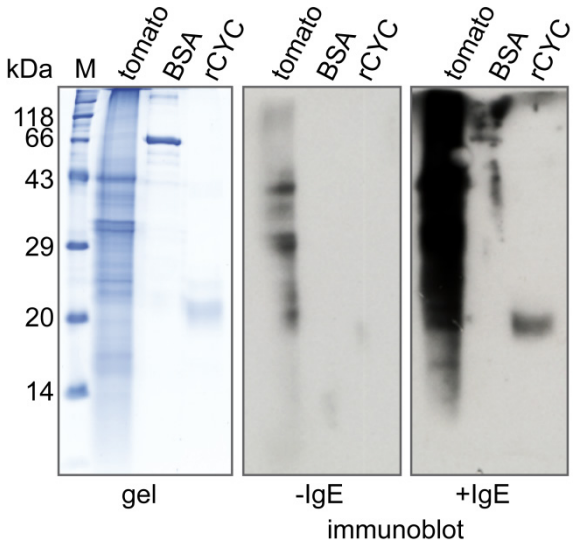

Figure 4.32: Coomassie stained protein gel and corresponding immunoblots with tomato fruit protein extract, BSA and recombinant cyclophilin. Immunoblots were developed with (+IgE) and without (-IgE) a serum pool of ten tomato-allergic subjects (dilution 1:10). A peroxidase-conjugated anti-human IgE antibody (dilution: 1:5,000) was used for detection (exposure to film: 30 min). M: protein weight marker.

The immunoblots with the serum pool indicated that at least one tomato-allergic subject reacted to the rCYC. For further characterisation sera from single subjects were investigated in 1D immunoblots according to their reaction to the rCYC. Simultaneous development could be achieved by using an AP-conjugated secondary antibody. Seven among twelve tomato-allergic subjects reacted with the rCYC (subject 2, 5, 7, 8, 9, 11, 12; black arrows in figure 4.33). The two negative controls (without any serum and with a serum of a non-allergic subject) also showed slight reactions to the recombinant protein (grey arrows in figure 4.33). To confirm these reactions and tomato cyclophilin as a new allergen, inhibition assays were conducted.

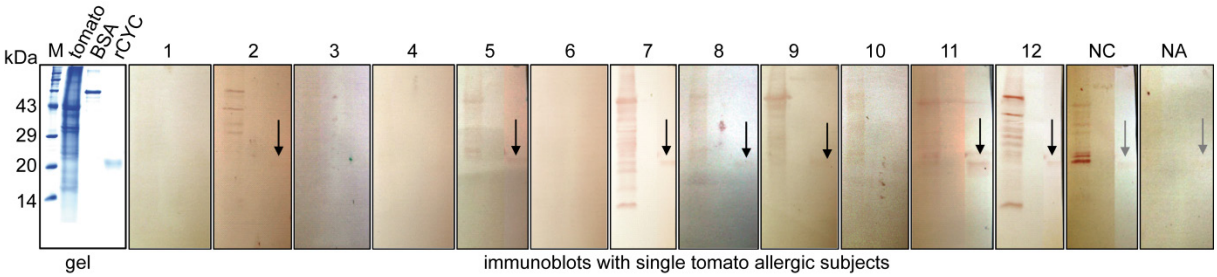

Figure 4.33: Coomassie stained protein gel and corresponding single immunoblots with tomato fruit protein extract, BSA and recombinant cyclophilin. Sera of tomato-allergic subjects (1 - 12, dilution 1:10), the non-allergic subject (NA) and a negative control without any serum (NC) were used for immunoblotting. Arrows show positive reactions to rCYC. Immunoblots with sera and alkaline phosphatase-conjugated anti-human IgE antibody (dilution 1:2,000) were incubated for 1 h with Fast Red/Naphtol. M: protein weight marker.

4.6.4 Immunoblot inhibition with recombinant cyclophilin

Immunoblot inhibition was carried out with the sera from tomato-allergic subject 8 and 11. These two sera were selected because they reacted with the rCYC in the 1D immunoblots and enough serum was available for further investigations. Inhibition was carried out with 50 µL of denatured rCYC incubated with the sera at 4°C overnight, but no inhibition of reaction to the rCYC could be achieved (figure 4.34).

To save serum and be able to test more different reactions in one single experiment, further analyses were carried out with ELISAs instead of immunoblots.

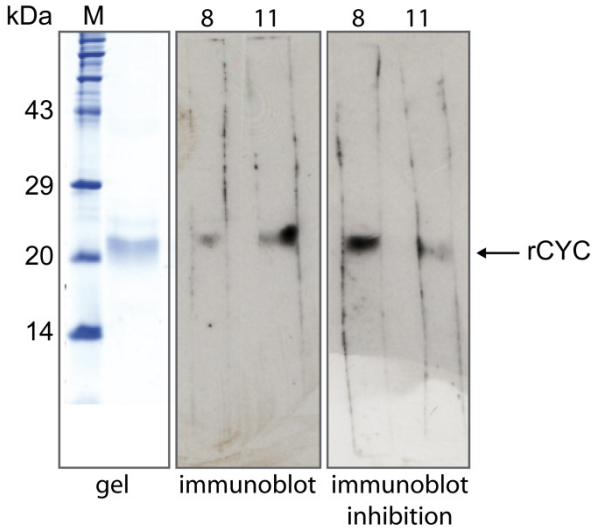

Figure 4.34: Immunoblot inhibition with sera from the tomato-allergic subjects 8 and 11. Coomassie stained protein gel with rCYC (20 µL), corresponding immunoblots (sera dilution 1:10), and immunoblot inhibition. For immunoblot inhibition sera were incubated before with 50 µL rCYC. A peroxidase-conjugated anti-human IgE antibody (dilution: 1:5,000) was used for detection. Both blots were simultaneously developed and exposed to film for 1 min. M: protein weight marker.

4.6.5 Optimisation of ELISA with recombinant cyclophilin

All ELISAs were conducted with natively purified rCYC which was coated on the intended ELISA plate. A chessboard titration was conducted to work out the right protein concentration and to find the

exact dilution for subjects' sera. rCYC concentrations from 0 - 62.4 µg/mL and serum pool (n=10) dilutions of 1:5, 1:10, 1:20, 1:30, 1:40, and 1:50 were tested. The POD-conjugated secondary antibody used in this assay was kept at a dilution of 1:1,000, as recommended by the manufacturer. Increasing OD values could be observed with an increasing amount of rCYC coupled on the plate, regardless of the serum dilution (figure 4.35). The plate was completely covered with rCYC after applying the protein extract in a concentration of 15 µg/mL; this concentration was therefore used in all further ELISAs. Serum dilution was kept at 1:50 in further assays because less dilution revealed only minor, negligible differences. As increasing OD values were, however, observed even without adding any serum, unspecific binding of the anti-human IgE antibody to the rCYC was supposed. Therefore, two other anti-human IgE antibodies were evaluated in different concentrations. Secondly, relatively high OD values were observed even without any rCYC on the plate, possibly due to unspecific binding of serum IgEs directly to the plate. Therefore, different blocking agents and concentrations were evaluated.

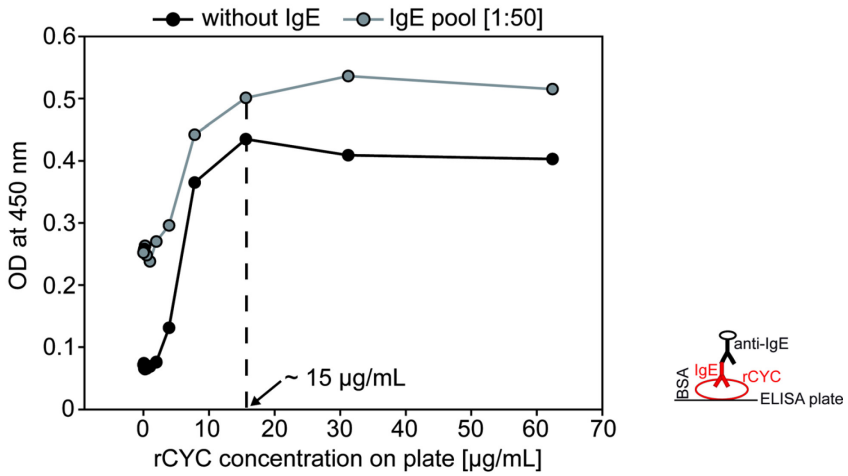

Figure 4.35: ELISA chessboard titration with different recombinant cyclophilin concentrations linked to the plate. OD values (at 450 nm) for a serum pool of tomato-allergic subjects (n=10, dilution of 1:50) and a negative control (without any serum, only with peroxidase-conjugated anti-human IgE antibody) are shown. A peroxidase-conjugated anti-human IgE antibody (dilution 1:1,000) was used to detect positive reactions. The optimal concentration of rCYC is indicated by the dotted line (15 µg/mL).

Three different POD-conjugated secondary antibodies were evaluated. Only the one used in the chessboard titration produced reliable results, and therefore, was analysed in different concentrations to reduce the high background signal (figure 4.36). Higher dilutions did not result in lower background signals, therefore, the dilution was kept at 1:1,000 in the following assays. In any case, differences between specific and unspecific reactions were high enough. Additionally, the pre-incubation of the POD-conjugated secondary antibody with rCYC to capture unspecific reactions did not result in lower background signals. Another strategy to reduce these unspecific reactions was to apply the rCYC in its denatured form, because immunoblots using the same anti-human IgE antibody showed no reaction at all in negative controls without any serum. Unfortunately, denatured rCYC on ELISA plates resulted in even higher background signals than its native form (data not shown).

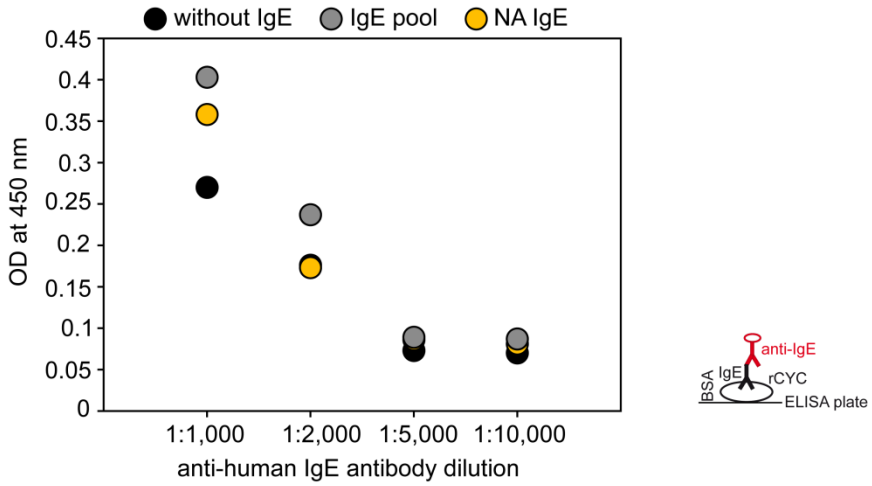

Figure 4.36: ELISA results of different peroxidase-conjugated anti-human IgE antibody working dilutions. 15 µg/mL rCYC was linked to the ELISA plate. OD values (at 450 nm) for a serum pool of tomato-allergic subjects (n=10, dilution of 1:50), a negative control (without any serum, only with peroxidase-conjugated anti-human IgE antibody), and a serum of a non-allergic subject (NA) are shown.

Secondary, unspecific reaction to the ELISA plates without rCYC could not be eliminated with different blocking agents and concentrations. Milk powder did not show any blocking capability (data not shown). Figure 4.37 shows two different concentrations of BSA (1.5% and 3%) as blocking agent. The differences were negligible, but 3% BSA was used for further investigations.

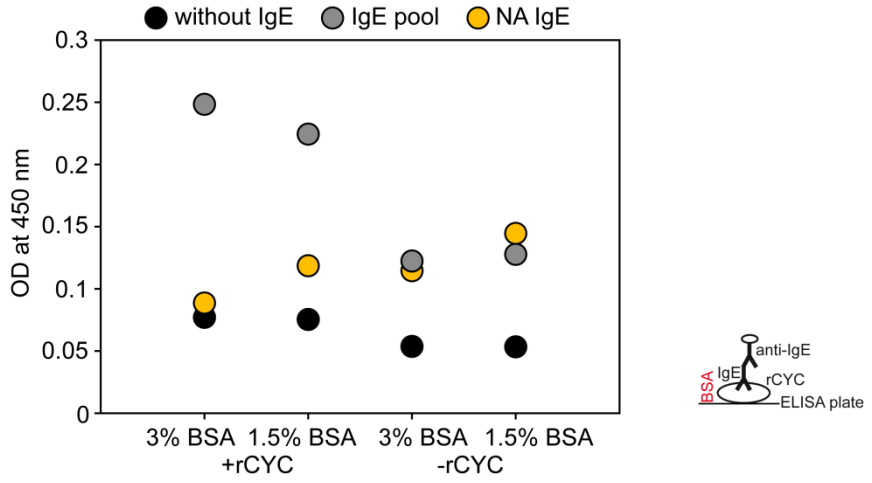

Figure 4.37: ELISA results of two different BSA blocking dilutions. 15 µg/mL (+rCYC) or no rCYC (-rCYC) was linked to the ELISA plate. OD values (at 450 nm) for a serum pool of tomato-allergic subjects (n=10, dilution of 1:50), a negative control (without any serum) and a serum of a non-allergic subject (NA) are shown. A peroxidase-conjugated anti-human IgE antibody (dilution 1:1,000) was used to detect positive reactions.

4.6.6 ELISAs with recombinant cyclophilin and single tomato-allergic subjects

Subjects 2, 5, 7, 8, 9, 11, and 12 positively reacted to rCYC in the immunoblot analyses (figure 4.33), therefore, their specific IgE to this protein was determined by ELISA. Since unspecific reactions could not be completely excluded, investigations with the sera from the single subjects were accompanied by different negative controls (without rCYC on the plate, without serum, and with serum of a non-allergic subject). All subjects seem to have specific IgE for rCYC and immunoblot results could be

confirmed (figure 4.38). However, in a second assay all twelve tomato-allergic subjects, the non-allergic subject included, showed a reaction to the rCYC (figure 4.39). A positive correlation (r=0.39) between the results of these two experiments (of subjects 2, 5, 7, 8, 9, 11, 12) and the relatively low standard deviations between the three technical replicates showed reproducibility of those ELISAs.

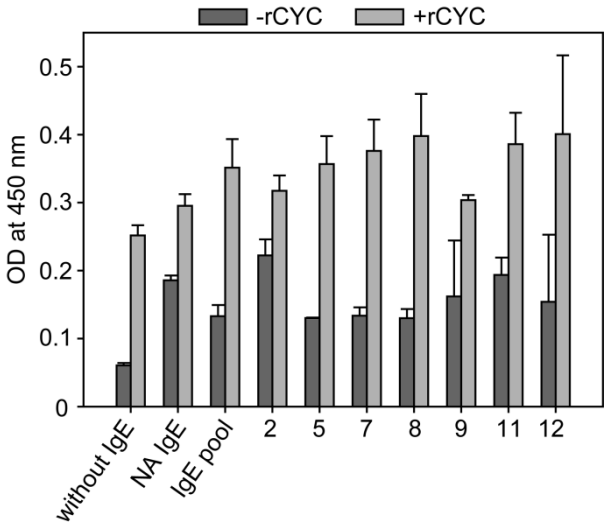

Figure 4.38: First ELISA investigation on specific IgE to recombinant cyclophilin of single tomato-allergic subjects. 15 µg/mL (+rCYC) or no rCYC (-rCYC) was linked to the ELISA plate. OD values (at 450 nm) for a serum pool of tomato-allergic subjects (n=10, dilution of 1:50), single subjects (dilution 1:50), a negative control (without any serum, IgE), and a serum of a non-allergic subject (NA) are shown. A peroxidase-conjugated anti-human IgE antibody (dilution 1:1,000) was used to detect positive reactions. ELISA reactions were performed in triplicate.

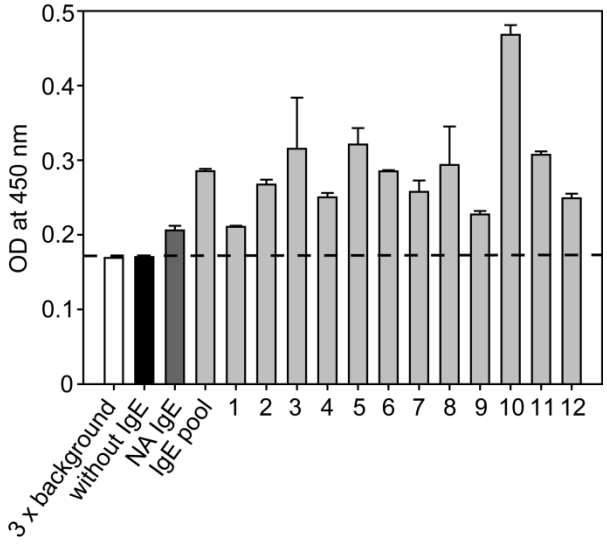

Figure 4.39: Second ELISA investigation on specific IgE to recombinant cyclophilin of single tomato-allergic subjects. 15 µg/mL rCYC was linked to the ELISA plate. OD values (at 450 nm) for a serum pool of tomato-allergic subjects (n=10, dilution of 1:50), single subjects (dilution 1:50), a negative control (without any serum, IgE), and a serum of a non-allergic subject (NA) are shown. A peroxidase-conjugated anti-human IgE antibody (dilution 1:1,000) was used to detect positive reactions. ELISA reactions were performed in triplicate. The dotted line shows values below the defined background.

4.6.7 ELISA inhibition with recombinant cyclophilin

ELISA inhibition was carried out to verify the positive reactions of the sera to rCYC and to exclude all unspecific reactions. Therefore, subject sera 5, 7, 8, 9, 11, and 12, positively reacting to rCYC in immunoblot analyses, and a non-allergic subject serum were investigated. Subjects 7, 8, and 9 showed an inhibition of at least 40% (7: 46%, 8: 45%, 9: 40%). Almost no inhibition was observed with subject number 12 (19%). The non-allergic subject showed an inhibition of 48%. Subjects 5 and 11 showed a clear inhibition with the rCYC for at least 60%. Inhibition assays of these two subjects were additionally conducted with BSA instead of rCYC as inhibitor, resulting in an absent inhibition (figure 4.40).

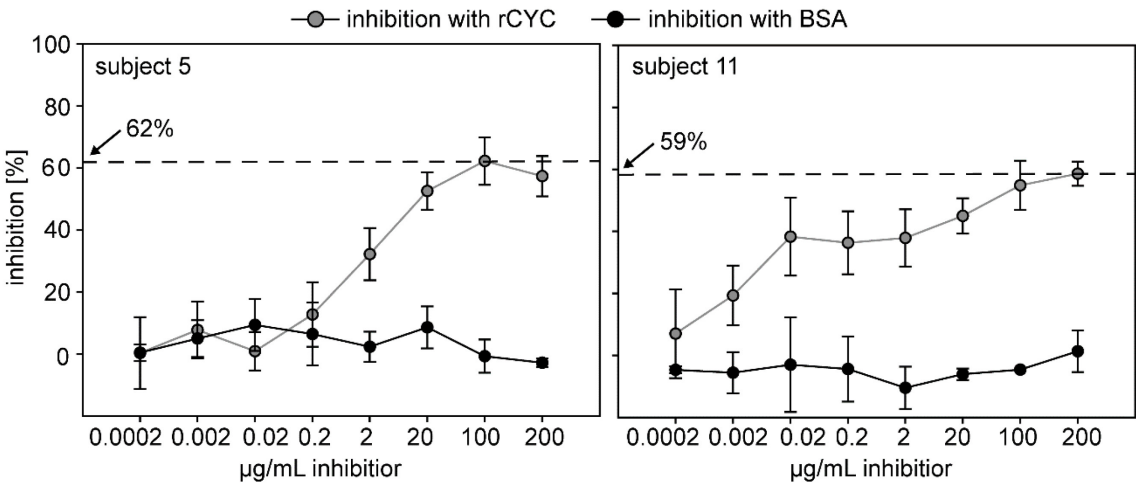

Figure 4.40: ELISA inhibition conducted with sera from tomato-allergic subjects 5 and 11. Inhibition is shown in per cent and different concentrations of rCYC (µg/mL) were evaluated as inhibitor. BSA was also tested as inhibitor, as the negative control. The inhibition assays were carried out in five technical replicates on two independent plates. The dotted line shows the respective percentage of inhibition.

4.6.8 Clinical allergy tests with recombinant cyclophilin

Skin prick and basophil tests were carried out by Josefine Grünhagen and Dennis Ernst in the Charité Berlin. Data were analysed by the current author.

Nine tomato-allergic subjects (1, 3, 4, 5, 7, 8, 10, 11, 12) could be recruited for skin prick and basophil activation and degranulation tests with the rCYC. Basophils of subjects 5 and 11 showed a clear activation as well as degranulation, if treated with the rCYC in different concentrations, respectively (figure 4.41). Basophils of subject 8 were slightly activated. The other six subjects (1, 3, 4, 7, 10, 12) as well as the non-allergic subject showed no basophil activation or degranulation after rCYC treatment. As already observed in previous analyses (data not shown) basophil activation measurements seemed to be more sensitive than degranulation measurements, but overall showed the same results. The skin prick test with rCYC (~30 µg/mL) was positive for subject number 11. All other subjects did not react to this recombinant protein.

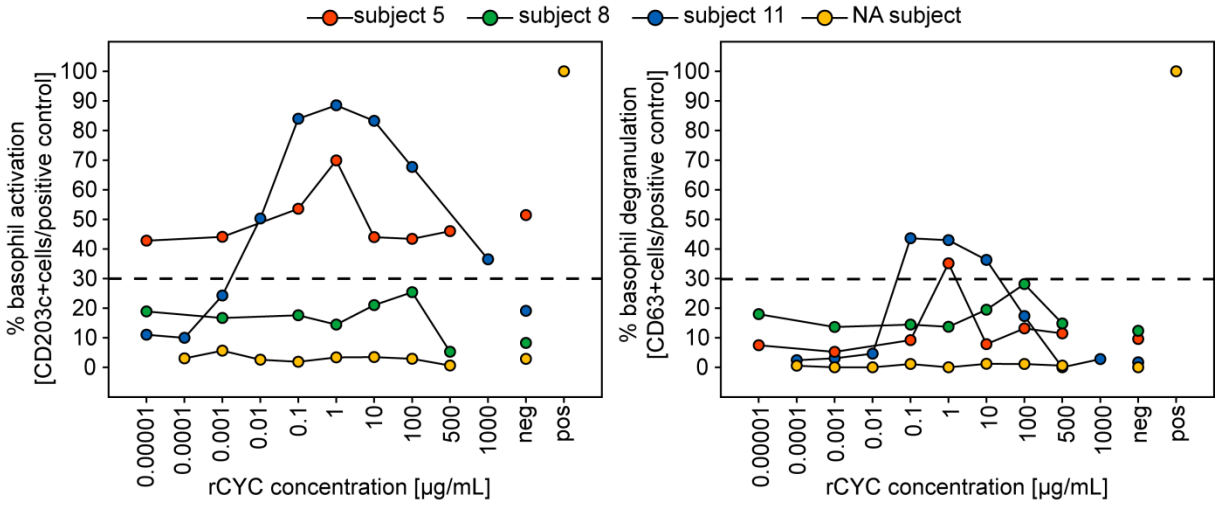

Figure 4.41: Basophil activation and degranulation tests with recombinant cyclophilin. Dose response curves of the three tomato-allergic subjects 5, 8, and 11 are shown. Serum of a non-allergic subject served as negative control. Basophil activation is shown in %CD203c^{+} cells normalised to positive control. Basophil degranulation is shown in %CD63^{+} cells, normalised to positive control. The dotted line shows 30% of basophil activation or degranulation, in clinical practice considered as a positive reaction.

4.6.9 Comparison of immunoblots, ELISAs and clinical allergy tests with recombinant cyclophilin

The results obtained in the different *in vivo* and *ex vivo* tests conducted with tomato-allergic subjects and the rCYC are summarised in table 4.12. In immunoblot analyses seven out of twelve tomato-allergic subjects reacted to the rCYC. The reaction of at least two out of six subjects to rCYC could be inhibited in ELISA inhibitions, and therefore, seem to be specific. The clinical tests with rCYC showed positive reaction in at least two out of nine subjects (basophil tests) or in one out of nine subjects (skin prick test).

Subject 9 could not be recruited for the clinical tests but showed positive reactions in immunoblots and ELISAs. Reaction of subject 5 and 8 to the rCYC could be inhibited in ELISA inhibition and basophil activation could be detected with the rCYC. Absent wheals after skin provocation could be explained by the lower sensitivity of skin prick tests. Obvious results were obtained for subject 11, reacting in all supplied tests to rCYC. In contrast, subjects 1, 3, 4, and 10 were negative in reaction to rCYC in all tests.

Table 4.12: Comparison of immunoblot analyses, ELISA inhibition, skin prick tests (SPT) and basophil activation (BAT) and degranulation (BDT) tests with recombinant cyclophilin.

subjects	immunoblot	ELISA inhibition	SPT (wheal diameter)	BAT >30%	BDT >30%
NA	- /+	48%	0 mm	-	-
1	-	nd	0 mm	-	-
2	+	nd	nd	nd	nd
3	-	nd	2 mm	-	-
4	-	nd	0 mm	-	-
5	+	62%	0 mm	+	+
6	-	nd	nd	nd	nd
7	+	46%	0 mm	-	-
8	+	45%	0 mm	-	-
9	+	40%	nd	nd	nd
10	-	nd	0 mm	-	-
11	+	59%	9 mm	+	+
12	+	19%	0 mm	-	-
sum (positive)	7/12	5/6	1.5/9	2/9	2/9

NA: non-allergic subject, nd: not determined, +: positive reaction, -: negative reaction

Summary and connection

Tomato cyclophilin could be successfully overexpressed and provoked reactions of tomato-allergic subjects. Unfortunately, overexpression of the other putative tomato allergens MAN, HSC70, NP24, and PepMVCP could not be achieved in this study. Considering the high commercial impact of the PepMVCP, being a putative allergen, it was purified from tomato and separately tested in immunoblots (next chapter).

4.7 *Pepino mosaic virus* coat protein - An allergen candidate

PepMVCP could be successfully purified from tomato fruits and leaves. 1D SDS protein gel showed different bands with one at 30 kDa, the molecular weight of the PepMVCP (figure 4.42).

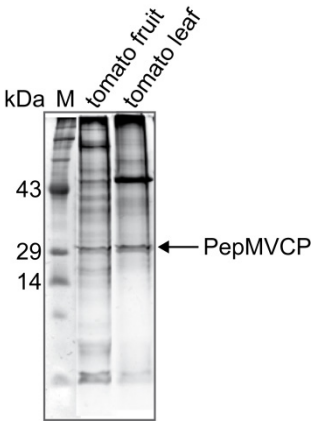

Figure 4.42: Silver stained protein gel of *Pepino mosaic virus* isolation from tomato fruits and leaves. M: protein weight marker.

The potential PepMVCP, purified from tomato leaves (figure 4.42), was trypsin digested and identified by mass spectrometry and Mascot search with a Mascot score of 439 and 28% sequence coverage (figure 4.43). The calculated nominal mass was 25.1 kDa and the pI 6.7. The identified sequence ADL63132 (NCBI) has two amino acid exchanges compared to the NCBI accession AAY51623 (PepMVCP of CH2 genotype), identified in immunoblots and used for overexpression.

```
  1 MENQPTASNP SDVPPTAAQA GAQSPADFSN PNTAPSLSDL KKIKYVSTVT
 51 SVATPAEIEA LGKIFTAMGL AANETGPAMW DLARAYADVQ SSKSAQLIGA
101 TPSNPALSRR ALAAQFDRIN ITPRQFCMYF AKIVWNILLD SNVPPANWAK
151 LGYQEDTKFA AFDFFDGVTN PASLQPADGL IRQPNEKEIA AHSVAKYGAL
201 ARQKISTGNY ITTLGEVTRG HMGGANTMYA IDAPPEL
```

Figure 4.43: *Pepino mosaic* coat protein identification via mass spectrometry. Purified protein (figure 4.42) was used for the identification. Matched peptides are indicated in red and sequence coverage with PepMVCP (ADL63132) was 28% and Mascot score was 439. Green letters indicate sequence difference to AAY51623 (PepMVCP of CH2 genotype).

After verifying the 30 kDa band as PepMVCP, an immunoblot with a tomato-allergic subjects' serum pool (n=10) was carried out. The serum pool showed no reaction to the PepMVCP from tomato leaves (figure 4.44) and additional tests with single sera also obtained only negative results (data not shown). Accessory immunoblots with PepMVCP from tomato leaves adjusted in its natural surroundings of tomato fruit could also not confirm the reactions to PepMVCP in the 2D immunoblots (arrows in figure 4.14, data not shown). In conclusion, these subjects might not react to PepMVCP and former putative reactions in 2D immunoblots might result from a reaction to a different protein, possibly hiding under the dominant PepMVCP.

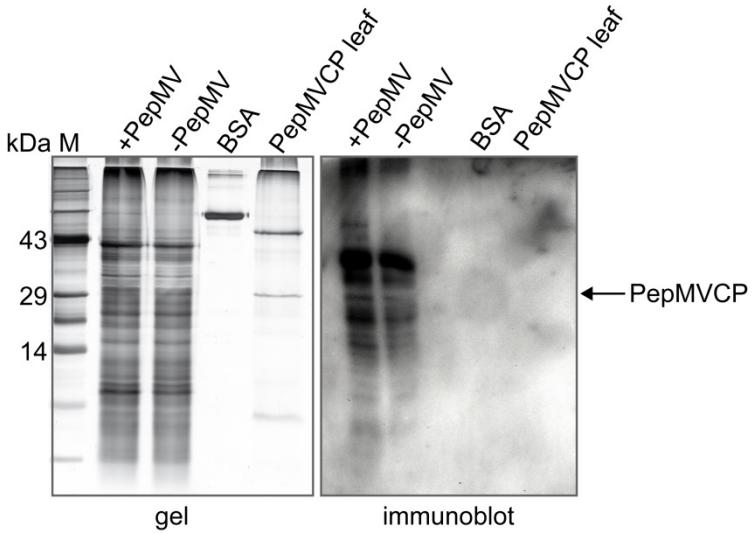

Figure 4.44: Silver stained protein gel and corresponding immunoblot of *Pepino mosaic* coat protein. Protein extracts of PepMV-infected (+PepMV) and non-infected control (-PepMV) tomato fruits (10 µg), BSA (1 µg) and PepMVCP purified from leaves (10 µg) are shown. Immunoblots were developed with (+IgE) and without (-IgE) a serum pool of ten tomato-allergic subjects (dilution 1:10). A peroxidase-conjugated anti-human IgE antibody (dilution: 1:5,000) was used for detection (exposure to film: 10 min). M: protein weight marker.

5. DISCUSSION

5.1 Several factors influence the allergenic potential of fruits and vegetables

Allergic reactions to fruits and vegetables are an increasing problem among modern societies (Gao *et al.*, 2012). Genetically modified plants with reduced allergenic potential have been developed (Le *et al.*, 2006;Lorenz *et al.*, 2006;Peters *et al.*, 2011), but food produced from such plants is not yet accepted by the European population. Therefore, other strategies to develop and to define hypoallergenic food have been investigated. Such strategies are based on the assumption that particular genetic and environmental factors influence the allergenic potential of fruits and vegetables.

Plant food allergens seem to be unequally distributed among different genotypes resulting in variations of their allergenic potential. A few studies reported for example alterations in the allergenicity of different apple cultivars and identified low allergenic cultivars, like 'Santana' and 'Elise', consumable for people with mild apple allergy (Bolhaar *et al.*, 2005;Ricci *et al.*, 2010;Vlieg-Boerstra *et al.*, 2011;Pasquariello *et al.*, 2012). Different allergic reactions towards certain cultivars or fluctuations in major allergen levels have also been shown for kiwi (Le *et al.*, 2011;Maddumage *et al.*, 2013). Cultivars of hazelnut (Wigotzki *et al.*, 2000), soybean (Dae-Yeul and Ye-Jin, 2010), as well as bell pepper (Jensen-Jarolim *et al.*, 1998) and tomato (Dölle *et al.*, 2011a;Lopez-Matas *et al.*, 2011a;Bencivenni *et al.*, 2012) have additionally been shown to provoke distinct allergic reactions. However, it has to be considered that these studies used various methods and inclusion criteria for the evaluation of allergenicity. Ripening status, storage duration, and cultivation conditions of the investigated fruits and vegetables probably also influenced their allergenicity. The developmental status of a tomato fruit, implicating changes in protein pattern, can influence its allergenic potential and weaker skin prick tests reactions were observed with green in contrast to red tomato fruits (Dölle *et al.*, 2011b). The major apple allergen Mal d 1 increased after storage and under certain environmental cultivation conditions (Matthes and Schmitz-Eiberger, 2009;Kiewning *et al.*, 2012). In contrast, different cultivation conditions of tomato plants, as far as it has been investigated, did not impact the allergenic potential of their fruits (Dölle *et al.*, 2011b).

Biotic factors, like symbionts or pathogens, probably affecting the allergenic potential, are less well studied. Pathogenic attacks or bio-protective supplements activate the defence system of plants and induce the expression of defence- and pathogenesis-related (PR) proteins (van Loon *et al.*, 2006). PR-proteins are well-known candidates for allergens and many plant food allergens belong to PR-protein families (Hoffmann-Sommergruber, 2000;Ebner *et al.*, 2001;Midoro-Horiuti *et al.*, 2001;Hoffmann-Sommergruber, 2002;Breiteneder and Radauer, 2005). The impact of biotic factors on the allergenic potential of healthy vegetables, like e.g. the worldwide highly consumed tomato, therefore, is a major concern.

5.2 Symbiotic mycorrhizal fungi influenced allergen-encoding gene expression but not the allergenic potential of tomato fruits

modified from Schwarz et al. 2010, Mycorrhiza

Arbuscular mycorrhizal (AM) fungi are widely distributed in natural and agro-ecosystems (Smith and Read, 2008) and used as biological agents to improve plant nutrition and plant health (Jeffries *et al.*, 2003). In the well-investigated symbiotic system tomato with *Funneliformis mosseae* (= *Glomus mosseae*), the protection against root and shoot pathogens has already been shown (Cordier *et al.*, 1998;Pozo *et al.*, 2002;Taylor and Harrier, 2003;Fritz *et al.*, 2006). Pozo and colleagues showed that pathogen damage after mycorrhization was not only reduced in mycorrhized parts but also in non-mycorrhized parts of inoculated root systems. They postulated that this acquired resistance resulted from a combination of locally and systemically induced defence mechanisms (Pozo *et al.*, 2002). Taylor and Harrier showed local and systemic changes in gene expression in tomato roots and leaves after mycorrhiza inoculation (Taylor and Harrier, 2003). Defence mechanisms are less well studied in tomato fruits and PR-protein-encoding gene expression in fruits after mycorrhization is shown in this study for the first time. Eight known and putative tomato allergens, including PR-proteins, were investigated and RNA accumulation of seven allergens was significantly enhanced in fruits of mycorrhiza-inoculated tomato plants independent of the genotype. Genes for PR-proteins, e.g. basic 1,3-β-glucanase and a chitinase, have already shown to be up-regulated in roots of the investigated genotypes '76R' (wild type) and 'RMC' (mutant not colonised by AM fungi) upon mycorrhization (Gao *et al.*, 2004). The genes for both enzymes were up-regulated also in tomato fruits of the current study (figure 4.1). RNA accumulation of Sola l 3, a lipid transfer protein and confirmed tomato allergen, was enhanced in tomato fruits and has already shown to be induced in rice roots after mycorrhization (Blilou *et al.*, 2000). Induction of PR-proteins might be part of the induced resistance after mycorrhization, based on changes in phytohormone levels (Blilou *et al.*, 1999;Pozo and Azcon-Aguilar, 2007). Recently, Salvioli and colleagues showed an influence on the transcriptome as well as on the amino acid composition in fruits of mycorrhizal tomato plants (Salvioli *et al.*, 2012). However, changes on transcript levels were weak and they could not observe an induction of major PR-protein-encoding genes. The post-harvest separation of fruit tissue could have already induced some PR-genes, possibly hiding mycorrhiza-induced resistance. Own experiences emphasise these suggestions and are discussed in chapter 5.3.1.

Likewise, the mycorrhiza mutant 'RMC' showed enhanced PR-protein-encoding gene expression after mycorrhiza inoculation. Nevertheless, this phenomenon can hardly be explained by systemic induced resistance because it needs a fully developed symbiosis (Slezack *et al.*, 2000). 'RMC' mutants indeed block the invasion of *Funneliformis mosseae* to the root cortex, but the AM fungus is able to form appressoria and penetrate epidermal and hypodermal cells. PR-gene activation in a mycorrhized 'RMC' mutant has already been shown by Gao and colleagues and it seemed that the presence and epidermal penetration of mycorrhiza is sufficient to trigger a defence response (Gao *et al.*, 2004). Additionally, it is reported that defence mechanisms are often only induced in early stages of mycorrhiza colonisation, the status where 'RMC' mutants generally remain (Harrison and Dixon, 1994;Garcia-Garrido and Ocampo, 2002). Upon inoculation with AM fungi, the induction of defence-related genes in roots was stronger and the expression lasted longer on a high level in the mutant 'RMC' than in the wild type '76R' (Gao et al. 2004). For some of the genes, a slightly higher induction in the mutant than in the wild type was also observed in fruits, but the difference was not significant (figure 4.1).

The increase of allergen relevant gene expression in mycorrhized tomato fruits did not lead to stronger reactions in clinical allergy tests (figure 4.2). This on the one hand might be due to the high inter-individual differences in skin prick test reactions, ranging from wheal diameters of 0 to 11 mm, and on the other hand due to the appropriate small subject cohort of ten tomato-allergic subjects. Immunoblots with individual subjects' sera were carried out to examine the high individuality in allergic reactions and are discussed in chapter 5.4. The individual variation was even higher towards tomatoes from mycorrhizal plants and slightly higher reactions to 'RMC' could only be observed in fruits from non-mycorrhizal plants. As mentioned in chapter 5.1, many studies claim a difference in the allergenic potential between various cultivars of fruits and vegetables. This was also shown for the two tomato cultivars 'Reisetomate' and 'Matina' (Dölle *et al.*, 2011a). However, the investigated genotypes '76R' and 'RMC' are nearly isogenic (Barker *et al.*, 1998) and RNA accumulation of relevant allergens in tomato fruits significantly differed only between mycorrhizal and non-mycorrhizal plants and not between the two genotypes. Another reason for increased allergen-encoding gene expression, not affecting the clinical response of tomato-allergic subjects, might be that increased gene expression must not necessarily lead to higher protein expression of the respective proteins. Additionally, post-translational control, like glycosylation or phosphorylation, influences the activity and life span of proteins and might be another reason for the absence of differences in skin prick tests.

In comparison to other studies using the same genotypes, e.g. (Gao *et al.*, 2001), the mycorrhization rate of the wild type '76R' was low, but anyhow a systemic reaction could be observed at least on RNA accumulation level. The low mycorrhization rate was probably due to high phosphate fertilisation used for the cultivation under practice conditions (Gerdeman, 1968;Gianinazzi-Pearson and Diem, 1982). However, higher mycorrhization rates do not necessarily have a higher impact on the defence-related transcriptome (Liu *et al.*, 2007). For further investigations on the impact of the plant defence response on the allergenic potential a strong pathogen was used.

5.3 Impact of pathogenic *Pepino mosaic virus* on tomato plants with main focus on the allergenic potential

modified from Welter et al. 2013, PLOS One

The *Pepino mosaic virus* (PepMV) is one of the major diseases of greenhouse tomato crops worldwide and tomato fruits commercially available in supermarkets are known to be naturally or intentionally infected with PepMV (Hanssen *et al.*, 2010a;Schwarz *et al.*, 2010a). Considering this information, there was an urgent need to analyse fruits from PepMV-infected tomato plants for their allergen expression and to investigate their allergenic potential during the harvest period.

At first, an assessment study was carried out to evaluate the necessity for a clinical study, to learn handling of the strong and easy transferable virus, and to establish a method for RNA accumulation analyses of allergens in tomato fruits. The focus of the second and main PepMV study was projected to the evaluation of the activated defence response of a PepMV-infected tomato plant and its impact on the allergenic potential of fruits through potential higher allergen expression. Besides this, particular aspects of this study were the analyses of major greenhouse grown tomato plants weeks after inoculation with the virus. Cultivation of plants under conditions similar to commercial cultivation in horticulture and the analyses of tomato allergy relevant fruits highlight the differences

to former studies, which were carried out under experimental conditions and mainly focussed on early infection stages in vegetative organs.

5.3.1 Allergen-encoding gene expression differed among fruit tissues and ripening stages

RNA accumulation analyses were conducted with different tomato fruit tissues of the cultivar 'Matina' showing varying expression pattern of putative tomato allergen-encoding genes related to their function (table 4.5). PepMV-infected and non-infected control fruits revealed no difference in gene expression (data not shown). Unfortunately, and even when tissues were frozen in liquid nitrogen directly after separation, this separation process probably had already induced wound reactions of the plant, including the activation of some PR-proteins. A previous study reported induction of the PR-genes *PR-1*, *PR-2,* and *PR-5* already 15 min after wounding (Reymond *et al.*, 2000); the approximate time which was needed for the separation. This wound reaction probably led to the induction of PR-genes also in non-infected control plants and blurred the effect of the PepMV infection.

Nevertheless, the proportional distribution in the different fruit tissues could be assessed. *Sola l 3* for example was mainly expressed in peel and seeds and less in pulp, confirming results from previous studies, detecting *Sola l 3* particularly in the peel of tomato fruits (Fernandez-Rivas and Cuevas, 1999;Lorenz *et al.*, 2006). Other defence-related allergens, e.g. NP24, were also accumulating in the peel, possibly because it is the first contact organ for fruit pathogens. Together with the seed storage protein vicilin, the mannosidase showed a high RNA accumulation in seeds. Contrary, the transcripts of tomato allergens related to certain ripening processes mainly appeared in fruit peel and pulp. Sola l 2, a β-fructofuranosidase, plays an important role in the glucose metabolism, and therefore, was mainly expressed in pulp and peel and less in seeds or the florescence (Elliott *et al.*, 1993;Kondo *et al.*, 2001). Kondo and colleagues showed higher protein expression of Sola l 2 in red-ripe tomato fruits in contrast to green ones (Kondo *et al.*, 2001). However, analyses of different ripening stages revealed no significant differences in *Sola l 2* gene expression of green and red-ripe tomato fruits (figure 4.7). Gene expression is not generally correlated with protein expression and higher protein concentrations can also be based on less degradation of respective proteins (Abreu *et al.*, 2009;Vogel and Marcotte, 2013). This circumstance is discussed in more detail in chapter 5.3.6.

The developmental analyses were actually intended to balance high individual differences between the biological tomato replicates, possibly resulting from external invisible but differing ripening stages. However, the two developmentally regulated genes, expansin (Anjanasree and Bansal, 2003;Payasi *et al.*, 2009) and β-fructofuranosidase (Yelle *et al.*, 1991;Westphal *et al.*, 2003) showed no different RNA accumulation according to the fruit maturation and individual differences were similarly high in the single ripening stages (figure 4.7). Therefore, different developmental stages cannot explain the high individual differences and it is not possible to relativize them with expansin. Another explanation for the high individual differences among the biological replicates might be the commercial cultivation of plants, causing more diverse individuals in contrast to those grown under strictly controlled conditions.

5.3.2 Allergen-encoding gene expression after *Pepino mosaic virus* infection revealed no difference between 'Reisetomate' and 'Matina'

The two tomato cultivars 'Matina' and 'Reisetomate' were inoculated with two different PepMV isolates to identify the ideal combination with the highest impact on plant defence. Unfortunately, the trial got additionally infected with a pathogen from the genus *Pythium* (Oomycota). Thereby, initially non-infected control plants were infected with this pathogen and probably also activated their defence. Except for the CO_2-fixing photosynthetic enzyme ribulose-1,5-bisphosphat-carboxylase-oxygenase (rubisco), no significant alteration in gene expression was observed in tomato fruits after PepMV infection (figure 4.6). Repression of photosynthesis and down regulation of rubisco has already been shown in virus-infected plants, and also in tomatoes infected with PepMV (Hull, 2002;Dardick, 2007;Hanssen *et al.*, 2011). Furthermore, one of the most common effects of mosaic-causing viruses in plants is the reduction of the most abundant plant protein rubisco (Hull, 2002). In contrast, *Phytium* infection was shown to have no impact on photosynthesis (Sutton *et al.*, 2006;Panova *et al.*, 2012). It seemed to be that rubisco is only affected by PepMV, in contrast to the PR-proteins, activated after viral and fungal pathogen attack.

In this assessment study quantitative real-time PCR for relevant allergen-encoding genes could be established and RNA accumulation results were shown to be suitable to common literature data. Adequate reference genes were identified based on literature and experimental data. Mascia and colleagues recommended glyceraldehyde 3-phosphate dehydrogenase and ubiquitin but advised against *18S rRNA* as reference genes for virus-infected leaf and root material (Mascia *et al.*, 2010). Cytoplasmic ribosomal RNA synthesis indeed is known to be inhibited after virus infection under certain conditions, but viruses generally have more impact on chloroplastic ribosomes (Hull, 2002). Additionally, this is mostly referred to data gained from leaf material and *18S rRNA* was proved to be an acceptable reference gene for virus-infected tomato fruits.

This assessment study was conducted to evaluate tomato cultivars and PepMV isolates for further investigations. Recommendations based on PR-protein expression analyses were negligible because of the *Phytium* infection and decisions were made based on practicality of cultivation (chapter 4.2, summary and connection): 'Matina' was used and inoculated with the aggressive PepMV isolate CH2.

5.3.3 Distribution and symptomology of *Pepino mosaic virus* varied in different plant organs and at different time points after inoculation

PepMV is systemically distributed in the whole infected plant (Schwarz *et al.*, 2010a). Systemic movement and long-distance transport of plant viruses is mainly organised through the phloem with the flow of metabolites from source to sink tissues (Hull, 2009). PepMV accumulation was higher in fruits in comparison to leaves (figure 4.8), probably because fruits are major sink organs (Hanssen *et al.*, 2011).

Viral abundance in fruits was higher at 3 than at 10 weeks after inoculation (WPI). Fluctuating virus titres have also been described for other viral diseases and plants are known to go through cycles of recovery and reinfection (Hull, 2002;Hull, 2009). Interestingly, higher viral loads at 3 WPI were accompanied with the occurrence of PepMV symptoms on fruits, disappearing at 10 WPI (figure 4.9). Hanssen and Thomma also reported a common recovery from PepMV symptoms after an initial infection (Hanssen and Thomma, 2010), and Hanssen and colleagues suggested a relationship with the observed transient transcriptomic response (Hanssen *et al.*, 2011). This correlates with the

observation of other viral diseases where symptoms are often transient and plants can recover from the first infection shock. At later stages symptoms can reappear (Dardick, 2007), probably accompanied by fluctuating defence responses, including differential expression of PR-proteins.
In the precedent assessment study PepMV symptoms were only visible on leaves. The reason for that might be the early inoculation with PepMV of young tomato plants. Late infection with PepMV is thought to be more harmful for the plants and especially threatens the fruit yield (Spence *et al.*, 2006). Therefore, the second main trial was infected after the first fruit development to achieve the maximum effect of PepMV on the fruits.

5.3.4 Allergen-encoding gene expression of tomato leaves and fruits at different time points was not generally affected by *Pepino mosaic virus*

Since 1970, when the first defence-related proteins have been discovered in tobacco after *Tobacco mosaic virus* infection (Gianinazzi *et al.*, 1970;van Loon and van Kammen, 1970), it is known from many studies that PR-proteins are activated after viral infections (e.g. (Bol *et al.*, 1990;Elvira *et al.*, 2008)). However, these studies mainly refer to investigations on vegetative plant tissue and it is known that PR-proteins are expressed in all plant organs (van Loon and van Strien, 1999). A few studies investigated PR-protein expression in respect to tomato allergy (Diaz-Perales *et al.*, 1999;Foetisch *et al.*, 2001;Kondo *et al.*, 2001;Weangsripanaval *et al.*, 2003;Palomares *et al.*, 2005) but investigation on tomato fruits after a pathogen attack are rare and mostly refer to post-harvest pathogens and treatments (Charles *et al.*, 2009;Jiang *et al.*, 2009;Zhu and Tian, 2012;Tzortzakis *et al.*, 2013). Among the known tomato allergens a few prominent defence- and PR-proteins exist (table 4.1). Therefore, the investigations on PR-protein and allergen-encoding gene expression of tomato fruits are not only interesting for allergy research but also give new insights in plant-pathogen interactions and plant-virus research.
One of our hypotheses was that the expression of genes, encoding defence- and PR-proteins, which interact with the IgEs of tomato-allergic subjects, is influenced by the spread of PepMV in the plants. In tomato leaves most investigated defence-related genes showed higher RNA accumulation at 10 WPI, even if induction levels were weak (figure 4.12). Most studies, reporting differences in PR-gene accumulation after virus attack, referred to hours or a few days after infection (van Loon and van Strien, 1999;van Loon *et al.*, 2006;Fakhro *et al.*, 2011;Naqvi *et al.*, 2011). This has also been described for young PepMV-infected tomato plants cultivated under controlled conditions in the climate chamber, showing induced expression of defence-related genes in leaves four days after inoculation. Thereafter, the number of regulated genes continuously declined up to twelve days after inoculation (Hanssen *et al.*, 2011). It appears that after an initial strong response of the plant to PepMV infection, adaptation to the permanent presence of the virus leads to constitutive low expression of numerous defence-related genes. This constitutive low-level expression could be part of systemic-acquired resistance of plants, preventing further infections by biotrophic pathogens (Hammerschmidt, 2009).
On the contrary, no general up-regulation of PR-genes and allergen-encoding genes was detected in tomato fruits of the same PepMV-infected plants at 10 WPI (figure 4.10). PR-proteins are particular abundant in leaves (Edreva, 2005) and organ-specific defence-related gene expression has been shown before, e.g. in tomato leaves in comparison to roots after pathogenic infection (Aime *et al.*, 2008). Based on an EST approach citrus showed organ-specific expression of genes coding for

members of the PR-protein family in leaf, fruit, root, stem, flower, and seed (Campos *et al.*, 2007); also in *Brassica rapa* defence-related chitinase-encoding genes were differentially expressed in roots, stems, leaves, and flower buds (Ahmed *et al.*, 2012).

Differences in RNA accumulation of the investigated allergen-encoding genes in fruits were also observed between the time points 3 and 10 WPI (figure 4.10). This might be explained by a fluctuation in the plant defence response to an unsteady virus quantity over time, as it is discussed above (chapter 5.3.3). Furthermore, plant pathogen interaction or the expression of PR-proteins in general might be also influenced by plant age and surrounding environmental conditions (Edreva, 2005;Zeier, 2005;De-la-Pena *et al.*, 2010;Quintero and Bowers, 2011;Cheng *et al.*, 2012). De-la-Pena and colleagues for example showed enhanced expression of defence-related proteins in *Arabidopsis thaliana* during flowering (De-la-Pena *et al.*, 2010). This indicates dependency of the plant defence system on certain developmental stages, which can be also observed in PepMV-infected tomato plants. Cheng and colleagues reported about the post-harvest sensibility of tomato fruits to temperature stress and showed in a microarray study the induction of more than 100 genes after heat exposure (Cheng *et al.*, 2012). Tomato fruits investigated in the current study were cultivated in a greenhouse during summer and were temporarily exposed to high temperatures. This might be another reason for the fluctuating PR-gene expression.

The differences in PR-protein and allergen-encoding gene expression between leaves and fruits, as well as between young and major plants, have already been shown for different apple cultivars infected with *Erwinia amylovora* (Mayer *et al.*, 2011). The authors showed an up-regulation of major apple allergen *Mal d 1.01* transcripts, encoding a PR-10, in leaves from seedlings after *E. amylovora* infection. In contrast, increased PR-10 protein-encoding gene expression could not be detected in apple fruits of infected major trees.

In summary, the lack or low induction of PR-protein-encoding genes after PepMV infection could be based on unstable greenhouse conditions (e.g. temperature), the investigation of relatively old plants, and the massive colonisation with herbivorous white flies (Puthoff *et al.*, 2010;Yang *et al.*, 2011), which is difficult to prevent in greenhouse tomato cultivation. All these factors are possible activators of plant defence even in non-infected control plants and might have blurred the effects of PepMV infection. It seemed to be that the above discussed *Phytium* infection (chapter 5.3.2) or the wounding response described for the assessment study (chapter 5.3.1) probably were not the only reasons for the low or absent increase of defence-related transcripts after PepMV infection. Together, these findings must be considered in further investigations, regarding defence responses of plants cultivated near to practice conditions and additionally regarding the impact of additional pathogen infection on the allergenic potential.

5.3.5 Storage of tomato fruits did not alter allergen-encoding gene expression and the allergenic potential

Since tomato fruits are commonly stored after harvest for a few days during transport, in the supermarket, or by the consumer himself, it might be of further interest if this circumstance affects the allergenic potential of those fruits. In apple the increase of the major apple allergen Mal d 1 after storage has recently been shown (Matthes and Schmitz-Eiberger, 2009;Kiewning *et al.*, 2012). However, in stored tomato fruits significant up-regulation of allergen-encoding genes could only be observed for *Sola l 3* (figure 4.11). The other investigated genes rather seemed to be down-regulated

at least in non-infected tomato fruits after storage. *Sola l 4*, the *Bet v 1,* and therefore, also a *Mal d 1* homologue, was not regulated at all after storage neither in PepMV-infected nor in non-infected control tomato fruits. Besides dealing with two completely different species, apple was stored for months, in contrast to tomato, which was and can only be stored for a short time period.

Interestingly, during post-harvest storage PepMV-infected tomato fruits seemed to stay longer in good shape than respective controls. One explanation might be the increased fruit resistance to e.g. post-harvest pathogens through PepMV-activated plant defence mechanisms. This has already been discussed for mycorrhiza-induced resistance (chapter 5.2) and for post-harvest-applied biocontrol yeast, protecting tomato fruits from other pathogens through the induction of defence-related proteins (Jiang *et al.*, 2009). Even if allergen-encoding genes, including PR-genes, are not significantly up-regulated after storage (figure 4.11), there is at least a trend towards an induced expression in stored PepMV-infected tomato fruits compared to non-infected controls (figure 4.12). However, basophil activation tests conducted with fresh and stored fruits revealed no significant differences (figure 4.17) and the storage of tomato fruits is probably irrelevant for its allergenicity.

5.3.6 Allergen expression differed between time points after *Pepino mosaic virus* infection - a comparison between different quantification methods

Protein expression was determined based on spot intensities on 2D gels and iTRAQ measurements. Relative protein concentrations were compared to respective RNA accumulation analyses of the same samples. Except for the PepMVCP quantification (figure 4.19), the two protein determination methods did not reveal comparable results and showed no correlation to the RNA accumulation data (figure 4.20). The iTRAQ measurements resulted in the quantification of three potentially relevant allergens; among those, two major tomato allergens (Sola l 2 and PG) showed an increased expression in PepMV-infected tomato fruits. Unfortunately, a repetition of these measurements in order to achieve a higher recovery rate failed. Further comparisons with the RNA accumulation data were conducted with the 2D gel-based protein quantification. However, prediction of respective protein abundance was not possible with the RNA accumulation of certain allergens.

The completely sequenced genome of tomato, allows gene expression analysis as a relatively straightforward and cost-effective tool. It is, however, known that transcription levels do not always reflect the protein content and that RNA levels cannot be consistently used to predict protein abundance (Nie *et al.*, 2007;Tan *et al.*, 2009). The lack of correlation might be on the one hand caused by inaccurate measuring techniques. On the other hand, the relationship between protein and mRNA concentration also depends on many other complex regulatory processes, like e.g. mRNA stability, translational regulation, and protein degradation (Abreu *et al.*, 2009;Vogel and Marcotte, 2013).

In summary, there was no general induction of defence-related allergens observed after PepMV infection neither on RNA nor on protein level. Immunoblots and basophil activation tests conducted with the same samples from the three time points after PepMV infection confirmed the results and did not show any significant difference, even if dose response curves tend to differ depending on time (figures 4.21, 4.22, and 4.23). This time dependence was also observed in RNA and protein abundance and has already been discussed in chapter 5.3.4. However, induction and repression levels of respective allergens are weak and gained from only one sample per time point.

It remains questionable if quantifying allergens on RNA accumulation level is the right tool to evaluate the allergenic potential from plants grown under commercial conditions. In any case RNA accumulation analyses with qRT-PCR is a well-established, easy, and fast method to get an idea about the regulation of genes after certain treatments of plants. Further conclusions about protein abundance have anyway to be treated with care. In allergy research, however, not even a quantification on protein level can finally replace clinical allergy tests due to the individual reactions to certain allergens (chapter 5.4, (Asero, 2013)).

5.3.7 Immunoblots with *Pepino mosaic virus*-infected tomato fruits and sera from tomato-allergic subjects revealed differences in the reaction to putative tomato allergens

As indicated in a dot blot (figure 4.13) tomato-allergic subjects' sera differently reacted to PepMV-infected tomato fruit protein extracts compared to non-infected controls in 2D immunoblots. All putative tomato allergens identified by immunoblots of PepMV-infected tomato fruits with sera from tomato-allergic subjects belonged to proteins involved in stress or defence responses of the plant (figure 4.14, table 4.9). Besides two already known tomato allergens: chitinase (Diaz-Perales *et al.*, 1999) and anionic peroxidase (Weangsripanaval *et al.*, 2003), and three others (polygalacturonase inhibitor protein, abscisic stress-ripening protein, and superoxide dismutase) were identified and are described in more detail in chapter 5.5, as new putative tomato allergens. This might be explained by an induced plant defence and a higher expression of these proteins in infected fruits and at least for the peroxidase a higher abundance could be observed (figure 4.20).

Conversely, the subjects' sera reacted with other PR-proteins (glucanase, NP24, and PR23) on the corresponding immunoblots of non-infected tomato fruits. This might be explained by constitutive expression of some PR-proteins, especially in fruits that are more likely to be attacked by insects or fungi (Ebner *et al.*, 2001), and has already been discussed in chapter 5.3.4. Unfortunately, it was not possible to quantify these proteins on 2D gels. Moreover, the alkaline proteins glucanase, vicilin, NP24, and PR23 are not even visible on protein gels of PepMV-infected tomato fruits (figure 4.14). Despite performing the immunoblots and corresponding gels in three replicates, technical problems, for example partial damage of the porous basic ends of the first dimension gels, cannot be completely excluded. Therefore, these alkaline proteins can be described as IgE-reactive proteins, but should be eliminated from comparison of reactions to PepMV-infected versus non-infected control fruit proteins.

Sola l 1, Sola l 2, and polygalacturonase, three of the major tomato allergens (Dölle *et al.*, 2011a), evoked comparable reactions with the subjects' serum pool on immunoblots with PepMV-infected and non-infected tomato fruits, even if they appeared differentially expressed on 2D protein gels (figure 4.20). Sola l 3, another confirmed tomato allergen, could not be detected in the current immunoblot analyses. Sola l 3, a lipid transfer protein, is a well-known protein, involved in plant defence responses (van Loon and van Strien, 1999) and RNA accumulation was significantly enhanced at 3 and 10 WPI in virus-infected fruits (figure 4.10). However, this protein has only shown to be an important allergen for particular allergic subjects, prevalently living in the Mediterranean area (Pastorello and Robino, 2004). Allergic subjects, recruited from Berlin and surroundings might not be sensitised to lipid transfer proteins and Sola l 3 was never detected in one of the former studies including these subjects.

5.3.8 *Pepino mosaic virus* infection of tomato did not impact the allergenic potential of fruits

Contrary to our hypothesis, standardised clinical allergy tests (double blind placebo controlled food challenges, skin prick tests, and basophil tests) on tomato-allergic subjects revealed no differences in reaction to PepMV-infected in contrast to non-infected fruits, reflecting the results of the molecular analyses. One reason might be the fluctuation derived from the practice conditions under which the plants were cultivated and the fruits, freshly harvested for every single subject, were produced. RNA accumulation of relevant tomato allergens seemed to be dependent on time (figure 4.10 and 4.20) and basophil dose response curves also tend to differ between 4 and 12 WPI (figure 4.22). Therefore, skin prick tests were not only carried out with fresh tomato material during the whole experiment (3 - 13 WPI) but also with frozen material from one single time point (3 WPI, figure 4.15).This did not alter highly variable results and individual subjects' reactions, indicating not only fruits as the reason for high variability, but also individual reactions to the same material. This is further discussed in chapter 5.4.

Independent of these considerations, basophil tests with tomato fruits harvested at different time points after PepMV infection failed to differ significantly. Different protein expression pattern of PepMV-infected tomato fruits might impact the reaction of single individuals, depending on respective sensitisation to certain allergens but obviously not a whole subject cohort, in which individual reaction might be perished. Skin prick and basophil tests might properly predict the likelihood of an allergic reaction, but not the severity of this reaction which can only be assessed by oral challenge tests (DBPCFC). DBPCFC, as the 'golden standard' allergy test, also showed highly variable individual reactions, especially in reaction to PepMV-infected tomato fruits (figure 4.16). In general reactions were weak, often below the defined limit, and therefore, are not further considered and discussed. It is known that tomato allergy rarely provokes severe allergic reactions (Larramendi *et al.*, 2008) and frequently occurring symptoms are often subjective, and therefore, can hardly be evaluated.

Concluding remarks on the impact of biotic factors on tomato and the allergenic potential of their fruits

One particularity of this study was the investigation of tomato fruits after symbiont colonisation or pathogen infection, since the general plant-microorganism interaction research on molecular level is mainly focused on leaves or roots. Another novelty was the investigation of tomato plants which were grown near to commercial cultivation conditions in the greenhouse. Experiments concerning plant-microbe interactions and the analysis of gene regulation and protein expression are usually conducted on young plants, cultivated under controlled conditions in climate chambers, where less other factors influence the plant defence system. These controlled conditions are perfect to study underlying mechanisms and to understand the whole defence system in basic research. However, for allergy research it was necessary to stick to the commercially applied cultivation conditions as close as possible, to assess the real impact on tomato fruits sold for consumption and to estimate the risk for tomato-allergic people. In this study it was shown that results gained from 'laboratory-like' plant-pathogen systems cannot be easily transferred to 'field-like' investigations. The expression of PR-proteins and allergens depend on plant age and organ, and on the surrounding environmental conditions; altogether modulating the complex regulation network of the plant's defence system. Especially, unpredictable changing environmental factors play a decisive role in commercial

cultivation systems for vegetable production and probably blur the effects of intentionally inoculated symbionts or pathogens, observed in basic research experimental approaches.

In summary, the hypothesised impact of a single biotic factor on the allergenic potential seems to be negligible and of minor risk for the allergic population. Overall, certain intentionally applied environmental conditions or biotic factors are too weak to cause visible and stable changes, first in protein pattern and second in the allergenicity of a commercially cultivated plant (figure 5.1). Therefore, future approaches for reducing the allergenic potential of such plants should concentrate on hypoallergenic genotypes, found by screening of existing cultivars, produced by classical breeding, or by genetic engineering.

The current study showed, moreover, a very high variation in the allergic reactions of the investigated tomato-allergic subjects. This high inter-individuality in sensitisation and the reaction to specific proteins has recently been summarised (Asero, 2013). Allergic reactions might, therefore, depend on individual sensitisation patterns and not only on the investigated tomato material itself. For this reason, it is recommended to choose the subject cohort size as large as possible to detect significant differences.

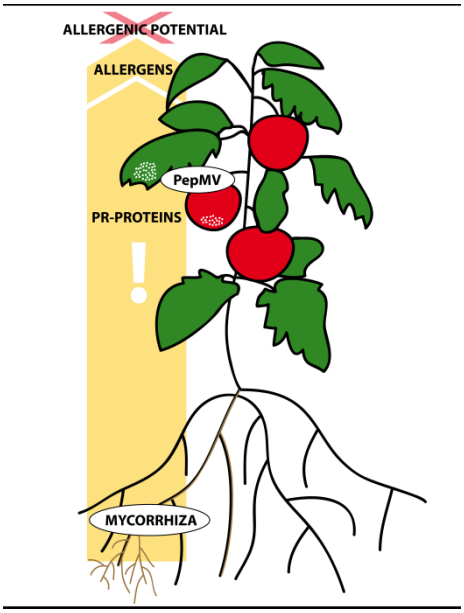

Figure 5.1: Illustration of the main statement of this study: Although plant defence response, including expression of PR-proteins, resembling putative allergens, can be activated by symbionts (AM fungal colonisation) and pathogens (*Pepino mosaic virus* (PepMV) infection), tomato fruits possess no higher allergenic potential.

5.4 Tomato-allergic subjects showed high inter-individual differences in their allergic reactions

modified from Welter et al. 2013, in revision of Clinical and Experimental Allergy

In clinical allergy tests tomato-allergic subjects showed highly variable reactions to tomato fruits from the same genotype or grown under the same conditions (e.g. wheal diameters of skin prick tests in figure 4.26). This led to the hypothesis that the IgEs of different subjects interacted with different tomato allergens and this subsequently resulted in the high variability of reactions in allergy tests. It is known that individual specific IgE reactivity can be highly diverse, although people show allergic reactions to the same food (Herian *et al.*, 1990). Therefore, previously achieved skin prick test data of the mycorrhiza study (figure 4.2) and of another recent study, dealing with the impact of different nitrogen fertilisation conditions of tomato plants on the allergenic potential (Dölle *et al.*, 2011b), were used to test this hypothesis. Skin prick test data were correlated with the amount of positive protein IgE interactions on immunoblots of single subjects. Although the IgEs of the subjects indeed reacted with different allergens among the tomato fruit extracts, no correlation could be detected between skin prick test reactions (wheal diameter) of nine tomato-allergic subjects towards fruits from different genotypes or produced under different conditions and the number of proteins interacting with the IgEs of these subjects (figure 4.26). This indicated that variations in skin prick test results among individual subjects cannot be explained by the amount of proteins they react to. Interestingly, single subjects showed reactions to different proteins in immunoblots but no reaction at all to the same tomato fruits in skin prick tests and vice versa. This might be explained by false-positive reactions in immunoblots caused by the necessity of only one allergen epitope for the reactions in immunoblots, and at least two for the cross-linking to specific IgE receptors, to provoke reactions in skin prick tests. On the contrary, the absence of any protein reaction in the immunoblot in combination with a positive skin prick test reaction might be explained by low abundant allergens probably undetectable with this immunoblot method. This is further discussed in chapters 5.8.2 and 5.8.3.

If it is not the quantity of proteins explaining high individuality in reaction, it might be the quality of proteins. Within the cohort of this study the serum of each subject reacted with eight out of ten identified putative tomato allergens in immunoblots. Therefore, candidate proteins, being responsible for the high individuality in clinical reactions, could not be determined. Interestingly, nearly every identified putative allergen of the non-mycorrhized mycorrhizal mutant 'RMC' provoked reactions with 100% of the subjects' sera. This is contrary to the corresponding non-mycorrhized wild type '76R', where only the pectinmethylesterase 1.9 showed reactions with all subjects' sera (figure 4.25). Since RNA accumulation of selected allergens showed no genotype-specific accumulation (figure 4.1), it might be speculated that this is due to different protein translation pattern or genotype-dependent modifications of the proteins, as it has been discussed in chapter 5.3.6. Anyway, it only resulted in slightly higher skin prick test reactions to the non-mycorrhized mutant 'RMC' which in summary over all subjects were not significant (figure 4.2).

The most prominent putative allergens in this study were the pectinmethylesterase 1.9 and the thaumatin-like protein NP24. Pectinmethylesterase 1.9 reacted with at least 88% of the subjects in all genotypes and growth conditions. Reactions to NP24 were indeed less frequent, but except for the genotype '76R' and the cultivar 'Counter' fertilised with an excess of NO_3, more than 80% of the subjects showed a reaction to this protein. Nitrogen is involved in protein biosynthesis, and therefore, the fertilisation of tomato plants with different forms of nitrogen, differentially available

for the plants, was thought to alter the protein content and composition. A recent study, concerning the allergenic potential of the fungus *Aspergillus fumigatus,* showed an increase of major allergen expression and the allergenicity with elevated CO_2 levels and higher nitrogen concentrations in the growth media (Lang-Yona *et al.*, 2013). Besides dealing with two completely different organisms, the fungus was grown under strictly controlled conditions in contrast to the tomato plants investigated here. Possibly, the effect of nitrogen fertilisation on tomato plants grown under commercial cultivation conditions is blurred by other influencing environmental parameters in the greenhouse, like it has already been discussed in chapter 5.3.4. This again raises the question, if 'laboratory-like' experiments are easily transmittable to field studies.

Recapitulating, this study showed that high inter-individual differences in allergic reactions cannot simply be explained by the quantity or quality of putative allergens reacting with the sera from the tomato-allergic subjects. Subjects showed individual IgE binding profiles and some proteins could be identified as major allergens at least for this cohort, but interestingly this seemed to be dependent on the tomato genotype and on the growth conditions. This high individuality in subjects' reactions to certain proteins demonstrates again the importance of identifying new putative allergens and their recombinant production for a more detailed allergy diagnosis and research.

5.5 Identification of known and new putative tomato allergens

modified from Welter et al. 2013, PLOS One and Welter et al. 2013, in revision of Clinical and Experimental Allergy

The identification of complete allergen profiles is important for allergy research and the production of hypoallergenic vegetables or fruits needs the knowledge about their major and minor allergens (Riascos *et al.*, 2010). In contrast to other vegetables, for tomato a number of allergens have already been described (chapter 1.5.4, www.allergome.org). In this study 13 additional putative tomato allergens could be identified based on immunoblot analyses. The identification of new tomato allergen candidates was a major concern of the immunoblot study and sera from tomato-allergic subjects were separately analysed on individual immunoblots to detect as many allergens as possible (chapter 4.4). Contrary, immunoblots of the PepMV study were carried out with a serum pool of ten tomato-allergic subjects (chapter 4.3.7). Nevertheless, the recovery rate of known and new tomato allergens on immunoblots was higher in the PepMV study. Probably this was due to the optimised POD-conjugated secondary antibody usage with chemilumescent detection, in contrast to the alkaline AP-conjugated secondary antibody with less sensitive colorimetric detection used before.

Tomato allergy is often caused by a cross-sensitisation through pollen allergens. The elicitors of such cross-reactivities are highly conserved pan-allergens widely present in all plant species. Two confirmed tomato allergens are well-known pan-allergens: Sola l 1, a profilin, and Sola l 4, a PR-10 protein and Bet v 1 homologue. However, these well-known tomato allergens were not detected on single immunoblots with the sera from the nine subjects of the immunoblot study. The absent reaction to Sola l 1 was probably a technical problem related to the detection limit of the AP-conjugated secondary antibody in 2D immunoblots. Sola l 1 was detected in nearly all previous 1D immunoblots of the VEGAL group (e.g. (Dölle *et al.*, 2011a) and with the serum pool in the PepMV study using the POD-conjugated secondary antibody. Sola l 4, as well as the lipid transfer protein Sola l 3, have never been detected in one of the previous immunoblots. The allergy to certain foods is clearly influenced by specific pollen exposure but also by dietary habits, differing in certain

geographical areas (Asero *et al.*, 2009). Reactions to Sola l 3 were reported to occur more often with IgEs of subjects from the Mediterranean area (Pastorello and Robino, 2004), as already discussed in chapter 5.3.7. Lipid transfer protein sensitisation is independent from pollen cross-reactivity and due to its heat- and proteolytic stability often correlated with severe systemic allergic reactions (Pastorello and Robino, 2004). The tomato-allergic subjects investigated here mostly showed oral allergy syndromes and mild allergic reactions (table 3.6), and therefore, are probably not sensitised to lipid transfer proteins. Sola l 4 has quite recently been detected as an allergen from tomato and is less well characterised (Ballmer-Weber and Hoffmann-Sommergruber, 2011). Therefore, it remains unknown if the absent reaction in these immunoblots resulted from absent sensitisations, if it was a problem of detection, or if this protein was less abundant in the investigated tomato fruit protein extracts. However, detection with the POD-conjugated secondary antibody was optimised for the PepMV study and Sola l 4 was, as a defence-related protein, probably not underrepresented in PepMV-infected tomato fruits. For these reasons it is most likely that absent reactions resulted from absent sensitisation of the current subject cohort to Sola l 4. Sola l 2, as a confirmed tomato allergen coding for a β-fructofuranosidase, was a major tomato allergen of the subject cohort and was identified in nearly all immunoblots. The absent reactions in immunoblots with the single subjects' sera were probably based on the above mentioned detection difficulties with the AP-conjugated secondary antibody. However, Sola l 2, as a glycoprotein, is discussed to be an allergen of tomato (Westphal *et al.*, 2003;Kaulfuerst-Soboll *et al.*, 2011). Indeed, it is known that some allergic subjects developed specific IgE antibodies against certain N-glycan structures, called cross-reactive carbohydrate determinants (CCD). Several studies stated that its clinical impact is of minor importance (Foetisch *et al.*, 2003;Altmann, 2007;Mari *et al.*, 2008). In contrast to this, another study postulated that such tomato glycoproteins, as Sola l 2, polygalacturonase, and pectinmethylesterase, are involved in clinical symptom development (Foetisch *et al.*, 2003). Considering this information, subjects of the current analyses were tested for CCD-specific antibodies. Subject number 5 responded negative for CCD-specific antibodies and at the same time reacted with all identified, putative allergens in the immunoblot analysis. Hence, there was no difference in IgE binding pattern which might be based on CCDs, as it has already been observed in a previous study of the VEGAL group (Dölle *et al.*, 2011a). Therein, four out of twelve tomato-allergic subjects had CCD-specific antibodies, but IgE binding patterns did not differ between CCD antibody positive and CCD antibody negative subjects. In table 3.6 it is shown that two out of ten subjects' sera, representing the serum pool used for allergen detection in the PepMV study, had CCD-specific antibodies. Therefore, in these immunoblots glycosylated, newly identified, putative tomato allergens might possibly be of minor clinical relevance. Anyhow, newly identified allergens on immunoblots with the total protein extracts should be regarded as candidates, which have to be individually investigated further, e.g. as recombinant allergens (chapter 5.7).

Pectinesterases and polygalacturonases are also known to have N-glycosylation sites (Foetisch *et al.*, 2003;Dölle *et al.*, 2011a). The polygalacturonase was always identified as one of the major tomato allergens in this and also previous studies (Dölle *et al.*, 2011a;Lopez-Matas *et al.*, 2011b). Different pectinesterases reacted with the IgEs of the subjects and are known allergens from various plants (Jimenez-Lopez *et al.*, 2012). Two of them, PME1.9 and PME2.1, have been described as putative tomato allergens before (Foetisch *et al.*, 2001;Kondo *et al.*, 2001). Additionally, a pectinesterase inhibitor U1 and a pectinacetylesterase were identified here as new putative tomato allergens. Pectinesterases have been first identified as allergens in tomato fruits but in the meantime they were

also detected among allergens from ash tree and grass (Niederberger *et al.*, 2002;Aina *et al.*, 2010). The IgE interaction to these proteins could therefore very well be based on the cross-reactivity to pollen (Lopez-Matas *et al.*, 2009). The detected mannosidases have not yet been described to be allergens of plants, but provoked reactions with the IgEs in the immunoblot and PepMV study. Allergenic extracts from mites contain a number of enzymatic activities including a mannosidase (Cardona *et al.*, 2006) and *Aspergillus fumigatus* also harbours an allergen which was identified as this enzyme (Singh *et al.*, 2010). It might therefore be that the subjects involved in the current analysis are not only sensitised due to contact with pollen, but also with house dust containing mite or mould proteins. The reaction to tomato mannosidase might be due to a cross-reaction between mites or moulds and tomato. However, in the immunoblot study investigating immunoblots of single tomato-allergic subjects only one individual was tested for specific IgE against *Aspergillus* and responded negative, even though reacting positive to mannosidase in the immunoblot. In contrast, eight out of nine subjects positive to mannosidase in the immunoblots also had specific IgE to birch (table 3.6). Therefore, a sensitisation through fungi is questionable and further investigations are necessary to confirm this suggestion. Mannosidases, as well as pectinesterases and polygalacturonases are functionally involved in the fruit ripening process (Bourgault and Bewley, 2002;Yamamoto *et al.*, 2005;Terefe *et al.*, 2009). Another putative new tomato allergen involved in ripening is the abscisic stress-ripening protein.

Recently, the seed storage proteins 11S globulin and vicilin have been described as putative tomato allergens (Bässler *et al.*, 2009) and proteins similar to those could be identified again with the IgEs of the tomato-allergic subjects. The glycolytic enzyme enolase was also detected to be a new putative allergen of tomato. Enolase is already known as a confirmed allergen from fungi and fish, but also from latex, and from corn- and grass pollen (www.allergen.org). Another enzyme involved in glycolysis, namely the fructose-1,6-bishosphate aldolase was newly identified with the subjects' serum pool and has never been described as an allergen. Further putative new candidates are the 1-aminocyclopropane-1-carboxylate oxidase homologue, involved in ethylene biosynthesis, and a signalling nucleoside diphosphate kinase. Besides those, an allergenic activity has never been observed for an aspartyl protease family protein (also known as nucleoid DNA-binding-like protein). The function of this protein in tomato is unknown, but the homologue in tobacco possesses proteolytic activity and might be involved in the degradation of denatured proteins under stress conditions (Murakami *et al.*, 2000).

Interestingly, numerous defence-related proteins were identified on immunoblots of the PepMV study: The thaumatin-like proteins NP24 and PR23 belong to the PR-protein family PR-5 and are known allergens in many fruits and vegetables (Sharma *et al.*, 2013). In tobacco these proteins inhibit hyphal growth and spore germination of a large number of economically important plant pathogens (Abad *et al.*, 1996). Chitinases are known allergens from tomato and also other fruits and vegetables and belong to the PR-protein families PR-3, PR-4, PR-8, and PR-11 (Diaz-Perales *et al.*, 1999;van Loon *et al.*, 2006). Chitinases are chitin-degrading enzymes and hydrolyse chitin polymers from e.g. cell walls of pathogenic fungi (Hahn, 1996;Ahmed *et al.*, 2012). The glucan-hydrolysing enzyme glucanase, a PR-2 family protein, is known to be induced after pathogen attacks (Bulcke *et al.*, 1989) and its allergenic activity has already been described (Fuentes-Silva and Rodriguez-Romero, 2006). Out of the two detected peroxidases the anionic peroxidase has already been shown to act as an allergen from tomato (Weangsripanaval *et al.*, 2003). Peroxidases are involved in the stress response of a plant by oxidising toxic reductans (Banci, 1997), by cross-linking cell walls (Perez-de-Luque *et al.*,

2006), and by generating reactive oxygen species (Daudi *et al.*, 2012). Another defence-related putative tomato allergen, newly identified here, is the polygalacturonase inhibitor protein, according to its name known to inhibit polygalacturonases of fungal plant pathogens**.**

The far most interesting new candidates are the superoxide dismutase, an enzyme of the anti-oxidative response, and different heat shock proteins, assisting protein folding as chaperones. The findings that heat shock proteins or superoxide dismutases could act as tomato allergens might be of particular interest in allergy research due to their wide distribution in nearly all studied organisms (Bowler *et al.*, 1994;Whitley *et al.*, 1999). Heat shock proteins are already known as confirmed allergens from fungi, mites, hazelnut pollen, and chick pea (www.allergen.org). A few Fe/Mn superoxide dismutases from fungi, latex, and pistachio have recently been identified as allergens (www.allergen.org). The first and only Cu/Zn superoxide dismutase has been confirmed as a pollen allergen from olive: Ole e 5 (Butteroni *et al.*, 2005). In this study a tomato Cu/Zn superoxide dismutase is described for the first time as a putative allergen from fruits or vegetables and should be taken as an indication for the occurrence as an allergen in other food stuff.

In summary, immunoblots are a useful tool for the detection of individual reactions to certain allergens and their identification is an essential basis for developing new diagnostic test systems with purified and recombinant allergens (Steckelbroeck *et al.*, 2008). Newly identified allergens need, however, verification by testing them individually as purified or recombinant allergens in immunoblots and clinical allergy tests.

5.6 Purified *Pepino mosaic virus* coat protein did not react with tomato-allergic subjects' sera

One obvious difference in the protein patterns between PepMV-infected tomato fruits and non-infected controls is the highly abundant PepMV coat protein (PepMVCP, figure 4.14). PepMVCP has been identified many times in database research after mass spectrometry measurements of IgE-interacting peptides, indicating its potential role as a new allergen. Further investigations were absolutely essential, because PepMV-infected tomato fruits are increasingly available in the supermarkets due to the legal cross-protection with avirulent PepMV isolates (chapter 1.3.3). Another potential risk for the allergic population might develop through genetically modified virus-resistant crops. Viral diseases threaten plant cultivation worldwide and resistant cultivars have already been developed. A popular method is the coat-protein mediated resistance described for many species (Abel *et al.*, 1986;Morroni *et al.*, 2008): The coat protein of a certain virus is constantly expressed in the respective virus threatened plant and therewith protects the plant from the viral disease. To some extent these genetically modified species are already available on the market, like for example the *Papaya ringspot virus* resistant papaya 'SunUp' (Gonsalves *et al.*, 1998;Gonsalves, 1998;Fermin *et al.*, 2011).

Immunoblots with purified PepMVCP were carried out but revealed no reaction with the serum pool of tomato-allergic subjects (figure 4.44). PepMVCP positive spots on immunoblots with the whole tomato protein extract of PepMV-infected fruits were probably only due to the high abundance of this protein in comparison to other tomato proteins. Viral coat proteins, e.g. from *Tobacco mosaic virus* have shown to represent half of total plant leaf protein (Hull, 2002). PepMVCP probably covered other underrepresented putative tomato allergen candidates reacting with the serum, and therefore, protein spots were identified as PepMVCP instead as another tomato protein. Recent

investigations on the allergenicity of e.g. the papaya 'SunUp' (Fermin *et al.*, 2011) or a C*ucumber mosaic virus* resistant tomato (Lin *et al.*, 2010) confirmed the current results and predicted no risk for a higher allergenic potential resulting from such genetic modifications. These investigations were based on bioinformatic comparisons with known allergen sequences and the proteolytic stability of respective coat proteins.

Viral coat proteins seem not to act as allergens. However, it has to be considered that the purification procedures might have destroyed potential conformational epitopes of PepMVCP and therewith its allergenicity. Furthermore, genetically modified papaya and tomato were not tested in clinical allergy tests and predictions based on database comparisons have to be routinely repeated due to the constant identification of new allergens.

5.7 Overexpression of *Pepino mosaic virus* coat protein, tomato heat shock protein, mannosidase and a thaumatin-like protein in *Escherichia coli* failed

As mentioned above the detection and verification of allergens is necessary for future allergy research, diagnosis, and therapy. With the knowledge about an allergen panel of certain fruits and vegetables it is possible to define low allergenic cultivars or to develop hypoallergenic crop plants with reduced allergenic potential, as it has been shown for profilin- (Le *et al.*, 2006) and lipid transfer protein- (Lorenz *et al.*, 2006) reduced tomato or the PR-10 silenced carrot (Peters *et al.*, 2011). Furthermore, the development and expansion of recombinant allergen-based immunoassays for allergy diagnosis needs the knowledge about new allergens (Shreffler, 2011). For the verification of an allergen, detected on an immunoblot with the total protein extract of a respective species, separation from the extract is necessary for its individual testing on allergic subjects. Therefore, putative allergens are purified from total protein extracts or recombinantly produced in an appropriate overexpression system.

In this study new putative tomato allergen candidates were identified and necessitated verification. Overexpression in *E. coli* is a common tool for producing recombinant allergens and in general is easy to establish. Four candidate putative allergens were selected for overexpression: the *Pepino mosaic virus* coat protein (PepMVCP), for further risk assessment like it is discussed in chapter 5.6, a heat shock protein (HSC70), because of its wide distribution and therefore major impact as an allergen, a mannosidase (MAN), because it is only known as an allergen from fungi, and a thaumatin-like protein (NP24), already described but not verified as a tomato allergen (www.allgome.org). Unfortunately, it was not possible to overexpress any of these proteins in *E. coli*. HSC70 and MAN already failed to be cloned into respective vectors (table 4.11). NP24 and PepMVCP could not be overexpressed in any of the investigated *E. coli* strains under various conditions. Personal contact with Yannis Livieratos (Crete, Greece, (Mathioudakis *et al.*, 2012)) confirmed the unattainable overexpression in *E. coli* at least for PepMVCP and HSC70. A lot of proteins can be overexpressed in *E. coli,* but it is also known that systems have to be carefully adapted according to the respective proteins. However, overexpression of respective proteins in *E. coli* depends not only on technical demands but might also fail due to particular protein characteristics. In consequence, other expression systems have to be evaluated. Reasons for ineffective overexpression in *E. coli* can be misfolding of the recombinant protein and its segregation into insoluble inclusion bodies, leading to proteolytic degradation (Schmidt and Hoffman, 2002). Different *E. coli* stains with additional tRNAs for eukaryotic protein

translation (e.g. Rosetta) or disulphide-bond allowing strains (e.g. Origami) were used in an attempt to overcome this problem, but without any success (chapter 4.5).
Nevertheless, viral coat protein overexpression in *E. coli* is generally possible and the *Cucumber mosaic virus* coat protein could be successfully overexpressed (Bang *et al.*, 2012). Additionally, an osmotin from tobacco with sequence similarities to the putative tomato allergen NP24 was also successfully overexpressed in *E. coli* (Sharma *et al.*, 2013). Further adaptations of the system, even if dealing with proteins from another organism, might finally enable the overexpression of these proteins in *E. coli*. However, due to limited time the focus was switched to cyclophilin, another putative tomato allergen.

5.8 Identification of cyclophilin as a new putative tomato allergen

Tomato cyclophilin (CYC) has been detected as a putative allergen on former immunoblots with tomato-allergic subjects' sera (data not shown), but did not interact with the IgEs in any of the current immunoblots. CYC has already been overexpressed in *E. coli* in 1990 (Gasser *et al.*, 1990). This CYC was also shown to cross-react with the birch pollen allergen Bet v 7, also coding for a cyclophilin (Cadot *et al.*, 2006). The reaction with tomato-allergic subjects' IgE, its pan-allergenic nature, and the confirmation that in *E. coli* recombinantly produced cyclophilins from other species function as allergens (Horner *et al.*, 1995a;Fluckiger *et al.*, 2002;Cadot *et al.*, 2006;Glaser *et al.*, 2006) strongly indicate tomato CYC as a new tomato allergen candidate.

5.8.1 Overexpression of tomato cyclophilin in *Escherichia coli*

CYC was successfully overexpressed in *E. coli*. It is known that his-tag purification of recombinant proteins attain the highest yields when conducted under denaturing conditions. Denaturants facilitate the solubilisation of possibly formed inclusion bodies through protein unfolding and ensure accessibility of the his-tag, necessary for the binding of rCYC to the Ni-NTA agarose column. Unexpectedly, the native purification of the his-tagged rCYC resulted in remarkably higher yields with around 3 mg/L bacterial cell culture. Reasons for that can only be speculated and might be due to incomplete and morbid protein unfolding under denaturing conditions possibly hiding the his-tag. Another possibility could be a heavy linkage of rCYC to the Ni-NTA agarose preventing the dissolving of rCYC from the column, even with high amounts of imidazole.

5.8.2 Tomato-allergic subjects' IgEs reacted with the recombinant cyclophilin on immunoblots

Immunoblots with rCYC and a serum pool of tomato-allergic subjects indicate the allergenic nature of tomato cyclophilin (figure 4.32). The absence of a reaction in immunoblots with the whole tomato protein extract and the same serum pool might be probably due to the low abundance of this protein (figure 4.18), difficult to detect in the small 2D immunoblots (figure 4.14).
The immunoblots developed only with the AP-conjugated secondary antibody without any serum showed false-positive reactions to rCYC (figure 4.33). This was possibly based on unspecific binding of this secondary antibody to rCYC in the absence of any immunoglobulins. Moreover, the sera from single tomato-allergic subjects showed no reaction at all to rCYC. Therefore, it was excluded that apparent positive reactions were only based on unspecific reactions of the secondary antibody, and

more likely were true IgE-rCYC cross-linkages. Besides specific IgE antibodies, blood serum contains also other immunoglobulins, like e.g. IgGs. It can be speculated that unspecific IgG binding to rCYC blocked the epitopes and therewith inhibited the unspecific binding of the secondary antibody in the presence of rCYC negative candidate sera. The serum of the non-allergic subject seemed to react to rCYC as well as to particular tomato proteins and sensitisation to tomato or a cross-reactive allergy could not be completely excluded. However, positive IgE-allergen cross-linkages on immunoblots can be based on only one allergen epitope. In contrast, for the manifestation of clinical symptoms and for basophil and skin prick tests two epitopes are needed to cross-link the IgE (Cadot *et al.*, 2000). Inclusion criteria for the non-allergic subjects were the absence of allergic symptoms and a negative skin prick test to tomato; immunoblots are not generally included when recording clinical history of allergy.

The POD-conjugated secondary antibody, on the contrary, did not show any unspecific reaction to rCYC (figure 4.32), and therefore, was used for the immunoblot inhibition. However, positive IgE-rCYC cross-linkages could not be inhibited with the denatured rCYC (figure 4.34). This was possibly due to wrong inhibitor-antigen ratio caused by the unknown concentration of denatured rCYC. To clarify these uncertainties, ELISA inhibitions were established with defined amounts of natively purified rCYC.

5.8.3 ELISA inhibition and clinical allergy tests confirmed the allergenic potential of the recombinant cyclophilin

First of all a chessboard titration was conducted to evaluate suitable dilutions of sera from the tomato-allergic subjects and the appropriate amount of rCYC which has to be coupled on the plate (figure 4.35). The increasing OD values with an increasing amount of rCYC without any serum were probably due to unspecific binding of the secondary POD-conjugated secondary antibody to rCYC. However, this secondary antibody was used for further investigations based on the facts that specific IgE-rCYC cross-linkage could anyhow be detected and other secondary antibodies as well as higher secondary antibody dilutions were even less suitable (figure 4.36). Another problem was the unspecific binding of the IgEs to the plate or even to the plate blocking agent BSA, resulting in signals even without rCYC-coupling (figure 4.37). Unspecific binding to BSA was firstly excluded because the immunoblot membranes were also blocked with BSA and did not show high background signals. However, the IgEs sometimes reacted with BSA when applied as the negative control on the immunoblots (figure 4.32). Higher BSA concentrations, which usually exclude any IgE binding on the plate's surface, could not reduce the high background signals. Milk powder as blocking agent was even less suitable and did not show any blocking capability or IgE reaction to this reagent was even higher than to BSA. Therefore, BSA was used for further assays and sufficient difference in respective values still guaranteed the detection of specific IgE binding to rCYC.

Since all investigated sera from the tomato-allergic subjects seem to have specific IgEs to rCYC (figure 4.39), ELISA inhibitions were carried out to confirm these reactions. Since the amounts of sera were limited, inhibition assays could only be conducted for six tomato-allergic subjects, positively reacting to rCYC in the immunoblots. For all these subjects' sera an at least 40% inhibition of the CYC-IgE interaction could be detected. Furthermore, the inhibition for two subjects was absent with BSA as an inhibitor, confirming positive and specific inhibitions through rCYC (figure 4.40). In addition, basophils of these two subjects were activated through rCYC (figure 4.41). Altogether, this strongly

indicated a specific IgE-rCYC cross-linkage for at least these two subjects. For 100% inhibition in ELISA higher amounts of rCYC would have possibly been necessary. Otherwise, technical problems, like the above mentioned unspecific bindings might be responsible for the missing total inhibition. In skin prick tests only one of the subjects showed a positive reaction to rCYC, probably because of the lower sensitivity of the skin in comparison to the basophil tests. It is known that basophil tests possess an extremely high analytical sensitivity and it is possible to detect even low allergen-specific serum IgE levels (Kleine-Tebbe *et al.*, 2006).

The non-allergic subject showed positive inhibition through rCYC as it could be expected from the immunoblots. However, as anticipated the basophil and skin prick test of the non-allergic subject was negative in respect to rCYC (chapter 5.8.2). Two subjects showed rCYC-dependent inhibition in ELISAs and no reaction to rCYC in basophil activation tests. The specific IgE titre to a certain protein, detected in ELISAs do not have to be directly associated with the biological activity of the protein. The affinity between IgEs and allergens is the basis for their biological activity and their capability to cause symptoms of allergy (Fromberg, 2006). Positive reactions in ELISAs probably imply lower affinity rates than required for basophil activation and the triggering of allergic symptoms. Therefore, specific IgE measurements are not necessarily correlated with the occurrence of allergic symptoms. Clinical allergy depends on a lot of different factors, e.g. total serum IgE, epitope-specificity and on specific IgE or IgG (Bousquet *et al.*, 2006). Additionally, *in vitro* reactivity to a certain protein on immunoblots only depends on monoclonal antibody binding but polyclonal cross-linking is necessary to activate e.g. basophil cells *in vivo* (Steckelbroeck *et al.*, 2008). Therefore, specific IgE measurements should not replace other allergy tests, like skin prick or basophil activation tests. DBPCFC, the 'golden standard' of allergy tests, cannot be used to evaluate recombinant proteins for their allergenic potential because risks of an anaphylactic shock cannot be excluded.

Cyclophilins have firstly been detected as allergens from fungi (Horner *et al.*, 1995b). Therefore, it can be speculated that subjects reacting to rCYC are sensitised through fungi and IgE reaction resulted from a cross-reaction between fungal and plant proteins. In the meantime cyclophilin has already become acquainted as an allergen from pollen, e.g. birch (Cadot *et al.*, 2000) or sycamore (Pazouki *et al.*, 2009). Cadot and colleagues showed cross-reactions of a Bet v 7 antibody to different pollen extracts and to tomato cyclophilin, but no reactions to the known allergen Asp f 11, a cyclophilin from *Aspergillus* (Cadot *et al.*, 2006). They suggested that non-plant cyclophilins from e.g. fungi do not immunologically cross-react with plant cyclophilins and the predicted pan-allergenic nature of cyclophilins is probably divided into two groups coming from plants and fungi. From the twelve investigated subjects only two out of nine tested subjects showed specific IgEs against *Aspergillus* and four of four tested subjects had specific IgE to birch (table 3.6). Interestingly, the only subject, who was reacting to rCYC in skin prick test, possessed specific IgE to *Aspergillus*, but unfortunately, was not tested for specific IgE to birch. However, it has already been suggested in chapter 5.5 that sensitisation through pollen is more likely and known to be a common cause for food allergy (Garcia and Lizaso, 2011).

Cyclophilin probably possesses one or more glycosylation sites and can act as a glycoprotein (Thalhammer *et al.*, 1992). As discussed in chapter 5.5, some investigated allergic subjects possess CCD-specific antibodies, reacting to protein associated N-glucans with proposed minor clinical importance. However, *E. coli* is not able to post-translationally modify proteins through glycosylation and complex transformations of *E. coli* strains would be necessary to make them to do so (Makino *et al.*, 2011). Hence, *E. coli* produces non-glycosylated recombinant tomato cyclophilins and occurring

IgE binding is restricted to epitopes from rCYC. In contrast, the allergenic activity of the confirmed major tomato allergen Sola l 2 was completely inhibited when recombinantly produced in *E. coli,* indicating that its glycosylation is a prerequisite for interacting with IgE (Westphal *et al.*, 2003). Recombinant proteins produced in *E. coli* are no identical copies of its natural representatives and proper folding is possibly missing because of the absence of the appropriate machinery. However, Cadot and colleagues used the same overexpression system and showed isomerase activity of recombinant tomato cyclophilin, indicating properly folded rCYC (Cadot *et al.*, 2006).

The chemical and physical properties of the recombinant tomato cyclophilin still have to be characterised, including e.g. its allergenic activity after heat exposure and its proteolytic stability, which is of major importance for the allergic subjects (Sampson, 2004). The allergenic stability of rCYC depends on its epitope structures which are not yet characterised. Subjects' sera showed IgE binding to rCYC even in immunoblots under denaturing conditions. Assuming that the protein was not refolded in the gel after denaturation and conformational epitopes were destroyed, it can be supposed that sequential epitopes are responsible for the IgE cross-linkage. However, as discussed above immunoblot reactions are not necessarily related to allergic reactions and positive reactions in skin prick and basophil tests. Most cross-reactions, like e.g. between Bet v 7 and rCYC (Cadot *et al.*, 2006), are based on conformational epitopes. Additionally, most of the investigated tomato-allergic subjects only showed mild symptoms after the ingestion of tomato (table 3.6), also indicating the reaction to mainly conformational epitopes (Bannon and Ogawa, 2006). rCYC probably possesses sequential and conformational epitopes but only the conformational epitopes are of clinical relevance, and therefore, should be investigated further. In previous studies the IgE binding sites of allergenic fungal and human cyclophilins were modelled and potential cross-reactive epitopes were found (Roy *et al.*, 2003;Glaser *et al.*, 2006). However, the lacking cross-reactivity between fungal and plant cyclophilins indicates the occurrence of different epitopes, responsible for the allergic reactions, and further investigations are necessary to find the cross-reactive epitopes of plant cyclophilins.

Concluding remarks on the identification of tomato cyclophilin as a new allergen candidate

In summary, 11% (1/9 in skin prick tests) to 22% (2/9 in basophil activation tests) showed clear positive reactions to rCYC in clinical allergy tests. The birch cyclophilin Bet v 7 was also described as an allergen with reaction of only one out of six subjects to the purified protein (Cadot *et al.*, 2000). Consequently, the observed results strongly indicate the identification of a new allergen candidate and warrant to propose cyclophilin as a new allergen from tomato. Therefore, data of the current study will be submitted to the International Union of Immunological Societies, the responsible organisation for the appropriate nomenclature of new allergens, for the official confirmation of cyclophilin as a new tomato allergen. Furthermore, these results together with the postulated cross-reactivity of tomato cyclophilin with birch cyclophilin (Cadot *et al.*, 2006) indicate that cyclophilin is a pan-allergen and that homologous proteins from other fruits and vegetables could also cause allergic reactions.

6. CONCLUSION AND OUTLOOK

Tomato is one of the globally most consumed vegetables (http://faostat.fao.org) and is beneficial for human health due to its manifold secondary metabolites. Unfortunately, some consumers have to avoid tomato in their daily diet because they suffer from local and systemic allergic reactions. Allergies are caused by proteins and among others, pathogenesis-related (PR) proteins have been identified as allergens (Hoffmann-Sommergruber, 2000;Ebner *et al.*, 2001;Midoro-Horiuti *et al.*, 2001;Hoffmann-Sommergruber, 2002;Breiteneder and Radauer, 2005). These PR-proteins act as a part of the plant's defence system and can be induced upon various biotic or abiotic stressors (van Loon *et al.*, 2006).

European tomato production is mainly conducted under greenhouse conditions, where in the recent years a viral pathogen could rapidly emerge: the *Pepino mosaic virus* (PepMV), meanwhile threatening the tomato production worldwide (Hanssen *et al.*, 2010b). Lately, a vaccination strategy with mild virus isolates, not affecting fruit quality and yield, has been developed and is already applied in commercial cultivation systems in two major tomato producing countries: Belgium and the Netherlands (personal contact to Inge Hanssen, Scientia Terrae, Belgium). This natural or intentional PepMV infection is known to activate certain defence mechanisms in the plant, including an increased expression of PR-proteins (Hanssen *et al.*, 2011). Plant defence responses can also be induced by symbiotic organisms, like e.g. mycorrhizal fungi (Hause and Fester, 2005), which are commercially utilised as biofertilisers and bioprotective agents against subsequent pathogen attacks (Gianinazzi *et al.*, 2010).

In this context it was speculated that the activation of the plant's immune system through certain biological elicitors, potentially increasing the content of putative allergens, in turn, affects the human immune system of allergic persons upon consumption of plant material. For this reason, this thesis was focussed on the investigation of PR-protein expression, including identified allergens, of tomato plants cultivated under practice conditions after mycorrhiza colonisation or PepMV infection, and the impact on the allergenic potential.

In the described experiments, mycorrhized tomato plants indeed showed elevated expression of allergen-relevant genes but this did not result in an increased allergenicity of their fruits. In contrast to that, PepMV infection did not provoke the predicted general induction of defence-related allergens in tomato fruits and consequently did not lead to a higher allergenic potential. The major hypothesis of this thesis, therefore, proved invalid. In turn, these observations discovered the significant notion, that the plant's defence response seemed to be neither generally comparable from plants grown under controlled conditions to those cultivated in commercial systems, nor from vegetative to reproductive plant organs. PR-proteins, normally known to be induced after pathogen attacks and e.g. observed in leaves of young PepMV-infected tomato plants grown under strictly controlled conditions (Hanssen *et al.*, 2011), might be constantly expressed under commercial cultivation conditions in the greenhouse. Whilst the VEGAL group recently could show that tomato cultivars provoked significant different reactions in clinical allergy tests, different environmental conditions were found to be negligible for the allergenic potential (Dölle *et al.*, 2011a;Dölle *et al.*, 2011b). In addition, within this thesis' study it has been shown that the storage of tomato fruits also had no significant impact on allergen-encoding gene expression and its allergenic potential.

The absence of differences in allergenicity of tomato fruits after the change of plants' outer circumstances might have two reasons: Apart from the commercial growth conditions, blurring the effects of intentionally applied biotic elicitors of defence, another reason might be the high inter-individual variability of tomato-allergic subjects' reactions. The clarification of these individual allergic reactions was another focus of this thesis and confirmed the issue to be more complex than solely the amount and the divergence of allergens that subjects react to. Additionally, respective candidate proteins which generally cause strong, mild, or no allergic reactions could not be identified. Thus, the allergic reactions are not predictable based on the protein profile of certain food stuff. Nevertheless, the analysis of individual reaction patterns resulted in the discovery of several new putative tomato allergens, among those the cyclophilin. The tomato cyclophilin was recombinantly produced and its allergenicity could be confirmed through clinical allergy tests on tomato-allergic subjects.

The knowledge about the plant's defence system is mainly derived from basic research, investigating single defence elicitors on plants cultivated under strictly controlled conditions. These controlled systems are necessary to discover underlying mechanisms and therewith, to assemble and understand the system as a whole. Hence, it is known that PR-proteins are induced as a part of the plant's defence system and help the plant to cope with potential subsequent pathogen attacks. Out of this, and the knowledge from basic allergy research of certain tomato PR-proteins identified as allergens, new hypotheses are developed. The results of this study point to the fact that it is difficult to generally transfer basic research results to application, underlining the growing importance of 'field-studies'. Confirmed hypotheses, out of applied research studies, can serve as a proof of principal. Likewise, rejected hypotheses -unfortunately this is often disregarded- also extend the knowledge of a certain field, as it is shown in this study. In particular, it was shown for the first time that PR-protein and allergen levels vary after viral pathogen attack in different tomato plant organs (leaves and fruits) several weeks after inoculation with PepMV. Moreover, results from different time points and organs are non-transferable, which generally should be considered regarding the defence response of a plant. The fact that plants grown under commercial greenhouse conditions might individually differ in PR-protein and allergen expression, regardless of the PepMV infection, makes it challenging to formulate a final statement about the allergenicity of PepMV-infected tomato fruits.
The most striking finding emerging from this study's results and the previous work of the VEGAL group is that changing environmental conditions as well as pathogenic attacks in commercial plant cultivation -valid at least for tomato- do neither additionally threaten the plant food-allergic population nor help them to overcome or reduce symptoms of allergy. The effects of changed conditions or even strong pathogen attacks are blurred in commercially cultivated major tomato plants, generally exposed to several kinds of stresses. Altogether, this might increase the allergenicity of their fruits but only when compared to tomatoes cultivated under strictly controlled conditions, which, however, will never be available for consumers in any supermarket.

Some additional aspects have to be considered regarding this work:

First, the observations concerning the defence mechanisms of the tomato plants are based on only a few PR-proteins, namely those presenting allergens. Certainly, more PR-protein families are involved in plant's defence and plants possess also other strategies to interact with their outer environment, which have not been evaluated, and therefore, are not further addressed here. Second, it has been shown that RNA accumulation of known allergens is not a reliable predictor for the allergenic potential of certain fruits or vegetables. Several important regulatory steps between allergen-encoding gene expression and the allergenic potential of its source material have to be considered. Not only translation to proteins and their degradation or activation play crucial roles, but also the individual reactions of the allergic subjects to these proteins do so. Third, all clinical allergy tests were carried out on a small subject cohort from a restricted region in Germany. Therefore, these results can only be carefully transferred to the general allergic population.

The critical reflection and the major results of this work prompt the following new perspectives for further investigations:

First, on plant-virus interactions: To obtain a more detailed picture of the plants' defence mechanisms, controlled systems have to be compared to commercial cultivation systems and analyses should consider not only short term stress response but also later behaviour of all plant organs. A trail which could meet all these requirements was planned in summer 2012 in cooperation with Inge Hanssen from 'Scientia Terrae' in Belgium. We intended to compare PepMV-infected and non-infected young tomato plants, grown in the climate chamber under controlled conditions, with major tomato plants, grown near to commercial cultivation in the greenhouse. Harvest time points reaching from days up to weeks after infection were planned on different organs of the plant. Later analysis of gene and protein expression of major defence- and PR-proteins should complement the data we gained so far and build a bridge between basic and applied research. Unfortunately, the trail broke down because of bad weather conditions and a repeat was not possible during the time of the current PhD thesis. However, for further plant-virus research it might be useful to re-address this problem and to perform a trial, as originally planned, to answer open questions and to make further funded statements on plant defence mechanisms in commercial crop cultivation systems.

Second, on the reduction of the allergenic potential of commercially cultivated fruits and vegetables: Based on the findings that commercially cultivated plants might generally be stressed, it is probably not worthy to screen further environmental or biotic factors and their impact on the allergenic potential. The potential differences are only minor and could - if at all - only be detected with large sampling sizes and subject cohorts, at best originating from different regions in Europe. However, in general it is difficult to find allergic subjects willing to expose themselves to painful clinical allergy tests and large European cooperation projects would be necessary to realise those ideas. Probably, the only condition worthwhile for further investigation is storage. Stored fruits and vegetables, with possible changes in the allergen pattern, like it is shown in apple (Matthes and Schmitz-Eiberger, 2009;Kiewning *et al.*, 2012), can be easily avoided by the consumers themselves. Therefore, future attention should possibly be given to further investigations on different storage conditions and post-harvest treatments and the impact on the allergenic potential of commercially available and commonly stored vegetables, like e.g. carrot and celery. On the other hand the most promising field for the identification of hypoallergenic food is probably the selection of low allergenic cultivars. The genetic background of a cultivar is defined in a certain way and the choice and breeding of promising

ones might lead to low allergenic cultivars. Especially, the screening of old cultivars and species, nowadays backcrossed with new ones, possibly have a potential to be less allergenic and might get another chance on the market. It seems to be necessary to step back from producer-friendly high yield and resistant cultivars to those with traits more relevant for the consumer, like e.g. lower allergenicity. Another possibility is to genetically modify those plants in the way that they produce less allergens, albeit genetically modified food is not yet accepted by the European population. To overcome the problem of not accepted genetic engineering other strategies, like e.g. tilling (targeting induced local lesions in genomes) can be applied. Tilling combines chemical mutagenesis with high-throughput screening for point mutations, and the resulting new cultivars do not differ from those obtained by traditional mutation breeding. This circumvents transgenic modifications and is, therefore, attractive also for agricultural application (McCallum *et al.*, 2000;Henikoff *et al.*, 2004). The inactivation of allergen-encoding genes, through random point mutations and their identification through modern screening methods might also discover hypoallergenic cultivars, allowed for commercial cultivation in Europe.

Nevertheless, for the identification of low allergenic cultivars and the development of hypoallergenic food it is essential to identify and verify all potential new candidate allergens and define them as minor or major allergens. Putative tomato allergens identified in this study need to be recombinantly produced and individually tested in clinical allergy tests. Afterwards, these candidates need further characterisation to develop more specialised diagnosis and therapies for more personalised medicine, necessary to cope with the high individuality. In more detail this would be the characterisation of epitopes, e.g. of the putative tomato allergen cyclophilin, newly identified here. This could be achieved through bioinformatic comparisons with known allergen epitopes in databases or partial allergenicity analyses of single peptides from cyclophilin.

Altogether, these basics in allergen identification and characterisation facilitate understanding the mystery of allergy. One day we will aid allergic people with more detailed allergy diagnosis and subsequent curing therapy; and the identified and developed hypoallergenic cultivars will possibly normalise their habits of nutrition.

7. SUMMERISATION

INTRODUCTION*:* Tomato (*Solanum lycopersicum*) is one of the most consumed vegetable worldwide and the most famous in Germany (25 kg/person/year). With its many health relevant compounds it is very important for daily diet. Unfortunately, increasing tomato consumption is accompanied by an increasing risk for tomato allergy. Among the European population the number of food allergic people is increasing and tomato allergy has been confirmed as particular important.

A lot of factors might influence the allergenicity of a certain vegetable and a few studies already reported differences in the allergenic potential of different cultivars. Nearly nothing is known about the impact of different biotic factors, e.g. the colonisation of plants with pathogens or with commercially used beneficial biological agents. Pathogens and symbionts can activate the plant's defence response and thereby the expression of defence- or pathogenesis-related (PR) proteins. PR-proteins show high homologies to allergens and allergenic activity could be confirmed in many PR-protein families. A well-known beneficial biological agent is the mycorrhizal fungus, interacting with 80% of all land plants. Arbuscular mycorrhiza is used as an additive in substrates for commercial cultivation of vegetables and also for the production of tomato. Contrariwise, pathogens often colonise plants in commercial cultivation systems and potentially influence the expression of certain allergens. One of the worldwide most important pathogen for greenhouse grown tomatoes is the *Pepino mosaic virus* (PepMV), of which mild isolates are applied for vaccination.

Within this thesis the impact of these two biotic factors, focussing on PepMV, on the allergenic potential of tomato was analysed. Additionally, the study gives new insights in plant-virus research and contributes to the identification of new tomato allergens.

METHODS: Tomatoes were cultivated in a greenhouse near to commercial cultivation conditions and inoculated with a beneficial mycorrhizal fungus or the viral pathogen PepMV. Allergen expression was analysed on RNA accumulation level with quantitative real time RT-PCR and on protein level with 2D protein gels. 2D immunoblot analyses and subsequent mass spectrometry identification of putative tomato allergens were carried out with tomato-allergic subjects' sera. Additional clinical allergy tests, like skin prick tests, double blind placebo controlled food challenges, and basophil activation and degranulation tests, were conducted to evaluate the clinical relevance. The new tomato allergen candidate cyclophilin was overexpressed in *Escherichia coli* and the allergenic potential of recombinant cyclophilin was analysed with immunoblots, ELISA inhibition, and skin prick as well as basophil tests.

RESULTS*:* After successful mycorrhizal inoculation, RNA accumulation analyses of known and putative tomato allergen-encoding genes revealed an induction of most of the investigated genes in mycorrhized plants. Skin prick tests with tomato-allergic subjects, however, revealed no stronger reaction to fruits derived from mycorrhized plants (Schwarz *et al.*, 2010b).

On the contrary and against the expectations, PepMV-infected tomato fruits showed no general increase of defence-related allergens on RNA and protein level weeks after pathogen inoculation. Clinical allergy tests confirmed these results and did not reveal the expected increase in allergenicity of PepMV-infected tomato fruits. However, high inter-individual variation to PepMV-infected and non-infected tomato fruits were observed. These inter-individual differences and the high variation

of plants grown under commercial greenhouse conditions made a final statement about the allergenicity of PepMV-infected tomato fruits difficult to render (Welter *et al.*, 2013).

To investigate the basis for the high differences between individual tomato-allergic subjects, immunoblot analyses were conducted. The hypothesis that individual variability is based on differential reactions of single subjects to particular allergens, in tomato fruits of plants with certain genetic background or cultivated under distinct conditions, had to be rejected (Welter *et al*., in revision of *Clinical and Experimental Allergy*).

Proteins appearing in the immunoblots of the different studies were analysed by mass spectrometry and 13 candidates with putative clinical relevance as tomato allergens could be newly identified, among those heat shock proteins, a superoxide dismutase, and a cyclophilin. These findings might be a matter of particular interest in allergy research because of the wide distribution of these proteins in nearly all studied organisms. Tomato cyclophilin was recombinantly produced in *E. coli* and its allergenic activity could be demonstrated in *ex vivo* as well as in *in vivo* allergy tests on tomato-allergic subjects. These analyses indicate a potential role for cyclophilin as a putative new tomato allergen.

CONCLUSION*:* Even if the selection of certain cultivars might have an effect on the allergenicity of tomato fruits, this study showed that biotic factors seem to be of only minor importance for the allergenic potential of commercially cultivated tomatoes. Moreover, new insights in late stage plant-virus interactions were obtained and the identification of new allergen candidates enlarged the spectrum of putative tomato allergens.

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