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Enhancement of tomato resistance to

Tuta absoluta by the expression of two

barley proteinase inhibitors

A thesis submitted in fulfillment of the requirements
for the Doctor of Philosophy on Biotechnology.

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CERTIFICAN

Que **Dª Rim Hamza** ha realizado bajo nuestra dirección el trabajo que con el título de *Enhancement of tomato resistance to Tuta absoluta by the expression of two barley proteinase inhibitors,* presenta para optar al grado de Doctor por la Universidad Politécnica de Valencia.

Y para que así conste a los efectos oportunos, firman la presente certificación en Valencia a 12 de septiembre de 2017.

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Abstract

Evolution has provided vast genetic diversity, enabling plants to surmount many biotic pressures. Plants have evolved various morphological and biochemical adaptations to cope with herbivores attacks. Despite that, yearly, around 40 % of worldwide crop production is lost due to pests and pathogens, with 13 % due to insects. *Tuta absoluta* has become a major pest threatening tomato crops worldwide and without the appropriated management it can cause production losses between 80 to 100 %. To cope with this threat, we need to strengthen plant defense arsenals. The incorporation to plants of defensive genes like proteinase inhibitors by means of genetic engineering is a promising alternative.

In the first chapter of this work we investigated the inhibitory activity of two trypsin inhibitors from barley; BTI-CMe and BTI-CMc. Besides, we succeeded to increase the BTI-CMc *in vitro* inhibitory activity by introducing a single mutation in its putative reactive site.

In the second chapter, we investigated the *in vivo* effect of (a serine proteinase inhibitor) BTI-CMe and a (cysteine proteinase inhibitor) Hv-CPI2 isolated from barley on *Tuta absoluta* and we examined the effect of

their expression on the tomato defensive response. We found that larvae fed on the double transgenic plants showed a notable reduction in weight. Moreover, only 56% of the larvae reached the adult stage. The emerged adults showed wings deformities and reduced fertility. We also investigated the effect of proteinase inhibitors ingestion on the insect digestive enzymes. Our results showed a decrease in larval trypsin activity. Proteinase inhibitors had no harmful effect on *Nesidiocoris tenuis;* a predator of *Tuta absoluta,* despite transgenic tomato plants attracted the mirid. We investigated whether or not plant defensive mechanisms were activated in the transgenic tomato plants and found that, interestingly, the expression of the barley cysteine proteinase inhibitor promoted plant defense, inducing the tomato endogenous wound inducible *proteinase inhibitor 2* (*Pin2*) gene. Moreover, glandular trichomes production was increased and the emission of volatile organic compounds was altered. Our results demonstrate the usefulness of the co-expression of different proteinase inhibitors for the enhancement of plant resistance to pests.

La evolución ha proporcionado una gran diversidad genética, permitiendo a las plantas superar muchas presiones bióticas. Las plantas han desarrollado diversas adaptaciones morfológicas y bioquímicas para hacer frente a los ataques de los herbívoros. A pesar de ello, anualmente, alrededor del 40 % de la producción mundial de cultivos se pierde debido a plagas y patógenos, siendo un 13 % debido a insectos. *Tuta absoluta* se ha convertido en una de las principales plagas que amenazan los cultivos de tomate en todo el mundo y sin la gestión adecuada puede causar pérdidas de producción entre el 80 y el 100 %. Para hacer frente a esta amenaza, necesitamos fortalecer los arsenales de defensa de las plantas. La incorporación a las plantas, mediante ingeniería genética, de genes defensivos como los inhibidores de proteinasas es una alternativa prometedora.

En el primer capítulo de este trabajo se investigó la actividad inhibitoria de dos inhibidores de tripsina procedentes de cebada; BTI-CMe y BTI-CMc. Además, se logró aumentar la actividad inhibitoria *in vitro* de BTI-CMc mediante la introducción de una única mutación en su putativo centro reactivo.

En el segundo capítulo, se investigó el efecto *in vivo* de un inhibidor de serin proteinasa (BTI-CMe) y un inhibidor de cisteín proteinasa (Hv-CPI2) aislado de cebada en *Tuta absoluta* y se examinó el efecto de su

expresión en la respuesta defensiva del tomate. Se encontró que las larvas alimentadas con las plantas transgénicas dobles mostraron una notable reducción de peso. Además, sólo el 56 % de las larvas alcanzó la etapa adulta. Los adultos emergentes mostraron deformidades de las alas y reducción de la fertilidad. También se investigó el efecto de la ingesta de inhibidores de proteinasa en las enzimas digestivas de los insectos. Nuestros resultados mostraron una disminución en la actividad tripsina larvaria. Los inhibidores de proteinasas no tuvieron efectos nocivos sobre *Nesidiocoris tenuis*(depredador de *Tuta absoluta*) a pesar de que las plantas transgénicas de tomate atrajeron al mirido. Se investigó si los mecanismos defensivos de las plantas se activaban en las plantas de tomate transgénico y se encontró que, curiosamente, la expresión de la cistatina de cebada promovía la defensa de la planta, induciendo el gen del *inhibidor de proteasa 2* endógeno del tomate, inducible por herida (*Pin2*). Además, aumentó la producción de tricomas glandulares y se alteró la emisión de compuestos orgánicos volátiles. Nuestros resultados demuestran la utilidad de la co-expresión de diferentes inhibidores de proteinasas para el aumento de la resistencia de las plantas a plagas.

L'evolució ha proporcionat una gran diversitat genètica, permetent a les plantes superar moltes pressions biòtiques. Les plantes han desenvolupat diverses adaptacions morfològiques i bioquímiques per fer front als atacs dels herbívors. Tot i això, anualment, al voltant del 40 % de la producció mundial de cultius es perd a causa de plagues i patògens, amb un 13 % a causa de insectes. *Tuta absoluta* s'ha convertit en una de les principals plagues que amenacen els cultius de tomaca a tot el món i sense la gestió adequada pot causar pèrdues de producció entre el 80 i el 100 %. Per fer front a aquesta amenaça, necessitem enfortir els arsenals de defensa de les plantes. La incorporació a les plantes de gens defensius com els inhibidors de proteïnases per mitjà de l'enginyeria genètica és una alternativa prometedora.

En el primer capítol d'aquest treball es va investigar l'activitat inhibitòria de dos inhibidors de tripsina aïllats a partir d'ordi; BTI-CMe i BTI-CMC. A més, es va aconseguir augmentar l'activitat inhibitòria *in vitro* de BTI-CMC mitjançant la introducció d'una única mutació en el seu lloc reactiu putatiu.

En el segon capítol, es va investigar l'efecte *in vivo* d'un inhibidor de serin proteinasa (BTI-CMe) i un inhibidor de cisteïn proteinasa (Hv-CPI2) aïllats d'ordi en *Tuta absoluta* i es va examinar l'efecte de la seva expressió en la resposta defensiva del tomaca. Es va trobar que les larves alimentades

amb les plantes transgèniques dobles van mostrar una notable reducció de pes. A més, només el 56 % de les larves va aconseguir l'etapa adulta. Els adults emergents van mostrar deformitats de les ales i reducció de la fertilitat. També es va investigar l'efecte de la ingesta d'inhibidors de proteinasa en els enzims digestius dels insectes. Els nostres resultats van mostrar una disminució en l'activitat tripsina larvària. Els inhibidors de proteïnases no van tenir efectes nocius sobre *Nesidiocoris tenuis*, un depredador de *Tuta absoluta*, tot i les plantes transgèniques de tomaca van atreure al mirid. Es va investigar si els mecanismes defensius de les plantes s'activaven a les plantes de tomaca transgènic i es va trobar que, curiosament, l'expressió de cistatina d'ordi promovia la defensa de la planta, induint el gen de l'*inhibidor de proteasa 2* endogen de la tomaca, induïble per ferida (*Pin2*). A més, va augmentar la producció de tricomes glandulars i es va alterar l'emissió de compostos orgànics volàtils. Els nostres resultats demostren la utilitat de la co-expressió de diferents inhibidors de proteïnases per a l'augment de la resistència de les plantes a plagues.

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List of abbreviations

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°C: Celsius degree

µF: microfarad

µg: Microgram

µl: microliter

µM: micromolar

ACC: 1-aminocyclopropane-1-carboxylic acid

ACO: 1-aminocyclopropane-1-carboxylic acid oxidase

ACS: 1-aminocyclopropane-1-carboxylic acid synthase

ANOVA: Analysis of variance

BAAMC: Nα-Benzoyl-L-arginine-7-amido-4-methylcoumarin hydrochloride

BBI: Bowman Birk inhibitor

bp: base pare

BSA: Bovine serum albumin

CaMV: Cauliflower mosaic virus

cDNA: Complementary Deoxyribonucleic acid

cm: centimeter

CPI: Cystein proteinase inhibitor

Ct: Cycle threshold

cv: Cultivar

DAHP: 3-Deoxy-D-arabinoheptulosonate 7-phosphate

DALY: disability adjusted life years

DAPI: 4',6-diamidino-2-phenylindole

DMAPP: dimethylallyl pyrophosphate

DNA: Deoxyribonucleic acid

dNTP: Deoxynucleotide

DTT: DL-Dithiothreitol

E4P: Erythrose 4-phosphate

EDTA: Ethylenediaminetetraacetic acid

eV: Electron Volt

FAO: Food and agriculture organization

g: gram

GAP: glyceraldehyde 3-phosphate

GC: Gas chromatography

GE: genetically engineered

GMO: genetically modified organism

GPP: geranyl pyrophosphate

h: hour

IAA: Indole-3-acetic acid

IPP: isopentenyl pyrophosphate

IPTG: Isopropyl β-D-1-thiogalactopyranoside

IR: Refractive index

JA: jasmonic acid

kDa: Kilo Dalton

Km: kilometer

KV: kilo volt

L : liter

LB : Luria-Bertani

L-BApNA: Nα-Benzoyl-L-arginine 4-nitroanilide hydrochloride

M: molar

MAPK: mitogen activated protein kinase

Mb: Megabase

MEP: 2-C-methyl-D-erythritol 4-phosphate

mg : milligram

Min: minute

ml: milliliter

mm: millimeter

mM: millimolar

MMLV: Molony Murine Leukemia Virus

MOPS: 3-N-morpholino propanesulfonic acid

mRNA: Messenger ribonucleic acid

MS: Mass spectrometry

MS: Murashige and Skoog

MVA: mevalonic acid

n/z: neutron/proton

N: normal

ng: nanogram

nm: nanometer

nptII: neomycin phosphotransferase II

OCI: oryzacystatin

OD: optic density

PBS: Phosphate buffered saline

PCD: Programmed cell death

PCR: polymerase chain reaction

PEP: phosphoenolpyruvate

pH: potential of hydrogen

PI: Proteinase inhihibitor

PPP: Pentose phosphate pathway

PVA: Polyvinyl alcohol

PVP: polyvinyl pyrolidone

PVY: Potato virus Y

Pyr: pyruvate

RH: Relative humidity

RNA: Ribonucleic acid

rpm: rotation per minute

RT-qPCR: Real time quantitative PCR

RV: reaction volume

Rv: reduction value

s: second

SDS: Sodium dodecyl sulfate

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SIPK: Salycilic acid-induced protein kinase

SPI: Serine proteinase nhibitor

SPM: Solid phase microextraction

SSC: Soluble solid content

TA: Trypsin activity

TBE: Tris-Borate-EDTA

TCA: Trichloroacetic acid

TEMED: Tetramethylethylenediamine

TEV: Tobacco Etch virus

Tris: tris(hydroxymethyl)aminomethane

U: Unit

v/v: volume/volume

V: volt

VOC: volatile organic compound

w/v: weigth/volume

W:Watt

WIPK: wound induced protein kinase

α AI-PV: *Phaseolus vulgaris* α-amylase inhibitor

Introduction

I-Plant-Pest interaction

I.1 Crop loss due to pest

Since the beginning of agriculture over 11,000 years ago, pests have been the major threat for crop production. Food crops are damaged by more than 10,000 species of insects. Despite of an annual investment of US$ 40 thousand million and the application of 3 million metric tons of pesticides worldwide (Pimentel, 2009), around 40 % of crop production is lost due to pests and pathogens (Oerke, 2005; Savary *et al.*, 2012). Insect pests are responsible for 10-16 % of agriculture yield loss before harvest and almost a similar amount at postharvest (Bebber *et al.*, 2013). The direct economical damage is estimated to US$ 2,000 thousand million per year (Pimentel, 2009). The economical loss generated by pests is not restricted to the direct yield drop, other costs such as pesticides application, biological control agents, poisoning medical treatments and environmental decontamination should be considered (Oliveira *et al.*, 2014). Direct invasive insects damages cost more than US$ 70 thousand million per year, globally. While the associated health costs are estimated to US$ 6,900 million per year (Bradshaw *et al.*, 2016).

On one hand, Pesticides are widely used in agriculture to prevent or reduce losses by pest and thus improve yield and quality (Oerke & Dehne, 2004; Cooper & Dobson, 2007).

They can also improve nutritional value (Boxall, 2001; Narayanasamy, 2006). Thus pesticides can be considered an efficient, labor-saving tool for pest control. On the other hand, pesticides can cause serious concerns about health (van der Werf, 1996; Soares & de Souza Porto, 2009). Indeed, harmful effects on non target organisms, humans and wild life populations have been reported (Hernández *et al.*, 2011). The exposure to pesticides can occur from residues on food and drinking water for general population (van der Werf, 1996; Soares & de Souza Porto, 2009) or when mixing and applying pesticides for farmers. This risk is increased in developing countries due to the use of toxic chemicals that are banned in others, incorrect application techniques and poorly maintained equipment (Ecobichon, 2001; Asogwa & Dongo, 2009). Long term pesticide exposure can lead to a broad range of health issues such as cancer, neurodegenerative disease (Bassil *et al.*, 2007; Kanavouras *et al.*, 2011; Parrón *et al.*, 2011), reproductive and developmental toxicity (Hanke & Jurewicz, 2004) and respiratory effects (Hernández *et al.*, 2011). In Europe, pesticides health impact is estimated to about 2,000 DALY per year corresponding to an annual cost of 78 million € with an average burden of 2.6 hours and 12 € per person over life time (Fantke *et al.*, 2012).

The use of genetic engineering for plant resistance improvement offers a promising alternative. Since the 1980's, scientists have used genetic engineering to improve certain traits in plants, such as resistance toward pests (Metz *et al.*, 1995; Zhao *et al.*, 2003; Zhang *et al.*, 2015; Chakraborty *et al.*, 2016). However, only few transgenic plants were commercialized, due to the legislation and social fear from their long-term impact on health and environment. Thirty years later, the National Academies of Sciences, Engineering and Medicine, after the examination of almost 900 researches, concluded that genetically engineered crops had no harmful impact neither on human health, nor on the environment. Even more, the report indicated that insect-resistant genetically modified crops have had benefits on human health by reducing the number of insecticide poisonings (National Academies of Sciences & Medicine, 2017).

I.2 Plant response to herbivores

For as long as 350 million years, plants and insects have co-existed and developed series of relationships which affected both organisms at different levels, from biochemistry to population genetics. Although some of these interactions are beneficial, such as pollination, the most common relationship consists of insect's predation of plants and plant defense

against phytophagous insects. According to the evolutionary theory of Ehrlich and Ravn (1964), insect feeding on plants has been a determining factor in increasing species diversity in both phytophagous insects and hosts.

Herbivory insects use diverse feeding strategies to obtain nutrients from their host plant. Rather than acting as a passive victim in this interaction, plants respond to phytophagous insects with the production of toxins and defensive proteins that target physiological processes in the insect. Herbivore-challenged plants also emit volatiles that attract insect predators and bolster resistance to future threats. Some species accumulate high levels of compounds which function as biochemical defense trough their toxicity or their physiological properties. Other plants do not waist resources accumulating defense compounds, but seek to minimize phytophagous insects damage through rapid growth and development.

I.2.1 Plant direct defensive response

Secondary metabolites

All plants exhibit constitutive or induced accumulation of toxic secondary metabolites as part of their defense against pests. Across the plant realm, a great variety of small molecules with toxic or anti-feeding properties on insects

have been identified, such as terpenoids, alkaloids (nicotine, morphine, strychnine, cocaine, etc.), furamocoumarine, cardenolides, tannins, saponins, glucosinolates and cyanogenic glycosides. Some of these compounds are toxic to the host plant. Therefore, they are usually stored as benign precursors that are activated by insects attack. Different toxins can have synergetic effect in defense against phytophagous insects. For instance, a combination of two monoterpenoids is almost ten times more toxic against *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) than would have been predicted from a simple additive effect (Hummelbrunner & Isman, 2001). In addition to possible synergetic effect, metabolic diversity in toxin production can also provide defense against multiple phytophagous insects with different feeding styles or resistance mechanisms.

Defensive proteins

-   **Plant lectins**

Plant lectins comprise all plant proteins that bind reversibly to specific mono or oligo-saccharides. A typical lectin is multivalent and therefore capable of agglutinating or clumping cells. About 500 different plant lectins have been isolated and characterized. Numerous reports have studied their insecticidal effect against Lepidoptera, Coleoptera, Diptera and Homoptera (Sharma *et al.*, 2009; Van Damme,

2014). Lectins specifically recognize typical glycans that are abundantly present on the surface of the epithelial cells exposed along the intestinal tract of higher and lower animals. When binding to these receptors, the lectins exert harmful or toxic effects. Feeding trials with insects and higher animals confirmed that some plant lectins provoke toxic effects ranging from a slight discomfort to a deadly intoxication (Peumans & Van Damme, 1995; Grossi-de-Sá *et al.*, 2015; Raja *et al.*, 2016).

- **α-amylase inhibitors**

α-amylase inhibitors are plant proteins highly present in seeds, able to form complex with cellular amylases and are supposed to play a role in plant defense against insects (Mehrabadi *et al.*, 2012). A major interest has been focused on the expression of the common bean *Phaseolus vulgaris* α-amylase inhibitor (α AI-Pv) in other plants (Campbell *et al.*, 2011). This α-amylase inhibitor forms a complex with insect and mammalian α-amylases but is not active against plants and bacterial ones. α-AI-Pv inhibits the α-amylases in the gut of different insects and consequently blocks its larval development (Barbosa *et al.*, 2010; Dias *et al.*, 2010).

- **Proteinase inhibitors**

Plant proteinase inhibitors (PIs) are polypeptides or proteins that occur naturally in a wide range of plants and are part of their natural defense arsenal against insects. They are mainly found in storage tissues like seeds and tubercles. These proteins are induced in response to different biotic (insect attack, pathogen, etc), (Chen *et al.*, 2014; Quilis *et al.*, 2014) and abiotic (salinity, cold, etc) stress (Kidrič *et al.*, 2014; Quain *et al.*, 2014). The defense role of PIs was first discovered by Green and Ryan (1972) who observed that the expression of PIs was induced in tomato and potato leaves in response to insect attacks. The induction of PIs is systemic, within few hours after wounding, PIs induction is observed in adjacent leaves, leading to an accumulation of these proteins in all plant tissues.

As PIs are primary gene products, they are excellent candidates for pest-resistance engineering. This was first demonstrated by Hilder et al. (1987) when expressing in tobacco a trypsin inhibitor from *Vigna unguiculata,* which conferred resistance to various insects (Lepidoptera and Orthoptera).

I.2.2 Plant indirect defensive response

Plants produce a blend of secondary metabolites after attack or egg deposition of herbivorous insects (Mumm & Hilker, 2006; Dicke *et al.*, 2009), including volatile organic compounds (VOCs). Herbivory induced plant volatiles (HIPVs) mainly comprise terpenoids, fatty acid derivates, phenyl propanoids and benzenoids (Dudareva *et al.*, 2004; Mumm & Dicke, 2010). Their emission can be either local or systemic (Heil & Ton, 2008). HIPVs can induce behavioural changes in different community members: carnivorous, arthropods, parasitoids, nematods, insectivorous birds and neighboring plants (Soler *et al.*, 2007; Bruinsma *et al.*, 2009). HIPVs can attract phytophagous insects natural enemies, increasing predation pressure on the pest (Takabayashi & Dicke, 1996) and acting as indirect defense. This phenomena was referred to as "cry for help" (Dicke *et al.*, 1990). For instance, when attacked by caterpillars, maize seedlings release volatiles attractive to the parasitoids *Cotesia marginiventris* and *Microplitis croceipes* (Turling et al., 1990; 1993). Also, the parasitoid *Cotesia glomerata* was attracted by HIPVs emitted by *Brassica nigra* plants infested by *Pieris brassicae* (Ponzio *et al.*, 2014). HIPVs can also act as repellents for herbivorous insects. They may be repelled for different reasons: the odour may indicate the presence of competitors, it may be a signal of the production of toxic

defensive compounds or it can reflect that the plant is particularly attractive to natural enemies of the phytophagous insect (Bernasconi *et al.*, 1998). The aphid *Rhopalosiphum maidis* preferred healthy, undamaged maize seedlings or clean air, over plants emitting HIPVs in Y-tube olfactometer (Bernasconi *et al.*, 1998). Also, the phytophagous insect *Pieris rapae* was rather attracted by healthy *Brassica nigra* plants than the jasmonic acid induced ions releasing HIPVs (Bruinsma *et al.*, 2008). Another study showed that male *Ceratitis capitata* were less attracted to citrus plants emitting low levels of limonene (Rodríguez *et al.*, 2011).

In addition to their role as carnivores' attractants and phytophagous insects repellents, HIPVs are also involved in plant-plant communication. This phenomena was first described by Baldwin and Shultz (Baldwin & Schultz, 1983) and called "talking trees". It suggests that damaged trees emit airborne signals that warn neighboring healthy plants and induce their defenses. More recent studies have confirmed this theory. It has been shown that neighboring plants "eavesdrop" on volatile signals emitted by damaged plants and undergo transcriptional modifications to tailor their defense (Baldwin *et al.*, 2002). Also, it has been demonstrated that airborne volatiles emitted by damaged willow trees reduced damages in neighboring plants (Pearse *et al.*, 2013).
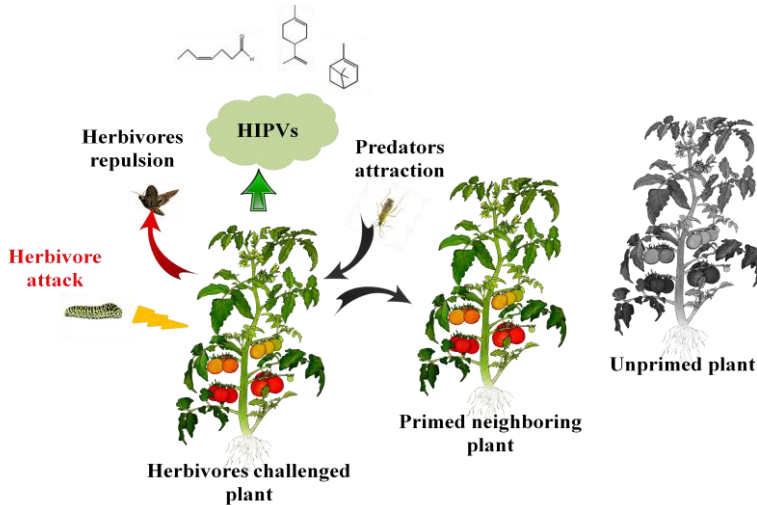
Figure 1: Herbivore-induced plant volatiles interactions.

II- Plant proteinase inhibitors

II.1 Plant proteinase inhibitors families

The different proteinase inhibitors characterized are specific for each of the four mechanistic classes of proteolytic enzymes. Based on the active amino acid in their reaction center, they are classified as serine, cysteine, aspartic and metalloprotease inhibitors (Belew & Eaker, 1976; Habib & Fazili, 2007). The activity of PIs is due to their ability to form stable complexes with target proteases blocking, altering or preventing access to the enzyme active site.

II.1.1 Serine proteinase inhibitors (SPIs)

SPIs are widespread throughout the plant kingdom (Odani *et al.*, 1986). An important number of these enzymes has been described and characterized in different plant species, being the most studied PIs.

In plants, SPIs have different physiological functions including the regulation of endogenous proteases and protection against pests. Moreover, they may act as storage proteins.

SPIs contain a cysteine residue as the catalytic active nucleophile in the enzyme active site. Serine proteinases such as trypsin, chymotrypsin and elastase are responsible for the initial digestion of proteins in the gut of the majority of higher animals (García Olmedo *et al.*, 1987; Hosseininaveh *et al.*, 2009; Saadati & Bandani, 2011; Jayachandran *et al.*, 2013). *In vivo*, they cleave long polypeptides chains into short peptides which are then degradated by exopeptidases to amino acids, the end product of protein digestion.

Different families of plant SPIs have been identified with diverse biochemical properties and different specificities.

- **Serpins**

This family is the most widespread of PIs. Serpin-like genes have been identified in almost all type of organisms: viruses,

bacteria, plants and animals (Irving *et al.*, 2000; Gettins, 2002; Rawlings *et al.*, 2004; Christeller & Laing, 2005; Law *et al.*, 2006). Multicellular eukaryotes, usually, possess several serpin genes (Roberts & Hejgaard, 2008). For instance, 29 serpin genes have been identified in *Arabidopsis thaliana* (Silverman *et al.*, 2001).

Serpins can inhibit trypsin-like proteins (Roberts *et al.*, 2003; Huntington, 2011) but they have no target in plants. They are probably involved in plant defense against pathogens (Hejgaard, 2005). It has been suggested that instead of interacting directly with pathogens, plant serpins may have a complex pathway up-regulating the host immune system (Law *et al.*, 2006). Serpins have mixed specificities toward proteases (Al-Khunaizi *et al.*, 2002; Hejgaard & Hauge, 2002; Huntington, 2011). Barley serpin is a potent inhibitor of trypsin and chymotrypsin (Dahl *et al.*, 1996a), but it also inhibits thrombin, plasma, Factor VIIa and Factor Xa (Dahl *et al.*, 1996b). Wheat serpin inhibits chymotrypsin and cathepsin G (Roberts *et al.*, 2003). Serpins have a molecular mass of 39-43 kDa. They are reversible "suicide" inhibitors. The cleavage of an appropriate peptide bond in the reactive centre loop of the inhibitor triggers a rapid conformational change so that catalysis does not proceed beyond the formation of an acyl-enzyme complex (Gettins, 2002).

- **Bowman-Birk proteinase Inhibitors (BBI)**

These SPIs were named after D.E. Bowman and Y. Birk, who were the first to identify and characterize a member of this family in Soybean (*Glycine max*) (Bowman, 1945; Birk, 1985). These inhibitors have, then, been identified in legumes, cereals and Poaceae (Odani *et al.*, 1986; Tanaka *et al.*, 1997; Laing & McManus, 2002; Prasad *et al.*, 2010; Dramé *et al.*, 2013; Kuhar *et al.*, 2013). These enzymes are generally found in seeds and are wound inducible in other plant tissues as leaves. In dicot plants, BBI consist of a single polypeptide chain of 8 kDa. The protein is double headed with two homologous domains bearing separated reactive sites. It interacts independently but simultaneously with two proteases which may be the same or different (Birk, 1985; Barbosa *et al.*, 2007). The first reactive site is usually specific for trypsin, chymotrypsin or elastase (QI *et al.*, 2005). The active site is stabilized by the presence of seven conserved disulfide bonds (Chen *et al.*, 1992; Lin *et al.*, 1993; da Silva *et al.*, 2001; Barbosa *et al.*, 2007). The monocot's BBI, have a different structure. They can have a single headed reactive site within a polypeptide of 8 kDa or a double headed reactive site forming a 16 kDa polypeptide (Tashiro *et al.*, 1987; Prakash *et al.*, 1996).

- **Kunitz family**

These PIs mostly inhibit trypsin, chymotrypsin and subtilisin (Park *et al.*, 2000; Laing & McManus, 2002) but they can also inhibit other proteases as cathepsin D and papain. Kunitz type inhibitors have been described in legumes, cereals and solanaceaous species (Laskowski Jr & Kato, 1980; Ishikawa *et al.*, 1994; Cruz *et al.*, 2013; Rufino *et al.*, 2013). These enzymes are produced under stress. They usually have a molecular mass of 18-22 kDa with two disulfide bonds and a single reactive site. These inhibitors are canonical and form a tight complex with the target protease that dissociates very slowly (Ritonja *et al.*, 1990; Migliolo *et al.*, 2010).

- **Potato inhibitors I**

These inhibitors have been described in different plants including potato tubers (Ryan & Balls, 1962), tomato fruit and leaves (Lee *et al.*, 1986; Margossian *et al.*, 1988; Wingate *et al.*, 1989) and squash phloem (Murray & Christeller, 1995). The inhibitors of this family generally lack any disulfide bonds, except inhibitors from potato tubers and cucurbits that show a single disulfide bond. They have a molecular mass of 8 kDa, are monomeric and show an inhibitory activity against chymotrypsin.

- **Potato inhibitors II**

The members of this family were identified in Solanaceae. They were first characterized in potato tubers (Dammann *et al.*, 1997), then were found in leaves, flowers, fruits and phloem of other Solanaceae species (Iwasaki *et al.*, 1971; Kim *et al.*, 2001; Luo *et al.*, 2009). These inhibitors were reported to inhibit chymotrypsin, trypsin, elastase, oryzin and subtilisin (Antcheva *et al.*, 1996; Xu *et al.*, 2004; Zavala *et al.*, 2004).

- **Cereal trypsin/α-amylase inhibitors**

The members of this family have serine proteinase and/or α-amylase inhibitory activity. These PI are active against heterologous α-amylases from insects, mites and mammals or trypsin-like proteases. They have been identified in different plants such as ragi (*Eleusine coracana*) (Shivaraj & Pattabiraman, 1981), coffee bean (Valencia *et al.*, 2000), *Phylanthus amarus* (Ali *et al.*, 2006), rye (Iulek *et al.*, 2000) or *Syzygium cumini* (Karthic *et al.*, 2008). The cereal trypsin/α-amylase inhibitors consist of a single polypeptide with a molecular mass of about 13 kDa containing five disulfide bonds (Christeller & Laing, 2005).

II.1.2 Cysteine proteinase inhibitors (Cystatins)

Plant cystatins are the second most studied class of PIs and have been identified and characterized in different plant species: cowpea (Flores *et al.*, 2001; Aguiar *et al.*, 2006), potato (Annadana *et al.*, 2003), cabbage (Lim *et al.*, 1996; Huang *et al.*, 2001), carrot (Sakuta *et al.*, 2001), chestnut (Connors *et al.*, 2002), Job's tears (Koh-Ichi *et al.*, 2002), etc. Cystatins have also been identified in the seeds of different crop plants, such as sunflower, rice, wheat, maize, soybean or barley (Misaka *et al.*, 1996; Yamada *et al.*, 2000; Gaddour *et al.*, 2001). Cystatins exist in both animals and plant organisms. The majority of plant cystatins are classified among the phytocystatin family. These PIs have a highly conserved region in the G58 residue, the glu-x-val-x-gly (QxVxG) motif and a pro-trp (PW) motif (Margis *et al.*, 1998; Martínez *et al.*, 2005). Studies of the papain inhibitory activity of oryzacystatin have identified this conserved motif as a primary region of interaction between the inhibitor and its cognate enzyme. The PW motif is believed to act as a cofactor. Phytocystatins have a dual role in plants, as defense proteins (Atkinson *et al.*, 2004; Ribeiro *et al.*, 2006; Álvarez-Alfageme *et al.*, 2007) and endogenous regulators involved in proteins turn over (Kiyosaki *et al.*, 2007; Weeda *et al.*, 2009). Phytocystatin expression is usually limited to specific organs and development phases such as germination (Bolter &

Jongsma, 1995) early leaf senescence (Huang *et al.*, 2001), cold and salt stress (Gaddour *et al.*, 2001; Belenghi *et al.*, 2003; Van der Vyver *et al.*, 2003).

II.1.3 Aspartic proteinase inhibitors

Aspartic proteinase inhibitors are less studied due to their relative rarity of occurrence in plants. They have been described in sunflower and potato tubers, barley and cardoon flowers (*Cynara cardunculus*) (Park *et al.*, 2000; Lawrence & Koundal, 2002).

II.1.4 Metallocarboxypeptidase inhibitors

Metallocarboxypeptidase inhibitors have been identified in solanaceous plants (tomato and potato), medicinal leech (*Hiruda medicinalis*), rats and humans (Homandberg *et al.*, 1989; Normant *et al.*, 1995; Reverter *et al.*, 1998; Arolas *et al.*, 2005; Kehoe *et al.*, 2016). Metallocarboxypeptidase inhibitors consist of a protein of 38-39 amino acid residues with a molecular mass of about 42 kDa (Hass *et al.*, 1975; Hass & Hermodson, 1981). These PIs inhibit strongly broad spectra of carboxypeptidases from both animals and microorgamisms but not from yeast or plants (Haukioja & Neuvonen, 1985).

II.2 Role of proteinase inhibitors in plants

According to recent studies, plant PIs may actively participate in regulation of proteolytic processes, act as storage proteins and serve as an important element in plant defense against pests and phytopathogenic microorganisms (Mosolov & Valueva, 2005).

II.2.1 Effect on plant proteinases

Storage proteins are mainly represented by cysteine proteinases of papain and legumin family (Shutov & Vaintraub, 1987; Müntz & Shutov, 2002). The first inhibitor able of suppressing the activity of an endogenous cysteine proteinase was found in barley seed (Mikola & Enari, 1970). The amount of inhibitor in seeds decreases in the course of germination coupled with the increase in the proteinase activity (Enari & Mikola, 1967; Kumar *et al.*, 2006). This phenomenon was largely studied in rice, where oryzacystatins I and II suppress the activity of seed cysteine proteinases (Orzains), which cleave glutelin, the major storage protein in rice (Abe *et al.*, 1987; Arai *et al.*, 2002). Both oryzacystatin are synthesized in maturing seeds. With the onset of germination, the inhibitors undergo decomposition. The synthesis of cystatins in seeds is characterized by the highest intensity at developmental stages preceding the accumulation of storage proteins (Kuroda *et*

al., 2001). This suggests that these PIs prevent mature proteolytic degradation of the newly formed storage protein (Arai *et al.*, 2002; Sin & Chye, 2004).

II.2.2 Effect on programmed cell death

PIs may also play an important role in programmed cell death (PCD) which takes place in the course of development and aging of plant tissues (Beers *et al.*, 2000). The application of an exogenous trypsin was able of activating PCD during xylogenesis in zinnia (*Zinnia elegans L.*). This process can be suspended by the SKTI (Soybean Kunitz type proteinase inhibitor) (Li *et al.*, 2008). Another form of PCD in plants is the hypersensitivity response to phytopathogenic infections (Heath, 2000). This process has much in common with apoptosis in animals. Cysteine proteinases of the caspase family play an important role in the development of apoptosis (Hengartner, 2000). Synthetic peptide inhibitors of caspase were shown to suppress the process of PCD induced by *Pseudomonas syringae pv. phaseolicola* or tobacco mosaic virus in tobacco, suggesting the existence of plant caspase-like activity (del Pozo & Lam, 1998). This was later confirmed by different studies (De Jong *et al.*, 2000; Coffeen & Wolpert, 2004; Chichkova *et al.*, 2004; Vartapetian *et al.*, 2011). Moreover, it has been demonstrated that PCD in soybean, induced by the pathogen *Pseudomonas syringae pv.*

glycinea is associated with the synthesis of papain-like cysteine proteinases, correspondingly, the induction of cystatin synthesis blocked PCD (Solomon *et al.*, 1999). Also, in *Avena sativa*, a serine proteinase was associated with the activation of PCD (Coffeen & Wolpert, 2004).

II.2.3 PIs as storage proteins

The idea that PIs may serve as storage proteins is suggested by the high content in PIs in seeds and other storage organs and their dynamics in the course of seed maturation and germination (Shewry, 1995; Genov *et al.*, 1997; Shewry, 2003). It has also been demonstrated that certain PIs belong to the same protein families as storage proteins, suggesting a common origin. On the other hand, certain typical storage proteins of plant, exhibit activities of PIs. An example is the case of *Aspargus pea* 2S albumin, psophocarpin B (Roy & Singh, 1988; Agizzio *et al.*, 2003).

II.2.4 PIs as plant defense proteins

The defensive role of PIs was first discovered by Green and Ryan (1972) , showing that these proteins are able to inhibit insect gut proteases. Later, several PIs have shown a defensive effect against pests by direct assay or by expression in transgenic plants (Oliveira *et al.*, 2014; Medel *et al.*, 2015; Armstrong *et al.*, 2016). Phytophagous insects specificity

differs according to the predominant protease produced in their gut. More specifically, different taxonomic clades of arthropods seem to predominantly produce different protease types that function optimally at different gut pH (Jongsma & Bolter, 1997; Saikia *et al.*, 2010). Most coleopterans have and acidic midgut and produce primarily cysteine or aspartate proteases (Schlüter *et al.*, 2010), while lepidopterans have an alkaline midgut and produce primarily serine proteases (Srinivasan *et al.*, 2006; Saikia *et al.*, 2010). Thus it is expected that the presence of both SPIs and CPIs in the same plant could increase plant resistance to different types of phytophagous insects by affecting their specific gut proteases (Jongsma & Bolter, 1997; Abdeen *et al.*, 2005; Oppert *et al.*, 2005).

Expressing different PIs in transgenic plants confirmed the important role played by these proteins in plant defense against pests and pathogens. This was first performed by Hilder et al., (1987). The gene encoding the cowpea trypsin inhibitor (CpTI) was expressed in tobacco (*Nicotiana tabacum* L.). The damage caused by the tobacco budworm larvae was 50% lower than in the control plants (Boulter *et al.*, 1990). These plants were also more resistant to other insects of the Lepidoptera order (Xu *et al.*, 1996). Subsequently, other PIs were expressed in other plants conferring them resistance to a wide range of pests (Carrillo

et al., 2011; Saadati & Bandani, 2011; Rufino *et al.*, 2013; Quilis *et al.*, 2014).

II.3 Proteinase inhibitors mechanism of action

Many studies have been dedicated to investigate the mechanism of action of PIs. Different inhibition mechanisms have been suggested: canonical, indirect, adjacent and allosteric (Figure 2).
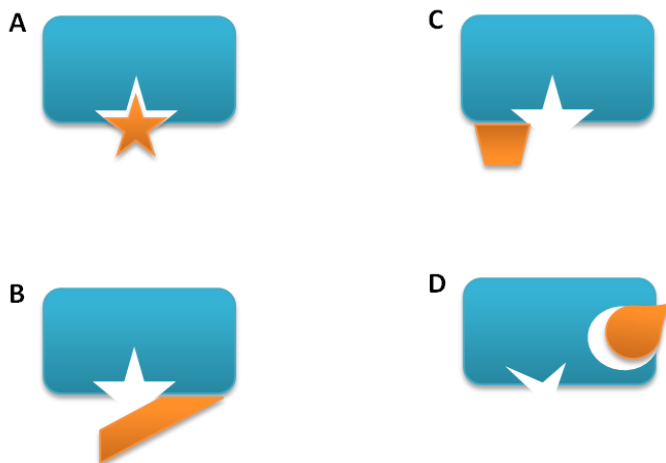
Figure 2: Different Proteinase inhibitor/Proteinase interaction modes. A: Canonical inhibition. **B:** Indirect blockage of active center. **C:** Adjacent / exosite binding. **D:** Allosteric interaction.

The most studied are SPIs, however it has been suggested that cysteine PIs and aspartate PIs act in the same way according to the mechanism proposed by Laskowski and

Kato (1980). The active-site substrate binding region of the protease binds to the corresponding substrate–like region (reactive site) on the surface of the inhibitor, leading to the inhibition of the protease. On the surface of each PI lies one or more (for multi-headed inhibitors) peptide bond known as reactive site which specifically interacts with the active site of a cognate enzyme. The value of Kcat/Km for the hydrolysis of this peptide bond by the cognate enzyme at neutral pH is very high (10^4-10^6 M^{-1} s^{-1}), (Estell *et al.*, 1980; Haq *et al.*, 2004) compared to a typical value for a normal substrate (about 10^3 M^{-1} s^{-1}). However the value of Kcat and Km for the inhibitor is both much lower than the value of normal substrate. Therefore, their hydrolysis is extremely slow and the system acts as if it was a simple equilibrium between the enzyme and free inhibitor on one hand and the complex on the other hand. The reactive site peptide bond of the inhibitor and, after hydrolysis, acquires a newly formed carboxyterminal residue designated as P1. Inhibitors with P1 Lys and Arg tend to inhibit trypsin and trypsin-like enzymes, while those with P1 Tyr, Phe, Leu and Met inhibit chymotrypsin and chymotrypsin-like enzymes. Inhibitors with P1 Ala and Ser inhibit elastase-like enzymes.

III. PIs herbivory induced signaling in plant

Mechanical wounding is not the only elicitor of signal pathway leading to PIs gene expression. Oligosaccharides fragments released from the plant cell wall, endopolygalacturonases, fungal cell wall chitosan oligomers and insect oral secretions also act as elicitors of the octadecanoid signal pathway involved in the induction of PIs gene expression. Early after plant is attacked by phytophagous insects, prosystemin is converted to systemin and together with the other wound signals binds to putative receptors in the plasma membrane. This generates a Ca^{2+} influx which depolarizes the cell membrane. Ca^{2+} is a second messenger known to be involved in multiple signal transduction pathways of environmental and developmental physiological changes (Lecourieux *et al.*, 2006). Subsequently mitogen-activated protein kinases (MAPKs), such as SIPK and WIPK, are activated. The MAPKs signaling cascade is a conserved pathway involved in different cellular responses in eukaryotes (Herskowitz, 1995; Chang & Karin, 2001; MapkGroup *et al.*, 2002). MAPKs activate a phospholipase that facilitate the release of α-linolenic acid from the chloroplast membrane. α-LeA is then converted to jasmonic acid via the octadecanoid pathway. First, S-adenosyl-L-Met is converted to 1–amincyclopropne-1-carboxylic acid (ACC) by the ACC synthase (ACS). Then

the ACC is oxidized to form ethylene by means of the ACC oxidase (ACO). MAPKs also promote the synthesis of ethylene. Studies in different plant species have shown that ethylene is necessary for the elicitation of PI's mRNA (O'Donnell *et al.*, 1996; Rakwal *et al.*, 2001; Jones *et al.*, 2005). Jasmonic acid together with ethylene and WIPK activate transcription factors responsible for the expression of PIs genes (Figure 3).
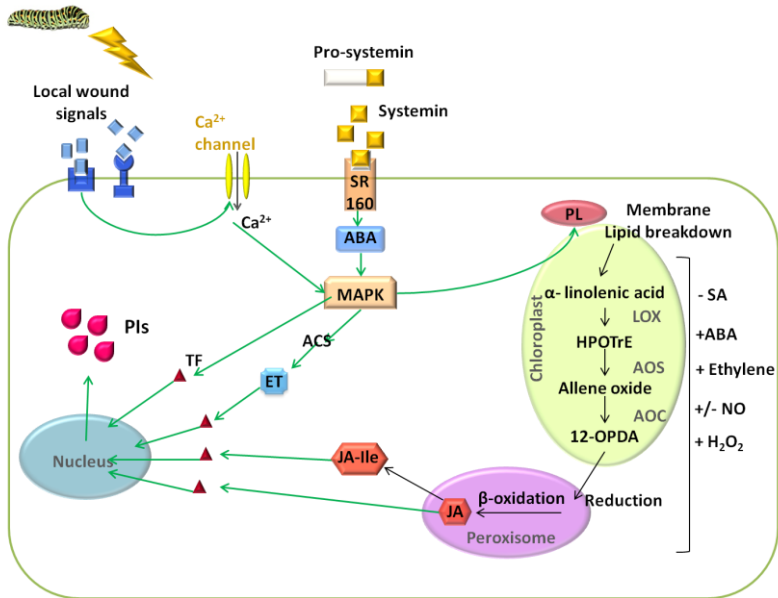
Figure 3: Intracelular wound signal transduction pathway leading to the induction of PI gene expression.

IV. Biotechnological applications of PIs in crop pest resistance improvement

The first transgenic plant harboring a foreign PI was generated by Hilder *et al.* (1987). The Cowpea trypsin inhibitor (CpTI) was expressed in tobacco. The transgenic plants showed enhanced resistance against the tobacco budworm *Manduca sexta* (Linnaeus) (Lepidoptera: Sphingidae). Later on, the same gene was transferred to different plant species like oil palm and potato, enhancing their resistance to bugworm larvae and tomato moth, and gregarious ectoparasitoids respectively (Bell *et al.*, 2001; Abdullah *et al.*, 2003). Afterwards, several transgenic plants expressing PIs from different origins have been produced. Since the economically important insect orders: Lepidoptera, Diptera and Coleoptera, use serine and cysteine proteinase for their digestive process, studies have particularly focused on genes encoding PIs active against these mechanistic classes of proteases. The potato proteinase inhibitor II was introduced in rice and the transgenic plants showed increased resistance to the pink stem borer (*Sesamia inferens* (Walker)(Lepidoptera: Noctuidae) in greenhouse trials (Duan *et al.*, 1996). In another study, Gutierrez-Campos et al. (1999) expressed the rice cystatin Oryzacystatin I in tobacco plants and induced resistance to two viruses: the Tobacco Etch Virus (TEV) and the Potato Virus Y (PVY). Other

studies have expressed the barley trypsin inhibitor BTI-CMe in wheat (*Triticum aestivum*) and rice (*Oryza sativa*). The transgenic plant showed a significant reduction of survival respectively on *Sitotroga cerealella* (Olivier) (Lepidoptera: Gelechiidae) larvae (Altpeter *et al.*, 1999) and the rice weevil *Sitophilus oryzae* (Linnaeus) (Coleoptera: Curculionidae) (Alfonso-Rubí *et al.*, 2003).

V. Insect adaptation to PIs

Most of the transgenic plants obtained considerably exceeded the wild counterparts in the resistance to insects and other pests. Nevertheless, insects can adapt to proteinase inhibitors in their diet through different mechanisms (Agrawal, 2001; Oppert *et al.*, 2005). These mechanisms include the stimulation of proteinase activity as well as the increase production of inhibitors-insensitive enzymes (Broadway & Duffey, 1986; Jongsma *et al.*, 1995; Gatehouse *et al.*, 1997; Mazumdar-Leighton & Broadway, 2001; Rivard *et al.*, 2004). This enzymatic response could occur within the same proteinase class, replacing one serine proteinase by another, or by producing a proteinase of a different class. Previous studies have shown that the presence of plant cystatins in the diet of *Callosobruchus maculatus* (Fabricius) (Coleoptera: Bruchidae) induced the production of aspartic protease activity (Zhu-Salzman *et al.*, 2003). In the insect gut,

proteinases are compartmentalized in regions providing maximal activity and better stability for each protein. Because of this compartmentalization, the shift in production of one proteinase from one class to another would not be straightforward and be rather difficult for the insect to accomplish (Oppert *et al.*, 2005). The larvae of *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), use primarily cysteine proteinase in food digestion (Oppert *et al.*, 2005). When fed an artificial diet including serine and cysteine PIs, a synergetic effect is observed, inhibiting the growth of *Tribolium castaneum* larvae (Oppert *et al.*, 2005). The combination of two inhibitors of different families could prevent the shift of the insect digestive enzymes from one class to another (Oppert *et al.*, 2005). Combining the expression of PIs of different classes may represent an interesting strategy to counter the insect response. To avoid insect adaptation, it is suggested to select PIs from unrelated plants. It has been shown that insects feeding on dicots cannot adapt to PIs from monocots and vice versa.

VI. Tomato proteinase inhibitors

In tomato leaves, wounding induces the accumulation of two non homologous serine proteinase inhibitors called PIN1 and PIN2. The tomato leaf inhibitors are very similar to potato tuber inhibitors I and II in subunit molecular weight,

composition and inhibitory activities against chymotrypsin and trypsin. However, unlike the potato tubers, tomato leaves exhibit only two isoforms of inhibitor I and a single form of inhibitor II. PIN1 with a molecular mass of 8.1 kDa, is a chymotrypsin inhibitor that weekly inhibits trypsin at its single reactive site (Johnson *et al.*, 1989; Haq *et al.*, 2004). Whereas PIN2 has a molecular mass of 12.3 kDa and contains two reactive sites one of which inhibits trypsin and the other inhibits chymotrypsin (Ryan, 1990). Both inhibitors are synthesized as precursors and undergo postranslational modifications to form the mature protein (Johnson *et al.*, 1989; Ryan, 1990). Expression of both genes is induced by jasmonic and abscicic acids (Wasternack *et al.*, 2006).

VII. Barley Proteinase inhibitors

VII.1 Barley serine proteinase inhibitors

Proteinase inhibitors belonging to the family of α-amylases and trypsin inhibitors have been identified. These proteins can be selectively extracted with chloroform/methanol mixture and therefore have been named CM proteins. Barley trypsin inhibitor CMe (BTI-CMe) is the best characterized member of this family (Rodriguez-Palenzuela *et al.*, 1989; Royo *et al.*, 1996; Alfonso-Rubí *et al.*, 2003). It belongs to the same subfamily as the trypsin inhibitor from Rye (RTI), maize (MTI) and Ragi (RBI) (Wen *et al.*, 1992). BTI-CMe is

encoded by the locus *Itr1*. Southern blot analysis of wheat/barley addition lines have assigned it to the 3HS chromosome. This inhibitor is highly active against trypsin and inactive against chymotrypsin, papain, pepsin, bacterial and fungal proteases and the endogenous barley proteases (Odani *et al.*, 1983; Lara *et al.*, 2000; Alfonso-Rubí *et al.*, 2003). The reactive site of BTI-CMe is the motif Glycine-Proline-Arginine-Leucine (GPRL). The same reactive site appears in RTI, MTI and RBI.

Another member of this family is BTI-CMc encoded by the gene *Itr2* located in the chromosome 7HS. The barley trypin inhibitor CMc presents low activity against trypsin (about the third of CMe) and no α-amylase activity. The later protein is invariant among barley varieties while BTI-CMe is polymorphic. Four allelic variants have been identified in domesticated barley BTI-CMe1-4.

VII.2 Barley cysteine proteinase inhibitors

In barley, thirteen cystatins have been identified: Hv-CPI1 to 13, encoded by the genes *Icy1* to *13*. These PIs have shown different gene structure, variation in mRNA patterns and important differences in the amino acid sequences (Martínez *et al.*, 2005; Abraham *et al.*, 2006). They share with animal cystatins motifs involved in the interaction with their target enzymes. Hv-CPI proteins share the reactive site QxVxG

located between β2 and β3 sheets with the exception of CPI9 where the third residue V is substituted by an I and CPI7-12-13 where the fifth G residue is changed either by S or E. The conserved W situated in the loop between β4 and β5 sheets is suggested to be compulsory for the interaction with the cognate enzyme is also conserved except in Hv-CPI7 and 11. All barley cystatins, except Hv-CPI7, inhibit *in vitro* papain and cathepsin L or B. Hv-CPI2 have shown a strong inhibitory activity against papain and phytopathogenic fungis *Botrytis cinerea* and *Fusarium oxysporum* (Abraham *et al.*, 2006). This inhibitor belongs to the group A of cystatins including Hv-CPI1 and OCI, suggested to have a wide range of target enzymes (Abraham *et al.*, 2006).

VIII. Tomato as a Solanaceae model plant

Tomato (*Lycopersicon esculentum*) represents the second horticultural most important crop after potato. According to the FAO (Food and Agriculture Organisation), about 145 million tons of fresh tomato fruits (FAOSTAT, 2011) are produced and cultivated annually in 4.5 million hectares worldwide. The release of the tomato genome sequence (Mueller *et al.*, 2009) and the development of efficient *Agrobacterium* transformation protocols (Ellul *et al.*, 2003; Di Matteo *et al.*, 2011) make this crop an interesting model plant. The miniature tomato Micro-Tom is a cherry type

tomato variety carrying two recessive genes conferring the dwarf genotype. This cultivar presents different attractive features like its short life cycle (70-90 days from sowing to fruit ripening), small size, and small genome (950 Mb) and therefore is considered a model cultivar for tomato genetics and functional genomics research. This variety was obtained by crossing Florida basket and Ohio 4013-3 varieties (Scott, 1989). Micro-Tom tomato has short life cycle, short internodes and small fruits. This phenotype is due to the presence of three mutations. The first one affects the Self-pruning (SP) gene that controls the determined/indetermined inflorescence phenotype (Pnueli *et al.*, 1998). The second is a punctual mutation of the Dwarf (D) gene that encodes for the 6-deoxocastasterone deshydrogenase implicated in the brasinosteroids biosynthesis pathway (Lima *et al.*, 2004). The mutation of the D gene results in small, dark and rough leaves. The third mutation is still uncharacterized and is responsible for the short internodes phenotype.

IX. South American tomato borer: *Tuta absoluta*

Nowadays, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) represents the most harmful tomato pest. This Lepidoptera was first described by Meyrick in 1917 in the Peruvian Andes (Meyrick, 1917). In the last decade, this Lepidoptera invaded several European countries and spread

to the whole Mediterranean basin. After this rapid invasion, 21.5 % of the cultivated surface and 27.2 % of tomato production are now infested by *Tuta absoluta* (FAOSTAT, 2011), resulting in an important environmental and economic issue (Desneux *et al.*, 2011).

IX.1 *Tuta absoluta* biology

Tuta absoluta is a micro-lepidoptera originated in South America that develops in tomato leaves, stem and fruit mesophyll, causing serious damages. Like all Lepidoptera, its life cycle is composed of four developmental stages: egg, larva, pupa and adult (Figure 4).

The egg has an ovoid shape of about 0.4 mm height and 0.2 mm diameter. Just after laying, the egg has a white-creamy color and gets darker before hatching (Estay, 2000). The larval stage is composed of 4 instars. Larvae of the fist instar has a creamy yellowish color with a dark head and measures about 1.6 mm. About 5-40 minutes after hatching, the larva starts mining and feeding on the leaves (Estay & Vásquez, 2002). When feeding in plant mesophyll, the larvae grow and acquire a darker green color. Larvae of the second instar are about 2.8 mm long. In the third instar, larvae become greener and increase their size reaching 4.7 mm. In the fourth larval instar, it acquires a dorsal red colored band. In this last larval instar, it can reach 9 mm (EPPO, 2011). When food is

available, the insect feeds continually and no diapause is observed. Just before the pupation (prechrysalis), it stops feeding and helped by a silk filament, falls to the soil, where it achieves its pupal development. The pupa measures 4.3 mm and has 1.1 mm diameter. Recently formed pupa show a green color that gets brownish before adult emergence. It is generally covered by a white silky cocoon (Apablaza, 1992). Adults measure about 7 mm with 10 mm wingspan for males and 11 mm for females. They present filiforme antennae (Larraín, 1987; EPPO, 2011). The brown abdomen is wider in females than in males (Estay, 2000). The duration of the developmental cycle depends mainly on temperature. It varies between 76.3 days at 14 ºC and 23.8 days at 27 ºC (Barrientos *et al.*, 1998). *Tuta absoluta* is a multivoltine specie with a high reproductive potential. The number of generation per year is between 10 and 12 (Barrientos *et al.*, 1998; EPPO, 2011).
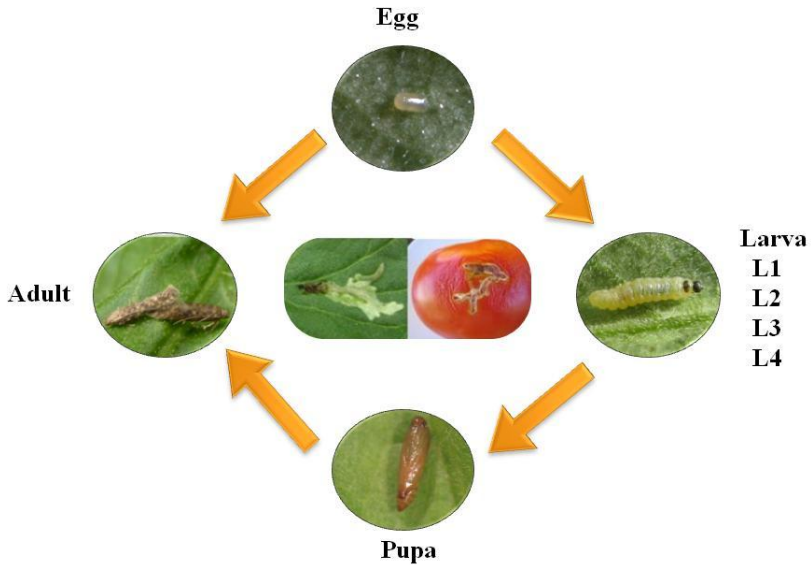
Figure 4: *Tuta absoluta* developmental cycle (egg, larva L1-L4, pupa and adult) and damages in tomato leaf and fruit.

IX.2 Symptoms and damages

Tuta absoluta adults are attracted by tomato volatiles. However, it can also damage other Solanaceae species such as potato, eggplant and *Solanum nigrum* L. (Vercher *et al.*, 2007; Viggiani *et al.*, 2009). The larvae can attack tomato plants of any development stage from seedling to mature plants (EPPO, 2006). Minutes after hatching, the larva penetrates between the two epidermises and starts feeding of the mesophyll digging translucide galleries (Uchoa-Fernandes *et al.*, 1995; Duarte *et al.*, 2015). Occasionally,

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the larva gets out of the gallery and attacks a new leaf, increasing the damage to the plant (Estay, 2000; Urbaneja *et al.*, 2008). Larvae prefer feeding on young leaves but they can also damage tomato flowers, stem and fruits (López, 1991; Desneux *et al.*, 2010).

Figure 5: Damages caused by *Tuta absoluta* in tomato. A: Damaged tomato leaf with galleries. **B:** Damaged tomato fruit. **C:** damaged tomato stem**. D:** damaged tomato flower.

IX.3 Geographical Distribution

For over 40 years, *Tuta absoluta* distribution was restricted to South America. After its first detection in South Spain in late 2006 (Vercher *et al.*, 2007) the pest spread to Europe, North Africa and Middle East. In about 5 years, *Tuta absoluta* spread approximately 4000 Km. Tomato fruit trading seems to be the first responsible for its rapid invasion.
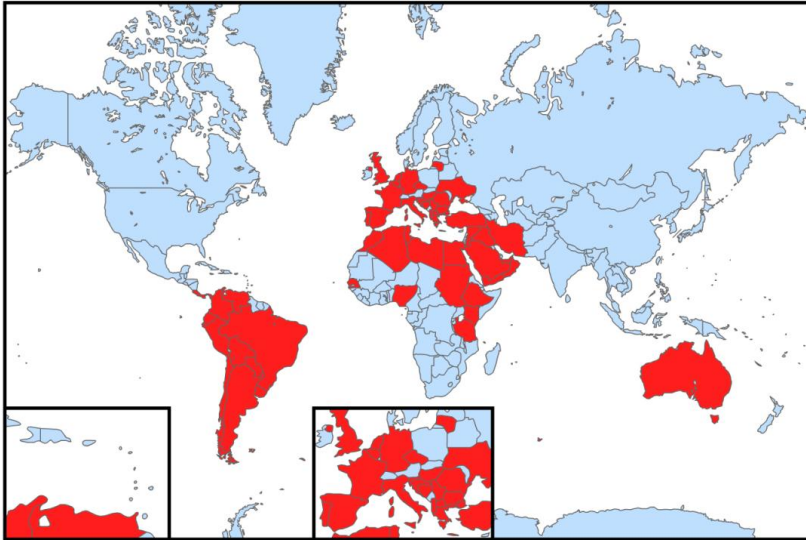
Figure 6: World distribution of *Tuta absoluta*.

IX.4 Biological control of *Tuta absoluta*

Since its introduction, chemical control has been the main method used to control *Tuta absoluta* leading to a multitude of undesired side effects on non target organisms (Arnó *et al.*, 2010; Biondi *et al.*, 2012a; Biondi *et al.*, 2012b). As an alternative, different integrated pest management strategies with parasitoids and predators have been tested.

IX.4.1 Parasitoids

In South America, about 50 parasitoids of *Tuta absoluta* eggs and larvae have been identified (Desneux *et al.*, 2010). Parasitoids are fundamentally hymenopterans: Encyrtidae,

Eupelmidae and Trichogrammatidae (*Trichogramma spp.* is the predominant). In the Mediterranean region, some egg parasitoids have also been reported (Desneux *et al.*, 2010). Among them, *Trichogramma achaeae* (Nagaraja and Nagarkatti) (Hymenoptera: Trichgrammatiddae), is commercially used for the control of *Tuta absoluta* (Cabello *et al.*, 2009). This parasitoid was able to reduce *Tuta absoluta* damage. However, it is not able of reaching adult stage parasitizing *Tuta absoluta* and subsequently cannot reproduce (Urbaneja *et al.*, 2012). Therefore its use should be combined with other control strategies.

IX.4.2 Predators

In South America, studies have reported that 79.8 % of the larval mortality in *Tuta absoluta* was caused by depredators like *Xylocoris* sp. (Heteroptera: Anthocoridae), *Cycloneda sanguine* (Linnaeus) (Coleoptera: Coccinellidae) (Miranda *et al.*, 1998; Urbaneja *et al.*, 2008).

In Spain, autochthonous mirids: *Nesidiocoris tenuis* Reuter and *Macrolophus pygmaeus* (Rambur) (Hemiptera: Miridae) are natural predators of *Tuta absoluta* feeding on eggs and larvae that appear spontaneously in attacked tomato fields
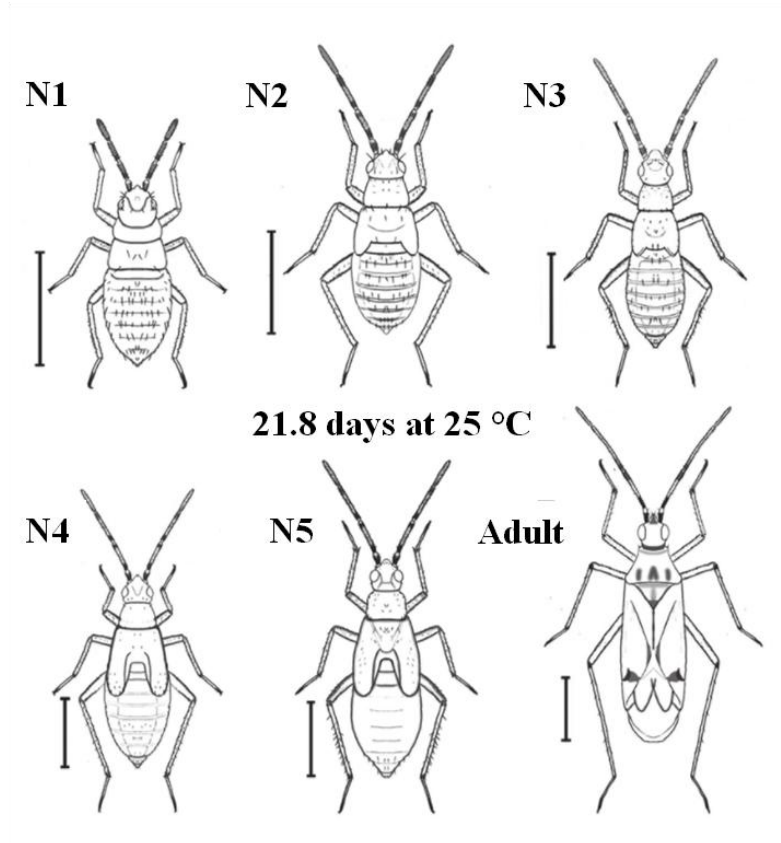
Figure 7: *Nesidiocoris tenuis* **and** *Macrolophus pigmaeus* **developmental stages.**

Both *N. tenuis* and *M. pygmaeus* have a hemimetabolic development, like other Hemiptera. Their development cycle comprises three stages: egg, nymph and adult. There are five wingless nymphal instars (N1-N5) before reaching adult

stage (Figure 7). After each nymphal instar, the insect grows in size and molts.

These two mirids are the most used depredators for the control of *Bemicia tabacci* (Gennadius) (Homoptera: Aleyrodidae) and *Tuta absoluta* in Europe. However this strategy requires high number of predators (Calvo *et al.*, 2009). This high population density can be reached between 5 and 8 weeks after mirids realease and this time is sufficient for *Tuta absoluta* to produce high damage to the crop. Moreover, *Nesidiocoris tenuis* and *Macrolophus pigmaeus* (Figure 8) are zoophytophagous. When predator density is elevated, it feeds on tomato plants. *Nesidiocoris tenuis* feeds on vascular tissues producing brown necrotic rings (Castañé *et al.*, 2011). These damages can provoke flowers and small fruits abortions, stem and leaves growth delay. *Nesidiocoris tenuis* can also feed on leaves and fruits causing yield and economical losses (Alomar & Albajes, 1996; Shipp & Wang, 2006; Sanchez, 2009; Arnó *et al.*, 2010).
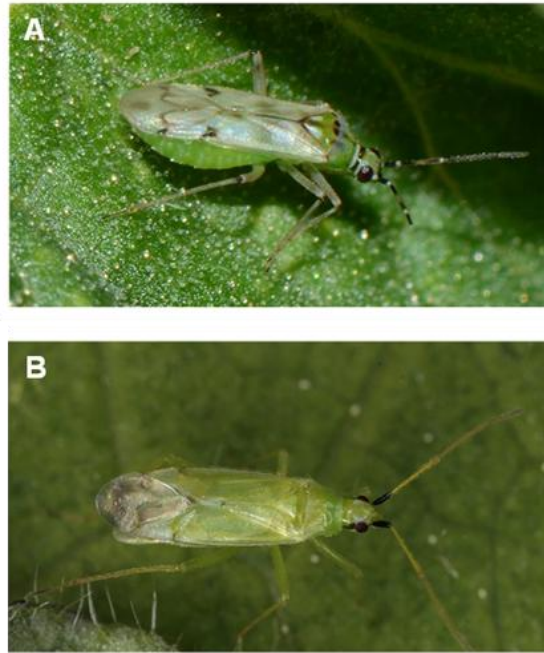
Figure 8: A: *Nesidiocoris tenuis* **adult. B:** *Macrolophus pygmaeus* **adult.**

Objectives

Genetic engineering is a powerful tool to improve plant pest resistance. It allows the increase of the genetic diversity of pest resistance traits and the reduction of the negative impact of arthropods on crop yield. As primary gene products, proteinase inhibitors are promising candidates to challenge pest attack. Tomato represents the second most important horticultural crop in the world. Over one quarter of its production is now infested by *Tuta absoluta*, causing environmental and economical concerns. Genetic engineering could be a useful strategy to improve tomato plant resistance and lower the losses caused by the Lepidoptera.

In this context, our **general objective** was to study the usefulness of the co-expression of two proteinase inhibitors as a molecular tool to enhance plant resistance.

Specific objectives:

- Improve the barley trypsin inhibitor BTI-CMc enzymatic activity by site directed mutagenesis.
- Study the effect of feeding two barley proteinase inhibitors on *Tuta absoluta*.
- Check the innocuity of the expressed proteinase inhibitors on *Tuta absoluta* natural enemy, *Nesidiocoris tenuis*.
- Investigate the impact of the foreign proteinase inhibitors expression on tomato endogenous defensive mechanisms.

Materials & Methods

I. Plant material and growth conditions

I.1 Plant material

In this work we used the barley *Hordeum vulgare* cultivar Rihane from the germplasm collection of the Regional commission for agricultural development (Gabes, Tunisia) to isolate proteinase inhibitor genes.

The ornamental tomato *Solanum lycopersicum* cv. Micro-Tom (IBMCP seed collection, Spain) was used to produce transgenic plants.

I.2 Growth conditions

Barley seeds were germinated in the darkness on vermiculite substrate under greenhouse conditions at 25–30 °C (day) and 18–20 °C (night) and were irrigated daily with Hoagland's solution (Hewitt, 1966).

Tomato plants were grown in pots with coconut fiber under standard greenhouse conditions and were irrigated daily with Hoagland's solution (Hewitt, 1966). Natural light was supplemented with Osramlamps (Powerstar HQI-BT, 400W) to get a 16 h light photoperiod.

II. Microorganisms

II.1. Bacterial strains

The bacterial strains used in this study are summarized in the table below:

Bacteria	Strain	Transformation method	Growth temperature
E. coli	DH5α	Heat shock	37 ºC
E. coli	DH10B	Electroporation	37 ºC
E. coli	BL21(DE3)pLysS	Heat shock	37 ºC
A. tumefaciens	LBA4404	Heat shock	28 ºC

Table 1: Bacterial strains used in this work.

II.2. Culture media

All bacterial strains were grown on Luria Bertani (LB) medium: 1 % tryptone, 0.5 % yeast extract and 1 % NaCl at pH 7.0. For culture on solid medium, 1.5 % of bacteriological agar (Pronadisa) was added.

II.3. Competent bacteria preparation

II.3.1 Thermo-competent cells

One colony of *Esherichia coli* (DH5α or BL21(DE3)pLysS) was resuspended in 5 ml LB medium and incubated overnight at 37 ºC under agitation at 200 rpm. The next day, the

bacterial suspension was diluted in 195 ml LB medium and incubated at 37 ºC in rotation until OD_{600}= 0.5 approximately. Then, the bacterial culture was transferred to pre-cooled tubes and centrifuged at 6000 rpm for 5 min at 4 ºC. The supernatant was then discarded and the pellet resuspended in 60 ml of TF1 solution (RbCl 1 M, MnCl 0.5 M, KAc 0.3 M, $CaCl_2$ 0.1 M and Glycerol 15 % v/v, pH 5.8). The solution was centrifuged 5 min at 6000 rpm at 4 ºC and the supernatant discarded. 16 ml of TF2 (RbCl 0.1 M, $CaCl_2$ 0.75 M, MOPS 0.1 M and glycerol 15 % v/v, pH 7) solution were later added to resuspend the pellet. The obtained bacterial suspension was aliquoted in 150 μl individual tubes, chilled in liquid nitrogen and stored at -80 ºC until use.

II.3.2 Electro-competent cells

One colony of *Agrobacterium tumefaciens* (LBA4404) or *E. coli* DH10B was suspended in 200 ml of LB medium and incubated under agitation at 200 rpm until absorbance reaches 0.5-0.7. The incubation was realized at 37 ºC for *E. coli* and at 28 ºC in presence of 2 ml $MgSO_4$ 1 M for *A. tumefaciens*. The bacterial culture was then transferred to pre-cooled tubes and centrifuged for 10 min at 6000 rpm at 4 ºC. The pellet was recovered and washed four times in decreasing volumes of ice cold glycerol 10 % (once with 200 ml, once with 100 ml and twice with 4 ml). Finally, the pellet

was resuspended in 2 ml of ice cold glycerol 10 % and the bacterial solution aliquoted in 40 µl tubes. The competent cells were then frozen in liquid nitrogen and stored at -80 ºC.

II.4 Bacterial transformation

- Heat chock transformation

An *E. coli* DH5α competent cells aliquot was first thawed on ice. Then, 1 µl of plasmid was added. The mixture was first incubated in ice for 30 min, then rapidly transferred to a water bath at 42 ºC for 90 s and then back to ice for 2 min. The transformed bacterial cells were later resuspended in 800 µl LB medium and incubated 1 hour at 37 ºC under 200 rpm agitation. The suspension was then plated on LB solid medium supplemented with carbenicillin (100 mg/L).

- Electroporation

E. coli DH10B and *A. tumefaciens* were transformed by electroporation. To an aliquot of competent cells, 1 µl of plasmid was added. The mix was then transferred to a pre-chilled electroporation cuvette (Biorad). The electroporation was performed at 200 Ω, 25 µF and 1.8 kV for *E. coli* and 400 Ω, 25 µF and 1.8 kV for *A. tumefaciens*. The bacteria were then resuspended in 800 µl LB medium and incubated 1 h at 37 ºC for *E. coli* and 3 h at 28 ºC for *Agrobacterium*. After the incubation, *E. coli* bacteria were plated on LB

medium with 100 mg/L spectinomycine and *Agrobacterium* on LB medium with 100 mg/L spectinomycine and 100 mg/L rifampicin.

III. Nucleic acids purification

III.1. Plasmid DNA extraction

- ### *Escherichia coli*

One colony of *E. coli* was inoculated in 3 ml of LB and incubated overnight at 37 ℃ under agitation (200 rpm). The next day, the plasmid DNA was extracted with the E.Z.N.A Plasmid DNA Mini Kit I (OMEGA, BIO-TEK) according to the manufacturer recommendations.

- ### *Agrobacterium tumefaciens*

One colony of *Agrobacterium tumefaciens* was inoculated in 3 ml LB medium supplemented with antibiotics and incubated for 2 days at 28 ℃ under agitation (200 rpm). Afterwards, the suspension was centrifuged at 12000 rpm for 5 min and the pellet resuspended in 150 µl of Solution I (50 mM Tris pH 8.0 with HCl, 10 mM EDTA, 100 µg/ml RNase A). Next, 150 µl of Solution II (200 mM NaOH, 1 % SDS) were added and the suspension mixed. The tubes were, then, incubated 5 min at room temperature (RT) and 1 min on ice. Later on, 150 µl of solution III (3 M Potassium Acetate, pH 5.5) were added and the solution mixed by inversion, then,

incubated 10 min on ice. After the incubation, the tubes were centrifuged at 13000 rpm at RT for 10 min and the supernatant recovered in a new tube. Then, two volumes of absolute ethanol were added and the tubes incubated 30 min at -20 ℃. The solution was later centrifuged 10 min at 13000 rpm and the supernatant discarded. The pellet was dried and dissolved in 20 µl of distilled water.

III.2. Genomic DNA isolation

Three tomato young leaves were collected and frozen in liquid nitrogen. The leaves were ground in 300 µl of extraction buffer (Tris HCl 0.2 M, LiCl 0.4 M, EDTA 0.2 M, 1 % SDS w/v). The samples were spinned at 13000 rpm for 5 min at RT and the supernatant transferred to a new tube. One volume of ice cold isopropanol was added and the solution mixed by inversion. The solution was centrifuged for 10 min at 13000 rpm and the supernatant discarded. The resulting pellet was dried and washed with 500 µl of 70 % ice cold ethanol. The recovered pellet was dried and resuspended in 200 µl of distilled water.

III.3. Total RNA purification

For barley RNA extraction, 500 µg of 12 days etiolated leaves were recovered and frozen in liquid nitrogen. For tomato RNA purification, 300 µg of young leaves frozen in

liquid nitrogen were used as starting material. The total RNA purification was performed with the RNeasy® Plant Mini Kit (OMEGA, BIO-TEK) according to the manufacturer recommendations.

III.4. Nucleic acid quantification

The purified DNA and RNA were quantified with a spectrophotometer NanoDrop® ND-100 at 260 nm.

IV. RNA retrotranscription

To eliminate any residual genomic DNA, 5 µg of the purified RNA were treated by DNase using the Turbo DNA Free™ kit (Ambion). Subsequently, 1 µg of treated RNA was retro-transcribed to cDNA using the PrimeScript™ reagent kit (Takara). cDNA first strand is synthesized from RNA by the Primescript™ enzyme, an MMLV (Molony Murine Leukemia Virus) and oligo dT primer.

V. DNA amplification by PCR

DNA fragments were amplified using 50 ng as template. The mixture was composed of 2.5 µl of buffer (10x), 1 µl dNTPs (10 mM), 1 µl of each specific primer (10 mM), 1 µl $MgCl_2$ (50 mM) and 1 U of Taq polymerase (Biotools®). The specific primers used for each fragment are described in Table 2. The amplification was achieved according to the

following program: a pre-melting at 94 ºC for 5 min, then 35 cycles of three steps (melting at 94 ºC for 30 s, annealing at primers specific temperature for 30 s and elongation at 72 ºC for 30 s) followed by a final elongation at 72 ºC for 5 min. For semi-quantitative RT-PCR, cycle's number was reduced to 30.

Primer	Primer sequence	Annealing T°
CMeT S	ATGTTCGGGGATATGTGTGCT	55 ºC
CMeT AS	TTACAAGACCACTTCATATCC	55 ºC
T35SF-Spe	ACTGACTAGTTGTGATATCCCGCGGCCAT	52 ºC
T35SR-Sal	ACTGGTCGACGCAGGTCACTGGATTTTGGT	52 ºC
P35SF-Sph-Sal	ACTGGCATGCACGTCGACCAAGCTGATCTC CTTTGCCCC	52 ºC
P35SR-SacII	ACTGCCGCGGCCGGAGTCCTCTCCAAATGA	52 ºC
SlActin8-F	CAAGTTATTACCATTGGTGCTGAGA	55 ºC
SlActin8-R	TGCAGCTTCCATACCAATCATG	55 ºC
Kan-dir	GACAAGCCGTTTTACGTTTG	56 ºC
Kan-rev	GATACTTTCTCGGCAGGAG	56 ºC
CMc-S	CTTAGGATCCTCATCCAGCATCTACACCTG CTA	55 ºC
CMc-AS	CAAAGCTTGTCGACAAGAACCACCGAAAG ATTCAG	55 ºC
PRL-S	GTGCCACGGCTCCCCATCGAG	52 ºC
PRL-AS	CTCGATGGGGAGCCGTGGCAC	52 ºC

Table 2: List of primers used and their annealing temperatures.

VI. DNA electrophoresis on agarose gel

Genomic and plasmid DNA fragments were separated by electrophoresis in agarose gel 0.8 % while PCR products were separated in 1-2 % gels. The agarose gel was prepared in TBE 1 % (Tris 0.89 M, Boric acid 0.89 M and EDTA 2 mM at pH 8). The same buffer was used for electrophoresis. The samples were mixed with 6x loading buffer at a final concentration of 1x.

VII. DNA digestion with restriction enzymes

1 µg DNA was digested in a mixture containing 5 U of restriction enzyme and 3 µl of the corresponding buffer (10x) in a final volume of 30 µl. The tubes were incubated 90 minutes at 37 ºC in a thermoblock (Eppendorf). The digestion was verified by electrophoresis on 1 % agarose gel.

VIII. Cloning techniques

VIII.1 Plasmids

The plasmids used in this study are summarized in the table below:

Plasmid	Features	Reference	Use
pGem T easy	AmpR, T-Overhangs for PCR Cloning	Promega	T-cloning
pCR™8/GW /TOPO®	SpecR, promoter T7, M13 site, attL1 and attL2 sequences	Invitrogen	Gateway entry vector
pK2GW7	SpecR (in bacteria), KanR (in plant), P35S, attR1 and attR2 sequences	(Karimi *et al.*, 2002)	Over-expression in tomato
pRSETB	IPTG induced T7 promoter, AmpR, His-Tag (x6)	Invitrogen	Protein expression in *E. coli*

attL and *attR* sequences are homologous recombination sites of the gateway system.
R: the antibiotic to which the plasmid confers resistance.

Table 3: **Plasmids used for cloning.**
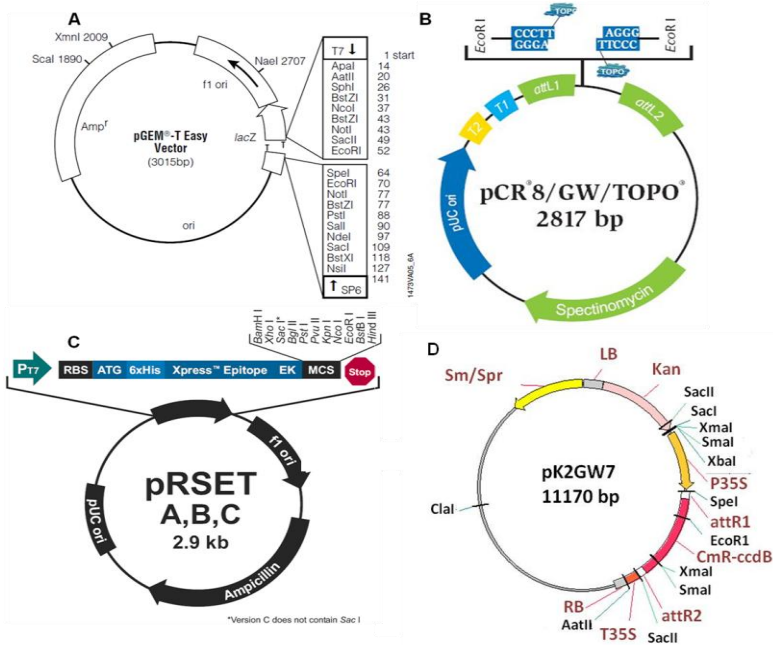
Figure 9: Maps of the plasmids used in this work. A: pGem T easy cloning vector; **B**: pCR8/GW/TOPO cloning vector; **C**: pRSETB *E. coli* expression vector; **D**: pK2GW7 plant expression vector.

VIII.2 DNA ligase mediated ligation

In order to assemble different fragments or to introduce them in pRSETB vector, restriction enzymes recognition sites were added by PCR and the fragment were ligated by T_4 ligase. The stoichiometric ratio insert:vector was 3:1. The following formula was used:

$$\textit{Insert quantity}$$
$$= \frac{\text{Vector quantity (ng)x Insert size (kb)}}{\textit{Vector size (kb)}} \text{ x molar ratio insert: vector}$$

The fragments were mixed with 1 U of T4 DNA ligase (Roche) and 1x ligation buffer. The mixture was then incubated at 16 ºC overnight.

The ligations with pGem® T-easy vector (Promega) were achieved according to the manufacturer instructions.

VIII.3 Fragments ligation by homologous recombination (Gateway™, Invitogen)

The gateway™ technology is based on the recombination capacity of the bacteriophage λ specific sites. These sequences are denominated "att" (Specific site **att**achment). The fragments were first assembled in pGem® T-easy vector (Promega). The obtained cassette was, then, amplified by PCR and cloned in the Gateway™ entry vector

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pCR8/GW/Topo™ that contains the recombination sites attL1 and attL2. The plasmids were later sequenced to check for mutations and the fragment orientation. Afterwards, the expression cassette was transferred to the pK2GW7 plant expression vector containing the attR1 and attR2 recombination sites. The recombination is catalyzed by the LR clonase enzyme (Invitrogen).

VIII.4 Sequencing

The fragments' sequencing was performed by the sequencing service of the Institute for Plant Molecular and Cell Biology (IBMCP) using a capillary sequencer (ABI 3100; Applied biosystems, Foster city, CA).

IX. Site directed mutagenesis

A punctual single base pair mutation was introduced into the *Itr2* gene using a PCR based strategy. A first amplification was realized with primers CMc-S and PRL-AS giving a fragment of 144 bp. And a parallel amplification was performed using PRL-S and CMc-AS primers, rendering a fragment of 272 bp. PRL-S and PRL-AS primers carry the mutated base in the middle of their sequence. Both fragments were mixed, denatured, re-annealed and used as template for a new PCR using CMc-S and CMc-AS primers to obtain the

complete fragment with the desired Leu to Arg mutation (Figure 10).

ACCAGCATCTACACCTGCTACGAAGGGATGGGTCTCCCCGTCAACCCGCTCCAGGGCTG
CMc-S

CCGTTTCTACGTGGCCTCGCAGACCTGCGGCGCCGTGCCGCTGCTCCCCATCGAGGTGAT
PRL-AS ←   → PRL-S

GAAGGACTGGTGCTGCCGGGAGCTGGCCGGCATCTCGTCCAACTGCCGCTGCGAGGGG

CTGCGCGTCTTCATCGACCGCGCGTTCCCTCCCAGTCAAAGCCAAGGCGCGCCCCCTCA

GCTGCCGCCGCTGGCGACCGAGTGCCCTGCGGAGGTGAAGAGGGACTTCGCCAGAAC

GCTCGCCCTGCCCGGGCAGTGCAACCTGCCGACCATCCATGGAGGCGCCTACTGCGTCT

TCCCATGAACGCTAGTTAGTTCATTGGGTCGTCGTGTGAATGAATGAATAAGCAGCATGT
CMc-AS

CGCGTTCGTGGATGCAGAAGTGCACAAGTGCAAAATAAAGTGAATCTTTCGGTGGTTCT

TGTCGACAAGCTTTGA

Figure 10: BTI-CMc site directed mutagenesis primers positions.

X. Protein expression induction

One colony of transformed BL21(DE3) pLysS was resuspended in 5 ml LB medium with 100 mg/L ampicillin. The culture was grown at 37 °C under 200 rpm agitation until OD_{600} reached 0.6. The culture was then diluted in 200 ml LB with antibiotic and grown until OD_{600} reached 0.5-0.8. Subsequently, the bacterial culture was cooled to room temperature and 0.5 mM IPTG was added. The induced bacteria were incubated 18 hours at room temperature under

agitation at 200 rpm. Cells were then harvested by spinning and resuspended in 10 ml bacteria lysis buffer (50 mM Tris pH 8, 30 % v/v glycerol, 0.1 % Triton x100, 100 µg/ml lysozyme). The bacterial suspension was mixed by pipetting and incubated 15 min at 30ºC until the suspension became turbid and viscous due to the release of bacterial DNA. The suspension was later sonnicated three times for 30 s. The solution was then spinned to eliminate cell debris and the supernatant recovered.

XI. SDS-PAGE protein separation

Protein electrophoresis separation was realized on Sodium Dodecyl Sulfate Poly-Acrylamide Gel Electrophoresis (SDS-PAGE) according to Laemmli (1970) method.

Protein separation was run on a 15 % polyacrylamide separation gel and 4 % stacking gel. The components of the separation gel were mixed in the order shown in the table below. 5 ml of the separation gel were poured between the glass plates and overlayed with 0.8 ml of absolute ethanol in order to ensure a flat surface and exclude air. After the gel had polymerized, the ethanol was poured off. The stacking gel was prepared and 2 ml were poured onto the top of the separation gel. Once dry, the gel was placed into the electrophoresis chamber. The separation was performed in 1 % running buffer. Protein samples were mixed with 4x

loading buffer. Then the mix was heated 10 min at 95 ºC for proteins denaturation, then cooled to room temperature and loaded in the gel. Separation was carried out at 120 V during 1 h. After the electrophoresis, proteins were fixed and stained with a Coomassie blue staining solution for 45 min under agitation. Distaining was performed as described by Hervieu (1997), by soaking in distilled water in microwave oven at 850 W for 15 min.

Separating gel 13.5% in 0.375 M Tris, pH 8.8	
Distilled water	2.9 ml
1.5 M Tris pH 8.8	2.5 ml
20% (w/v) SDS	50 μl
Acrylamide/Bis-Acrylamide (30% / 0.8 % w/v)	4.5 ml
10 % w/v Ammonium persulfate	50 μl
TEMED	5 μl
Total volume	**10.005**

Stacking gel 4 % in 0.125 M Tris, pH 6.8	
Distilled water	3.075 ml
0.5 M Tris pH 6.8	1.25 ml
20% (w/v) SDS	25 μl
Acrylamide/Bis-Acrylamide (30% / 0.8 % w/v)	0.67 ml
10 % w/v Ammonium persulfate	25 μl
TEMED	5 μl
Total volume	**5.05**

4x Sample loading buffer	
SDS	4%
Glycerol	40%
Tris pH 6.8	40 mM
EDTA	4 mM
DTT	320 mM
Bromophenol Blue	0.05% w/v

5x Running buffer	
Tris	15 g/L
Glycine	72 g/L
SDS	5 g/L
Distilled Water	qsp 1 L
pH	**8.3**

Table 4: Solutions used for SDS-PAGE protein separation.

XII. Recombinant protein purification

By means of the His-Tag present in the pRSETB vector, the recombinant proteins expressed in *E. coli* BL21(DE3)pLysS were purified by affinity chromatography on Ni^{2+} charged resin (GE, healthcare, Life sciences). The purification was achieved according to the manufacturer recommendations.

XIII. Trypsin activity assay

Trypsin activity assay was realized according to Erlanger et al. (1961) protocol with slight modifications. Commercial bovine trypsin (0.25 mg/ml) was mixed with 1.5 mM L-BA*p*NA substrate and 20 mM $CaCl_2$ in PBS buffer 67 mM pH 7.6. The different inhibitors were added at increasing concentrations: $3 \ 10^{-7}$, $6 \ 10^{-7}$, $1 \ 10^{-6}$ M. The mixture was incubated 10 minutes at 37 °C, then, the reaction was stopped by adding 30 % TCA. Subsequently, the solution was spinned and the absorbance measured at 405 nm. The amount of substrate hydrolyzed was calculated using a *p*NA reference curve. The results were represented as percentage of the remaining activity of trypsin without inhibitor.

Erlanger formula:

$$Trypsin\ activity\ (TA) = \frac{Absorbance\ at\ 405\ nm/\ min\ x\ 1000\ x\ RV}{8800\ x\ mg\ Protein\ in\ RV}$$

$$\%\ Remainig\ activity = \frac{TA\ control - TA\ inhibitor}{TA\ control}\ x\ 100$$

RV: Reaction volume
8800: extinction factor of *p*-nitroaniline.

XIV. Gene expression analysis

Gene expression level was estimated by RT-qPCR. The reaction was achieved in 96 well plates (Applied biosystems™) in a final volume of 20 µl. The mixtures contained 1 µg of cDNA, 10 µl Sybr Green PCR master mix (Applied Biosysytems™) and 0.3 µM of each specific primer. The reaction was realized in the thermocycler 7500 Fast-Real-Time PCR system connected to software provided by the manufacturer. The qPCR was performed according to the manufacturer recommended conditions. The amplification program consisted of a temper at 50 ℃ for 2 min and a denaturation at 95 ℃ for 10 min followed by 40 amplification cycles (denaturation at 95 ℃ for 15 s and 1 min elongation at 60 ℃). Three technical replicates were used for each sample. The relative expression levels were calculated according to the $2^{-\Delta\Delta Ct}$. Ct is the number of cycles required for the fluorescence signal to cross the threshold.

$$\Delta Ct = \text{Ct analysed gene} - \text{Ct reference gene}$$

$$\Delta\Delta Ct = \Delta Ct \text{ Sample} - \Delta Ct \text{ Reference sample}$$

The housekeeping gene *Actin8* of tomato (*SlAct8*), (Martín-Trillo *et al.*, 2011) was used as reference gene.

XV. Plant genetic transformation

Tomato plants were transformed according to the protocol described by Ellul *et al.,* (2003) with modifications.

XV.1. Seeds sterilization and germination

Approximately 100 tomato seeds were incubated 30 min in sodium hypochlorite (40%) with two drops of Tween-20. The seeds were next, washed in sterile distilled water 3 times for 5 min, 10 min and 2 hours respectively. Sterile seeds were then placed in petri dishes on sterile humid filter paper and incubated in darkness at 24 ºC ± 2 ºC for three days. After the incubation, the germinated seeds were transferred to germination medium (MG) and were grown 10 days at standard photoperiod conditions (16 h light, 8 h dark) at 24 ºC ± 2 ºC.

XV.2. *Agrobacterium tumefaciens* culture preparation

The transformed *Agrobacterium tumefaciens* strains were incubated at 28 ºC under agitation (200 rpm) with 100 mg/L

spectinomycin. The culture was refreshed every 48 h for 8 days. Subsequently, 5 ml of *Agrobacterium* culture were diluted in 1 L of LB medium without antibiotic, supplemented with 200 μM acetosyringone to promote the bacterial virulence. The bacteria were allowed to grow until OD$_{600}$ reached 0.5-0.6.

XV.3. Co-culture

10 days old cotyledons were cut on their edges and incubated with the *Agrobacterium tumefaciens* culture for 5 min. After the inoculation, the bacterial excess was removed on filter paper and the explants were placed in organogenesis IK4.0/4.0 medium supplemented with 200 μM acetosyringone. The co-culture was incubated 48 hours in the dark at 24 ℃ ± 2 ℃.

XV.4. Explants wash

After co-culture, the explants were washed for 10 min in IK 4.0/4.0 medium supplemented with 300 mg/L cefotaxime. They were then placed in petri dishes with the same medium without selection pressure and incubated at 24 ℃ ± 2 ℃ under standard photoperiod conditions. Three days later, the explants were transferred to a new organogenesis medium with selective antibiotic (100 μg/ml Kanamycin) and 1% zeatin, to allow organogenic callus formation. The medium

was changed every 15 days. Once differentiated leaflets are observed, the plantlet is separated from the callus and transferred to elongation medium (MEL). When the plantlet reached approximately 1 cm, it was transferred to rooting medium (ME).

XV.5. Plants acclimatization in greenhouse

When the roots of the transformed plant reached about 1 cm, the plantlet was transferred to greenhouse. The roots were washed with water to remove agar residues and the plant was transferred to a pot with coco fiber.

XV.6. Media and solutions

Mineral solution	mg/L
Macronutrients	
NH_4NO_3	1650
KNO_3	1900
$CaCl_2$ 2H2O	440
$MgSO_4$ $7H_2O$	370
KH_2PO_4	170

Micronutrients

H_3BO_3	0.83
$MnSO_44H_2O$	6.20
$ZnSO_4$	22.30
$Na_2MoO_4\ 4H_2O$	8.60
$CuSO_4\ 5H_2O$	0.25
$CoCl_2\ 6H_2O$	0.25

FeNa EDTA

$FeSO_4\ 7H_2O$	27.8
$Na_2\ EDTA$	37.3

Vitamins	mg/L
Riboflavin	0.25
Thiamin HCl	10
Pyroxidin HCl	1
Folic acid	0.5
Biotin	0.05
D-calcium pantothenate	0.5
Choline chloride	0.1
Glycine	0.5
L-Cystein	1
Malic acid	10
Ascorbic acid	0.5
Nicotinic acid	2.5

Germination medium (MG)	mg/L
Mineral solution (MS)	4.3
Sucrose	20
Agar	8

		Wash medium	Organogenesis induction medium	
	g/L	ML	IK 4.0; 4.0	IKZ 4.0; 4.0;1.0
MS	4.3	x	x	x
Sucrose	30	x	x	x
Myoinositol	0.1	x	x	x
Thiamin HCl	0.001	x	x	x
Vitamin SH	SH	x	x	x
IAA	0.004	x	x	x
Kinetin	0.004	x	x	x
Zeatin	0.001			x
Agar	8		x	x

Rooting medium	g/L
MS	4.3
Sucrose	20
Myoinositol	0.1
Thiamin HCl	0.001
IAA	0.0001
Agar	8

Table 5: Media and solutions used for tomato genetic transformation.

XV.7. Evaluation of the ploidy level in transgenic tomato plants

The ploidy level of transgenic tomato plants was determined by flux cytometry (Partec PAS II Ploidy analyzer) according to the method of Smulders *et al.* (1994). The cytometer was first calibrated using a control diploid tomato plant. A small leave was ground with 200 µl of nuclei extraction buffer (Partec). The resulting solution was filtered through a 50 µm nylon filter and 800 µl of nuclei staining solution were added. This buffer contains 1 mg/L DAPI Fluorochrome (4,6 diamino-2 phenyl-indole; DAPI Staining solution, Partec). It permits a fluorescent dying of DNA.

XVI. Tomato fruit characterization

XVI. 1. Morphological characters

Red mature fruits were collected from wild type and transgenic plants. Different morphological parameters were analyzed: Fruit shape, number of fruits per plant, number of seeds per fruit, fruit weight and parthenocarpy percentage.

XVI.2. Chemical characters

Soluble solids content (SSC)

Soluble solids content was determined by the mean of a hand refractometer. This instrument measures the refractive index

(IR) which indicates how much a light beam is slowed down when it passes through a liquid.

Tomato fruits were peeled and the pulp was triturated and filtered through miracloth. Before measuring tomato juice refractory index, the refractometer was calibrated using distilled water. Then two drops of tomato filtrate were analyzed. The obtained value is expressed in ° Brix and represents an estimation of the SSC present in the tomato extract.

Titratable acidity

The titratable acidity is an approximation of the total acid concentration contained within the fruit juice. It is measured by reacting the acids present in the solution with a base (NaOH) to a chosen end point close to neutrality indicated by an acid sensitive color indicator (Phenolphthalein).

To 5 ml of tomato filtrate, 15 ml of distilled water and 3 drops of phenolphthalein (1 %) were added. Subsequently, NaOH 0.1 N was added progressively until the solution turned pink. The titratable acidity was used to calculate the % of citric acid, the major acid in tomato fruits, according to the following formula:

$$\% \ \boldsymbol{Citric \ acid} = \frac{V1 \ x \ N}{V2} \ x \ K \ x \ 100$$

V1: Volume of NaOH (0.1 N) used.

V2: Sample volume (5 ml)

N: NaOH normality (0.1 meq/ ml

K: Citric acid Milliequivalent factor (0.064 g/meq)

Maturity and flavor Index

Maturity and flavor index were determined using the SSC and titratable acidity obtained values (Bisogni *et al.*, 1976; Navez *et al.*, 1999). According to the following formulas:

$$\boldsymbol{Maturity \ index} = \frac{°\text{Brix}}{\text{Titratable acidity}}$$

$$\boldsymbol{Flavor \ index} = \frac{°\text{Brix}}{20} \ x \ \% \ \text{Citric Acid} + \% \ \text{Citric acid}$$

XVII. Insect feeding trials

XVII.1. Insects and growth conditions

Tuta absoluta insects used belong to the artificial colony maintained in the Instituto Valenciano de Investigaciones Agrarias (IVIA, Valencia). The colony was started with adults captured from tomato fields near Castellón. The insects were maintained in cages (120x70x125). Weekly, 6 new

tomato plants are introduced into the cage. The colony is reared at 25 ºC ± 1 ºC, 60 ± 5 % RH at natural photoperiod.

Nesidiocoris tenuis adults were provided by Koppert (Nesibug, Koppert). When received, the insect were liberated in cages with tomato plants, and used in the next 72 h.

XVII.2. *Tuta absoluta* feeding trials

Three *Tuta absoluta* couples were placed with wild type tomato plants. After 48 h, white creamy eggs were collected. Twenty individual leaves from each transgenic line and the wild type control were placed in petri dishes on 2 % agar. A single *Tuta absoluta* egg was deposited on each leave and the development of the hatched larva was followed daily under binocular loupe. The plates were incubated at 24 ºC ± 2 ºC with a photoperiod of 16 h light/8 h darkness. During its development, the larvae were weighted 24 hours after each molting. The duration of each larval instar as well as the entire development cycle were registered for each insect.

XVII.3. *Nesidiocoris tenuis* feeding assay

Five plants of the CMe-CPI.3.3 transgenic line and wild type Micro-Tom tomato were placed in individual cages (bugdorm) with three couples of *N. tenuis* each. Bugs were provided, as alternative food, *Ephestia* kuehniella eggs (Entofood ®, Koppert) ad libitum. The different plants were

checked every two days, from eggs hatching to adults' emergence. Nymphal developmental time and the number of adults emerged were recorded.

XVII.4. Oviposition assays

T. absoluta adults emerged from the larvae fed on either transgenic or control plants, were collected and sexed according to the abdomen shape and color. Male adults present a thinner and darker abdomen (Vargas, 1970). Five couples were randomly formed from the emerged adults of each plant type. They were, then, transferred to plastic cups (370 cm^3) with a fresh tomato apical flush. According to the methodology described by Mollá *et al.* (Mollá *et al.*, 2014), the plastic cups were placed into small ones (230 cm^3) containing water. The tomato flush reached the water through a hole made in the inner cup. The bigger cup was covered with a fine mesh and fastened with a rubber band. Forty eight hours later, the tomato flush was removed and the number of deposited eggs was counted under a steromicroscope.

XVIII. Insect enzymatic assays

XVIII.1. Total protein extraction

About 40 mg of *Tuta absoluta* larvae of every instar from each treatment were pooled and ground in liquid nitrogen. The obtained powder was homogenized in 200 µl of ice cold

extraction buffer (0.1 M Tris pH 7, 0.1 % Ascorbic acid, 0.1 % L-cysteine, 0.5 M sucrose and 10 mg/ml PVP). The mixture was, then centrifuged at high speed for 15 min at 4 ºC. The supernatant was recovered and mixed with two volumes of ice cold 90 % acetone. The tubes were, then, incubated 2 h at -20 ºC and centrifuged 10 min at 4 ºC at high speed. Next, the pellet was washed twice with 90 % acetone, dried and resuspended in 100 µl of 0.5 M Tris buffer pH 8.

XVIII.2. Total protein quantification

Proteins concentration in the crude extract was determined using the Bradford method (Bradford, 1976). This method is based on the capacity of the Coomassie Brilliant Blue G250 dye to bind to proteins in acidic solution (via electrostatic and Van der Waals bonds) resulting in a shift of the maximal absorbance of the dye from 465 to 595 nm.

Increasing concentrations of BSA were used as standards (0.2; 0.4; 0.6; 0.8; 1; 1.5; 2 mg/ml). 3 µl of each sample were diluted in 97 µl of 1x Bradford reagent (Biorad). The mixture was incubated 5 min at room temperature, then, the absorbance was measured at 595 nm. The protein concentration in the samples was deduced from the standard curve.

XVIII.3 Enzymatic activity determination in crude extracts

The obtained crude extract was used to determine both trypsin and papain activity. Nα-Benzoyl-L-arginine 4-nitroanilide hydrochloride (BApNA) (Sigma) was used as chromogenic substrate for trypsin and pGlu-Phe-Leu p-nitroanilide (PFLNA) (Sigma) as substrate for papain. The trypsin-like and papain-like activity in the sample was determined by using a gradient of commercial trypsin (bovine trypsin, sigma) and papain (sigma) as standards. 5 µg of proteins of the crude extract were mixed with 5 µl of the corresponding substrate (10 mg/ml) and up to 100 µl Sodium phosphate buffer 67 mM pH 7.6 with 20 mM $CaCl_2$ for trypsin assays or 5 mM L-cysteine for papain assays. Each sample was incubated in duplicate at 37 °C for 30 min, and absorbance measured at 405 nm. As standards, we used the commercial trypsin and papain at six known concentrations (0.125 µg, 0.25 µg, 0.5 µg, 0.75 µg, 1 µg and 1.5 µg). Trypsin and papain activity was expressed as the percentage of trypsin-like or papain- like proteins from the sample's total protein content.

XVIII.4. Enzyme histochemistry

The fluorescent substrate Nα-Benzoyl-L-arginine-7-amido-4-methylcoumarin hydrochloride (BAAMC) (Santa Cruz

Biotechnology), specific to trypsin and papain was used to localize the targeted protease in the insect body. Larvae of the third instar, fed with wild type plant leaves, were sacrificed by freezing in liquid nitrogen, then included in the cryprotector gel NEG-50 (Richard-Allan Scientific) and frozen at -27 °C. Cryo-sections of 16 μm were realized with the cryostat (HM520 Microm). Sections were recovered on a poly-lysine coated slide and washed with 10 % polyvinyl alcohol (PVA) in PBS 67 mM pH7.6 to avoid macromolecules diffusion. Then, 50 μl of substrate solution (10 % PVA, 0.5 μl BAAMC 20 mg/ml, 2 mM $CaCl_2$ in PBS 67 mM pH 7.6) was applied to the section. The slide was incubated at 37 °C for 15 min, then washed 5 times for 1 min in 5 % PVA in PBS 67 mM pH 7.6 and one time with PBS 67 mM pH 7.6. Sections treated with BAMMC were examined for fluorescence using ultraviolet light with the Leica DM5000 microscope.

XIX. Olfactory response

The behavioral response of *T. absoluta* and *N. tenuis* adults to the transgenic plants CMe-CPI.3.3 volatiles was investigated in a Y-shaped tube olfactometer (Analytical Research Systems, Gainesville, FL) of 4.2 cm diameter, a 13.5 cm long base and two 5.75 cm side arms. The base of the tube was connected to an air pump providing a unidirectional airflow

at 150 ml/min. The side arms were connected to two glass jars of 5l volume, each one containing a different odor source: transgenic or wild type plant. Each container was connected to a flow meter and a water filter. Four fluorescent 60 cm-long tubes (OSRAM, L18 W/765, OSRAM GmbH, Germany) were placed 40 cm above the arms. Light intensity was measured with a ceptometer (LP-80 AccuPAR, Decagon Devices, Inc., Pullman, WA) at 2,516 lux. The environmental conditions were 23 ± 2 °C and 60 ± 10 % RH.

For each experiment, 40 adults; 20 females and 20 males were tested. Each insect was observed until it reached at least 3 cm up one of the side arms of the tube or until 10 min have passed. The insects that had not chosen any arm after 10 min were considered as "non responders" and were discarded from the analysis. After five individuals were tested, the olfactometer tube was flipped around to minimize spatial effect of arm choice, and after each 10 insects, the odor source was changed.

XX. Volatile compounds analysis

Volatile compounds were captured on a headspace solid-phase microextraction (HS-SPME) according to the protocol described by Bouagga et al. (2017) . Separation and detection were performed by means of gas chromatography coupled to a mass spectrometer (GC/MS). The adsorbing fiber coating

was PDMS/DVB-65 (65 μm Polydimethylsiloxane /Divinylbenzene; Supelco, Bellefonte, PA, USA). Fibers were mounted on a SPME fiber holder and injected trough the first septum of the sample container. The fiber was extended by pushing the plunger of the SPM filter holder and exposed to plant volatiles. For each plant, volatiles adsorption was performed during 3 hours. Each treatment had 6 replicates. After volatiles adsorption, the fiber is retracted into the needle and the SPME device removed. Desorption was performed by means of a CombiPAL autosampler (CTC Analytics) at 250 ºC during 1 min in splitless mode in the injection port of a 6890N gas chromatograph coupled to a 5975B mass spectrometer (Agilent Technologies). To prevent cross-contamination fibers were cleaned after desorption in an SPME fiber conditioning station (CTC Analytics) at 250 ºC for 5 min under a helium flow. Chromatography was performed on a DB-5ms (60 m, 0.25 mm, 1.00 μm) column with helium as carrier gas, at a constant flow of 1.2 ml/min. The GC interface and MS source temperatures were 260 ºC and 230 ºC, respectively. Oven programming conditions were 40 ºC for 2 min, 5 ºC/min ramp until 250 ºC, and a final hold at 250 ºC for 5 min. Data was recorded in the 35-300 m/z range at 5 scans/s, with electronic impact ionization at 70 eV. Chromatograms were processed by means of the Enhanced ChemStation E.02.02 software (Agilent Technologies).

Identification of compounds was performed by the comparison of both retention time and mass spectrum with those of pure standards. All the standards were purchased from Sigma-Aldrich. For quantitation, one specific ion was selected for each compound, and the corresponding peak area from the extracted ion chromatogram was integrated. The criteria for ion selection were the highest signal-to-noise ratio and being specific in that particular region of the chromatogram enough in order to provide good peak integration.

XXI. Statistical analysis

Statistical analysis was realized with the Graph Pad Prism 6 software. Duration of developmental instars was analyzed by ANOVA test, while larval weight and oviposition, for each transgenic line, were compared to wild type plants by t test. Chi-square tests of independence were applied to compare mortality percentage and olfactory response.

XXII. Bioinformatic tools

Protein 3D models were realized by Geno3D software (Combet *et al.*, 2002). Their visualization annotation and superposition were performed by Strap software (Gille & Robinson, 2006).

For DNA sequence alignment, Multalin software was used (Corpet, 1988). qRT-PCR primers were designed by Primer3Plus software (Untergasser *et al.*, 2007).

Chapter I: Improving BTI-CMc *in vitro* activity by genetic engineering.

Results

I. Site directed mutagenesis

A single base pair substitution from T to G permitted the substitution of a Leu (codon CTG) to an Arg (CGG) in the BTI-CMc putative reactive site. The obtained mutated fragment was first cloned into a pGem®-T-easy vector and then sequenced to check the presence of the desired mutation.

The change from Leu to Arg affects the protein at different levels. Leu is an aliphatic hydrophobic amino acid, while Arg is a positively charged one with a guanidinium group in its side chain. This shift affects the global charge of the protein, and the 3D structure, especially of the putative reactive site.

II. Expression constructs

The *Itr1 and Itr2* both native and mutated fragments were first cloned into a pGem®-T-easy vector and later transferred to a pRSETB expression vector (Figure 11). The plasmids were finally introduced into the *E.coli* BL21 pLysS DE3 expression strain.
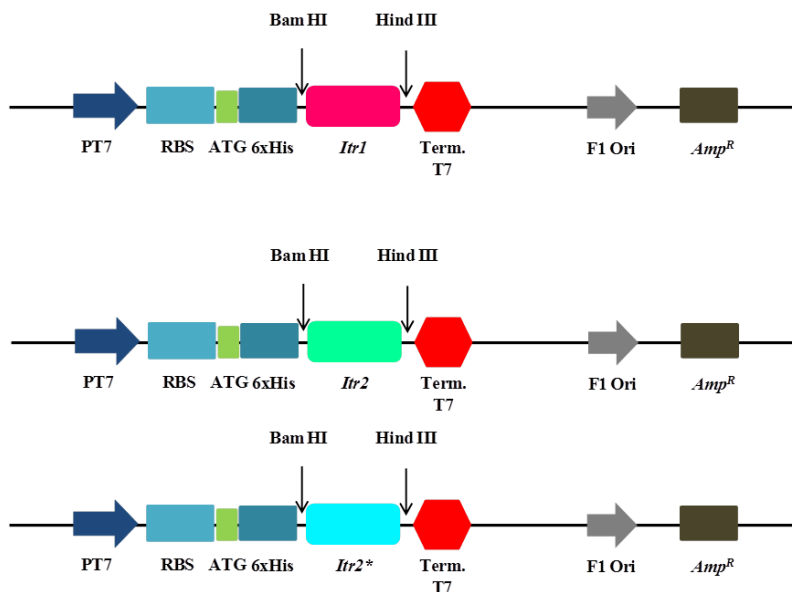
Figure 11: Expression constructs generated in pRSETB. PT7:
phage T7 promoter; **RBS**: Ribosome binding site; **ATG**:
transcription initiation codon; **6xHis**: Histidine tag; **Term T7**:
phage T7 terminator; F1 Ori: replication origin for β-lactamase
expression; **Amp^R**: β-lactamase coding gene.

III. Protein sequence analysis

The BTI-CMc sequence consists of 143 amino acids with a signal peptide of 24 residues. The alignment of the BTI-CMc protein sequence with the barley tetrameric α-amylase inhibitor BTAI-CMa subunit showed 48 % identity across the whole protein with 21 identical residues among the 29 of the N-terminal extremity (Figure 12A). However, it only showed 41 % identity with BTI-CMe, the other member of the barley trypsin inhibitors family (Figure 12B).

Figure 12: **Amino acid sequences alignment. A:** BTI-CMc and BTAI-CMa sequence alignment; **B:** BTI-CMc and BTI-CMe sequence alignment.

III. Protein structure: 3D models

The software Geno 3D was used to generate 3D models of each protein (Figure 13). The ragi bifunctional inhibitor of trypsin and α-amylase (1bip), which structure was previously determined by crystallography (Gourinath *et al.*, 2000), was used as template. The 3D structures of native BTI-CMc, BTI-CMc-PRL and BTI-CMe were compared. BTI-CMc and BTI-CMe models are highly similar despite of the differences between their amino acid sequences (only 36 % similarity). The 3D structure of both proteins is composed of 4 α-helixes. The putative reactive site of the protein is located in the loop connecting h1 and h2 helixes. 3D models of BTI-CMc and BTI-CMc-PRL were superimposed with that of BTI-CMe using Pymol software. When Leu is substituted by Arg in the putative reactive site of BTI-CMc, the loop acquires a more similar structure to that of BTI-CMe.
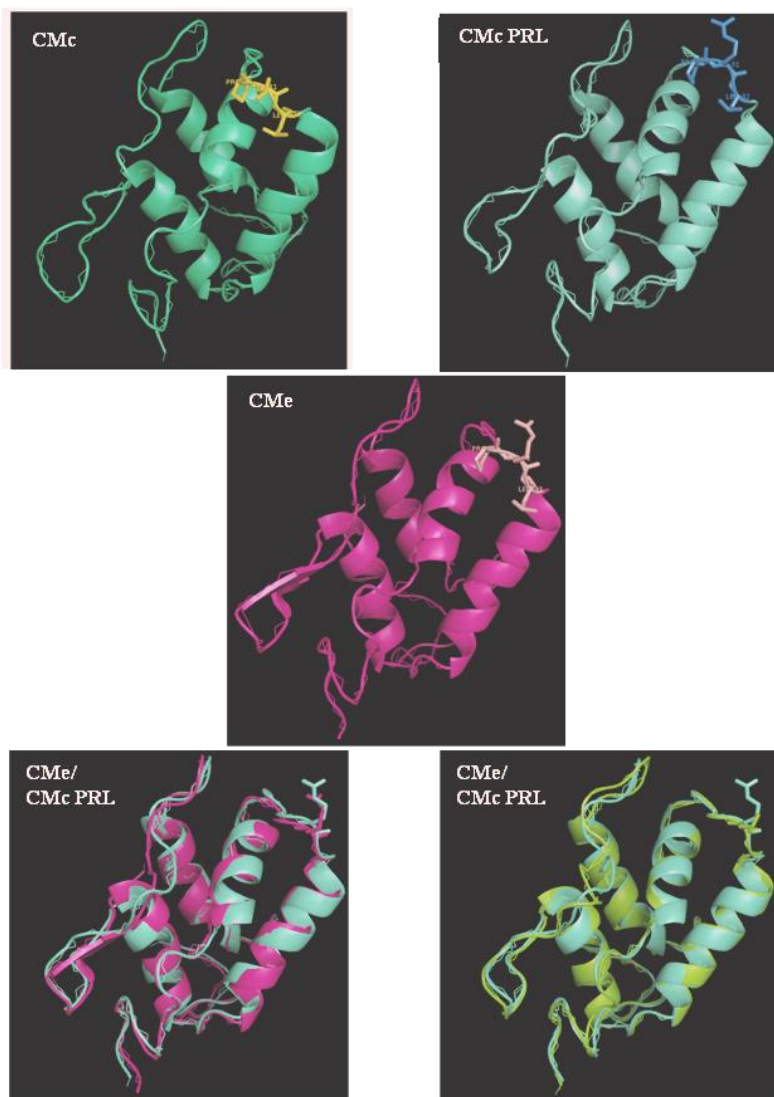
Figure 13: 3D modelization and comparison of CMc, CMc-PRL and CMe proteins.

IV. Protein expression

Protein expression in BL21pLysS DE3 cells was induced by the addition of IPTG to the bacterial culture. The cells were then harvested and lysed. Bacterial proteins were separated by SDS-PAGE to check for target protein induction. The protein extract after IPTG addition showed a wide band corresponding to the target protein molecular weight (15.84 kDa for BTI-CMe and 12.4 kDa for BTI-CMc) (Figure 14). The pRETB vector provides the protein of interest with a six histidine tag that allowed its purification by affinity chromatography on Ni^{++} resin.
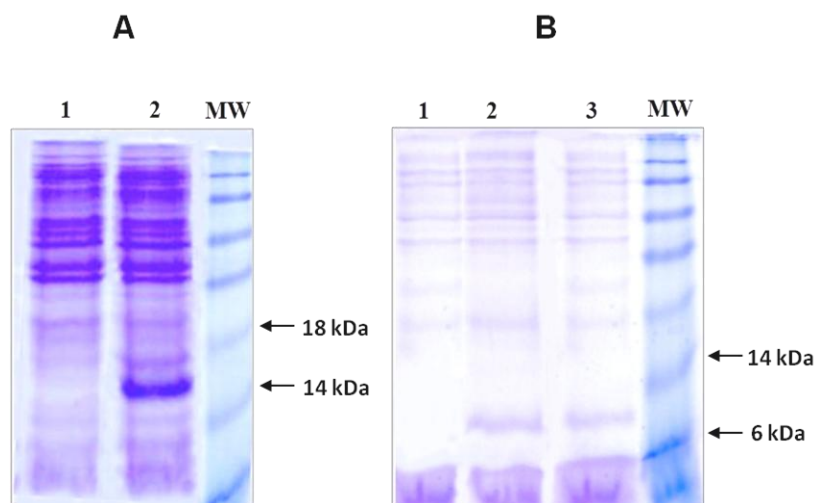
Figure 14: SDS-PAGE protein separation. A: *E. coli* expressing BTI-CMe proteins separation: 1: non induced bacteria; 2: IPTG induced bacteria; **B:** *E. coli* expressing BTI-CMc and BTI-CMc-PRL proteins separation: 1: non induced bacteria; 2: IPTG induced bacteria expressing native BTI-CMc; 3: induced bacteria expressing mutated BTI-CMc.

III. Trypsin activity

Purified proteins were quantified using the Bradford's method and their anti-trypsin activity was measured according to the Erlanger procedure (Erlanger, 1961). The results were expressed as the remaining trypsin activity after the inhibitor addition. The experiment was realized in triplicate and the mean value was represented (Figure 15). Trypsin inhibitory activity for the native BTI-CMc protein was about one third that of BTI-CMe (about 33 %). However, the engineered mutation from Leu to Arg in the putative active site of BTI-CMc increased its activity to a comparable level to BTI-CMe (about 82%).
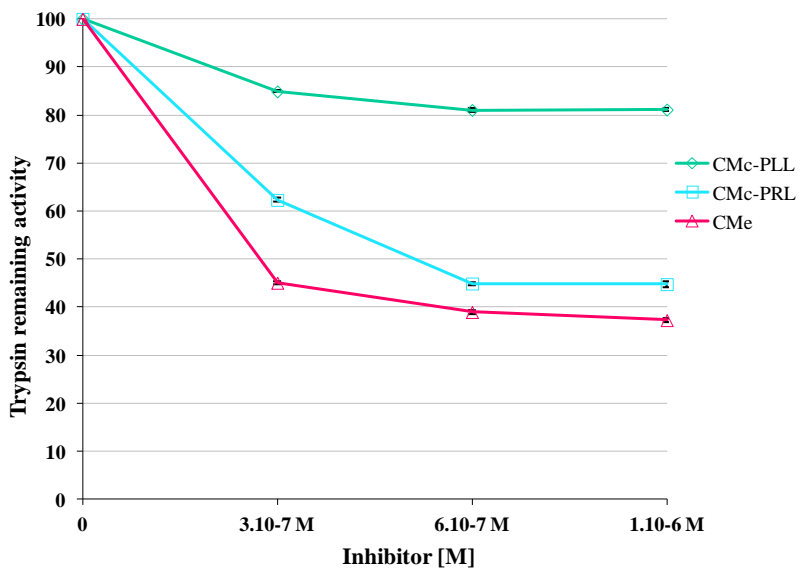
Figure 15: Trypsin remaining activity in presence of different concentrations of BTI-CMc, BTI-CMc-PRL and BTI-CMe.

Discussion

Cereal α-amylase and trypsin inhibitors are small proteins expressed in storage tissues and involved in plant defense against pests. These proteins can be selectively extracted by a mixture of chloroform/methanol and therefore are named CM-proteins. In barley, this family is represented by two members: BTI-CMe and BTI-CMc. The first one is a strong trypsin inhibitor carrying the characteristic PRL reactive site for trypsin inhibition conserved in other trypsin inhibitors from corn, rice and finger millet (Carbonero *et al.*, 1993). The second member, BTI-CMc, is a moderate trypsin inhibitor (33 % activity compared to BTI-CMe) (Barber *et al.*, 1986). BTI-CMc is considered a trypsin inhibitor although it only shows 36 % similarity with BTI-CMe, while it has 85 % identity with the wheat chymotrypsin WCI (Di Maro *et al.*, 2011) and 21 of its 29 N-terminal amino acids are identical to BTAI-CMa (Medina *et al.*, 1993), a subunit of the barley tetrameric α-amylase. Despite of this similarity, BTI-CMc doesn't show any α-amylase or chymotrypsin activity. *Itr2* and *Iat-1,* encoding for BTI-CMc and BTAI-CMa respectively, are both located in the short arm of the chromosome 7HS, suggesting that, perhaps, one gene originated as a duplication of the other. Accumulation of mutations could have generated a potential trypsin inhibitory loop common to members of the cereal α-amylase/trypsin

inhibitors. With the aim to investigate this possibility, we introduced a point mutation in the loop to match the canonical residue present in BTI-CMe. The trypsin inhibitory activity of BTI-CMc was improved, reaching 82 % of that of BTI-CMe. These results support the hypothesis that this loop is responsible for the trypsin inhibitory activity and necessary for the interaction with the substrate. The 3D structure prediction showed that BTI-CMe and BTI-CMc structures are very similar. Their protein backbone is formed by four α-helixes. According to these models and the interaction mechanism proposed for the ragi bifunctional inhibitor, we suggest that the binding reactive site of BTI-CMc is located on the loop connecting h1 and h2 helixes. Several serine proteinase inhibitors present an external loop as the primary binding segment with the target protease. According to the Laskowski (standard) inhibition mechanism, the chemical nature of this residue determines the specificity of the PI (Laskowski & Qasim, 2000). Although the amino acid residues are different between BTI-CMe and BTI-CMc, the loops are equal in length. Moreover, both proteins show a similar distribution of Cys residues in P6 and P10 positions respect to the P1 reactive site. While in BTI-CMe, the P1 residue corresponds to an Arg, a typical residue for trypsin inhibitors, Leu is found in the same position in BTI-CMc. P1 is known to be the most critical residue for the specificity of

proteinase inhibitors. Arg is a positively charged amino acid, frequently found in proteins active or binding site. It also has a complex guanidinium group on its side chain involved in hydrogen bonds formation and binding to negatively charged groups. However, Leu is an aliphatic, hydrophobic amino acid. Its side chain is non reactive and it is very rarely involved in protein function (Betts & Russell, 2003). The single point mutation from Leu to Arg affects the reactive loop at different levels: charge and conformation, impacting its ability to recognize and interact with target enzymes.

Cereal α-amylase/trypsin inhibitors are involved in plant defense against pests as well as in storage protein mobilization. In order to fix advantageous traits by mutations, proteins accumulate variations acquired through selective pressure processes. In this respect, differences in the reactive site can reflect a gained advantage either for plant defense against pests or metabolism. It is worth to mention that the PLL reactive site present in BTI-CMc was also found in homologous sequences in wheat. This finding makes unlikely that BTI-CMc is a degenerated, non functional inhibitor. The determination of its targets and functions remains to be elucidated.

Chapter II: Enhancing tomato defense against *Tuta absoluta* by expressing two barley proteinase inhibitors

Results

I. Genetic transformation of tomato

I.1. Genetic constructs

Different genetic constructs have been generated in order to express the proteinase inhibitors in tomato. Both *Itr1* and *Icy2* coding fragments were first cloned in the pCR8/Top/GW (pCR8-*Itr1,* pCR8-*Icy2*) (Figure 16 A, C). The fragments were, then, transferred by recombination to the Gateway™ plant expression vector (pK2GW7-*Itr1*, pK2GW7-*Icy2*) (Figure 16 B, D). This vector harbors the Cauliflower mosaic virus (CaMV) 35S promoter (P35S), the CaMV 35S terminator (T35S) and the *nptII* gene run by the PNOS promotor to confer kanamycin resistance to the transformed plants.
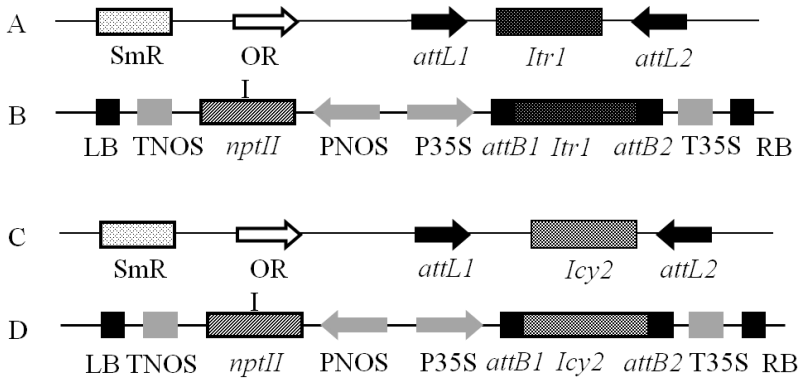
Figure 16: **Constructs used to express *Itr1* and *Icy2* genes in tomato. A:** pCR8-Itr1; **B:** pK2GW7-Itr1; **C:** pCR8-Icy2; **D:** pK2GW7-Icy2

In order to co-express both PIs in the same vector, we generated a multi-expression cassette containing both genes. First, we cloned *Itr1*, *Icy2*, the P35S promoter and the T35S terminator, each individually in a pGem-T-easy® vector, adding the necessary restriction sites. The resulting vectors: pGem-Itr1, pGem-P35S, pGem-Icy2, pGem-T35S are represented in Figure 16. P35S fragment was then sub-cloned in the pGem-Itr1 vector by restriction and ligation (pGem-Itr1-P35S). And T35S fragment was transferred to the pGem-Icy2 vector (pGem-Icy2-T35S). The next step consisted in gathering both fragments in the same plasmid generating the pGem-Itr1-P35S-Icy2-T35S vector. The obtained cassette was amplified by PCR and cloned in the Gateway™ entry

vector pCR8/TOPO/GW (pCR8-Itr1-P35S-Icy2-T35S). Subsequently, it was linearized and recombined with the plant expression vector pK2GW7 (pK2GW- Itr1-P35S-Icy2-T35S) (Figure 17).

The generated constructs were transformed in *Escherichia coli* DH10B. The plasmids were sequenced to confirm the absence of mutations and the orientation of the fragments. The checked vectors were used to transform *Agrobacterium tumefaciens* strain LBA4404. The recombinant bacteria were used to transform tomato plants in order to over-express the PIs.
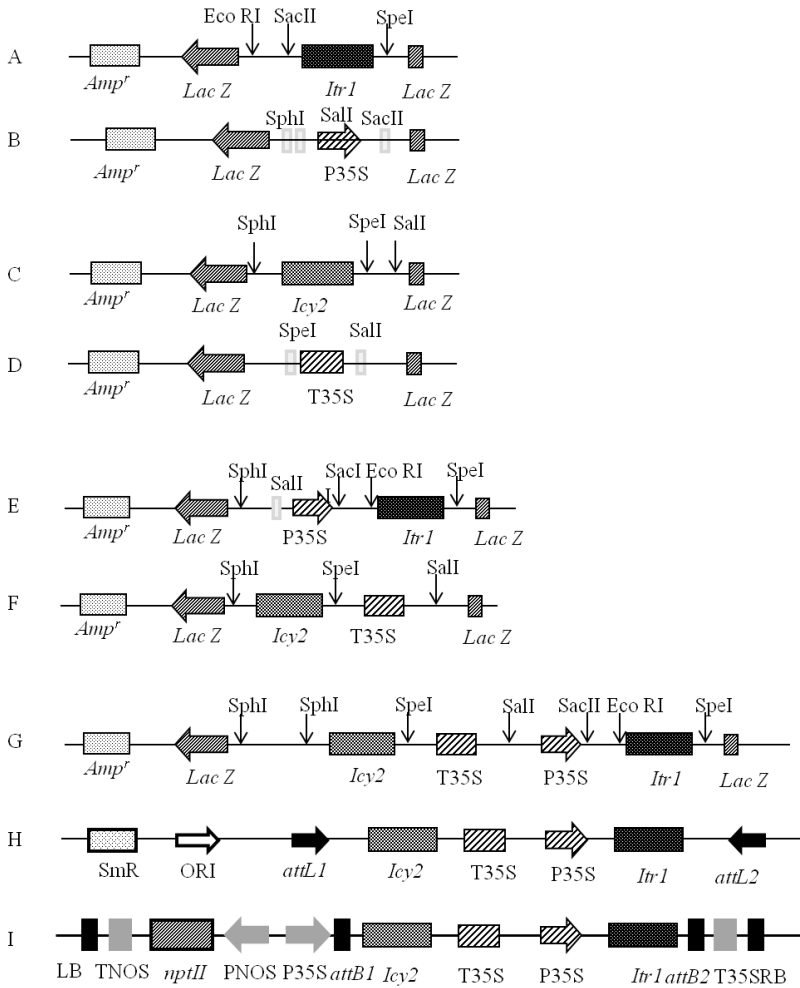
Figure17: Schematic representation of the genetic constructs used to co-express *Icy2* and *Itr1* in tomato. A: pGem- Itr1; **B:** pGem- P35S; **C:** pGem- Icy2; **D:** pGem- T35S; **E:** pGem-P35S-Itr1; **F:** pGem-Icy2-T35S; **G:** pGem-Icy2-T35S-P35S-Itr1; **H:** pCR8-Icy2-T35S-P35S-Itr1; **I:** pK2GW7-Icy2-T35S-P35S-Itr1.

I.2 Tomato genetic transformation

Tomato cotyledons explants are sensitive to Agrobacterium infection. After co-culture, the explants were transferred to a selective organogenic medium. Callus started to form on about 60 % of the explants. Two weeks later, callus started to differentiate showing small leaflets. The plantlets were allowed to grow until 1 cm height and were transferred to rooting medium, then acclimatized to soil conditions in green house. Six independent transgenic lines were obtained for plants expressing BTI-CMe and Hv-CPI2 individually and 8 transgenic lines co-expressing both transgenes. In order to discard any false transformant, PCR of the *nptII* gene was performed on the genomic DNA extracted from the transgenic plants. All the tested plants were positive for *nptII*. The ploidy of the transgenic plants was checked by flux cytometry using a diploid wild type Micro-Tom tomato DNA as positive control. All the transgenic plants were diploid.

I.3.Transgene expression analysis

Transgene expression level was analyzed by semi-quantitative PCR in the T1 plants. Transgenic plants where transgene expression was not detected after 30 PCR cycle were discarded. The retained lines were CPI2.1 and CPI2.4 expressing Hv-CPI2; CMe.4, CMe.2 and CMe.1 expressing

BTI-CMe and CMe-CPI.1, CMe-CPI.3 and CMe-CPI.4 expressing both PIs. The selected primary transformants were self-fertilized to produce T2 generation. The obtained seeds were segregated on a germination medium supplemented with kanamycin. The heterozygous lines with a single copy of the transgene segregate with a ratio 1:3 sensitive: resistant. The selected plants were tested for transgene expression levels by semi-quantitative PCR. CPI2.1.1, CPI2.4.5, CPI2.4.3, CPI2.1.6 and CPI2.1.11 showed the highest expression of Hv-CPI2. CMe.2.5, CMe.1.3, CMe.1.1, CMe.2.1, CMe.2.4 and CMe.2.3 had the highest expression of BTI-CMe, and CMe-CPI.3.3, CMe-CPI.3.1, CMe-CPI.3.11, CMe-CPI.3.13, CMe-CPI.3.7, CMe-CPI.3.2 and CMe-CPI.3.8 were selected for best co-expression of both transgenes. Seeds of these plants were recovered and segregated on kanamycin supplemented germination medium. The homozygous lines presenting a germination rate of 100 % were retained. First, a semi-quantitative PCR was performed to discard plants with low transgene expression level (Figure 18). The plants that showed the highest expression level in the semi-quantitative PCR, were submitted to qRT-PCR for more accurate analysis. According to the qRT-PCR results, among the transgenic lines expressing *Icy2* individually, CPI2.4.5 showed higher transgene expression level. For plants expressing *Itr1*,

CMe.2.1 was the line with higher expression. CMe-CPI.3.3 was the double transgenic line with higher expression level for both transgenes, *Itr1* and *Icy2*. It is noteworthy that CMe-CPI.3.3 expressed *Itr1* about 3 times more than CMe.2.1 and *Icy2* about 2.5 times more than CPI2.4.5 (Figure 19). The three transgenic lines were retained for subsequent experiments. They were self-fertilized and the seeds germinated to obtain the homozygous plants later used for insect feeding assays.
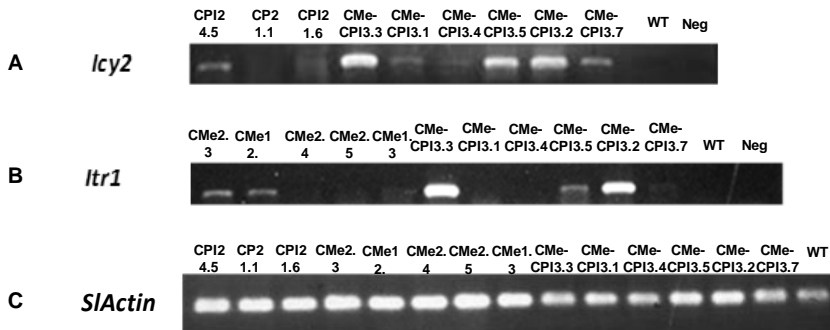
Figure 18: **Semi-quantitative PCR for *Icy* and *Itr1* genes in the homozygous plants. A:** Semi- quantitative PCR of *Icy2* gene: 1; **B:** Semi- quantitative PCR of *Itr1* gene; **C:** Semi-quantitative PCR of the constitutive gene *SlActin*.
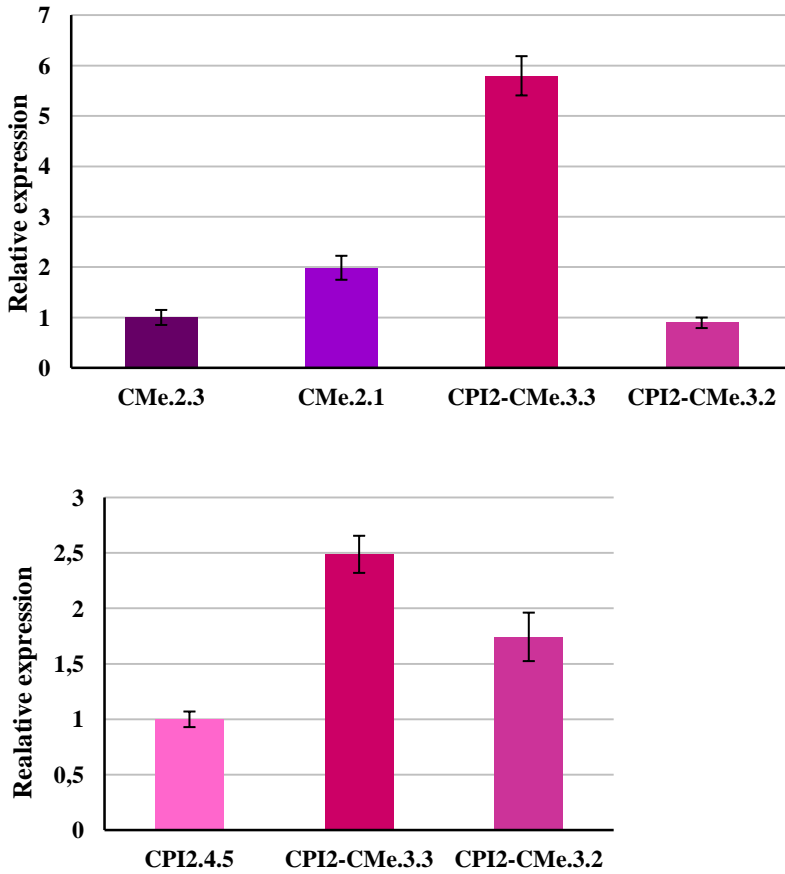
Figure 19: **Relative expression of *Icy2* and *Itr1* in the different homozygous transgenic lines.**

II. Tomato fruit characterization

II.1 Morphological characters

Mature fruits from transgenic CMe-CPI.3.3 plants were weighed collected and characterized. A reduction in weight was observed for the transgenic fruits. Tomato fruit mean weight was 3.8 g for wild type tomatoes and 2.9 g for transgenic ones (Figure 20 A).

The number of fruits produced per plant was also counted. We observed that the transgenic CMe-CPI.3.3 plants presented higher number of fruits when compared with the wild type. Transgenic plants produced a mean of 22.6 fruits per plant while wild type plants gave a mean of 17.2 (Figure 20 B).

Seeds were collected from those fruits and the mean number of seeds per fruit was determined. A reduction of the number of seeds per fruit was observed in the transgenic CMe-CPI.3.3 plants when compared to the wild type. Transgenic fruits presented a mean of 11.2 seeds per fruit versus 16.3 seeds per fruit for the wild type fruits (Figure 20 C).

The percentage of parthenocarpic fruits showed no significant difference between transgenic CMe-CPI.3.3 and wild type

plants with respective values of 19.3 % and 12.9 % (Figure 20 D).
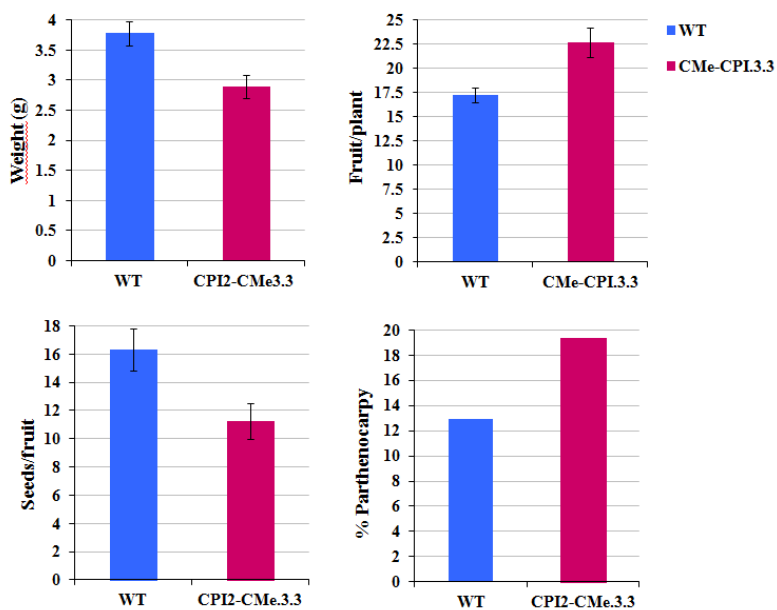
Figure 20: **Tomato fruit characteristics. A:** fruit weight; **B:** number of fruits/plant; **C:** Number of seeds/fruit; **D:** percentage of parthenocarpy.

Fruits diameter and height were measured. Fruit shape was determined by the ratio between fruit height and diameter. A ratio equal to 1 corresponds to round shape, ratio inferior to 1 indicates a flatten fruit form and values superior to 1, elongated fruits.

CMe-CPI.3.3 transgenic plants showed a slightly elongated fruits, heart-shaped, with a mean ratio height/diameter of 1.03, while wild type fruits were round to flatten with a mean ratio of 0.92 (Figure 21, white arrow).
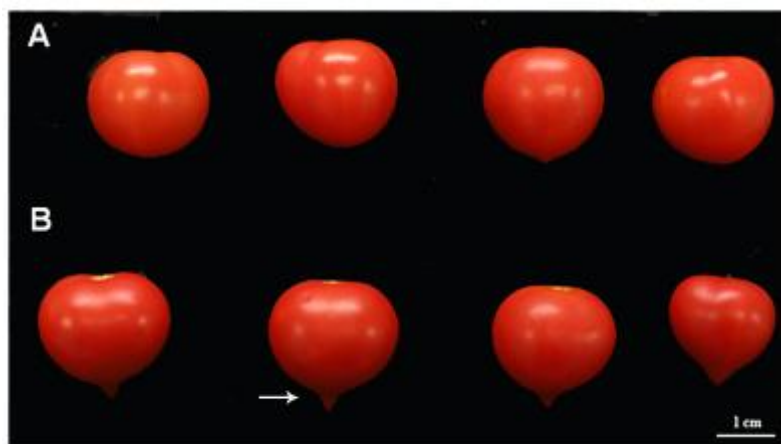
Figure 21: Tomato fruit shape. A: wild type tomato fruits; B: transgenic CMe-CPI.3.3 tomato fruits.

II.2 Chemical characters

The SSC of tomato fruits from the transgenic CMe-CPI.3.3 and wild type plants was determined by measuring the ° Brix. Both tomato fruits showed the same SSC (6 ° Brix).

With respect to the titratable acidity, transgenic and wild type tomato fruits showed similar % of citric acid with 1.49 % for wild type fruits and 1.46 % for the transgenic ones.

The later parameters allowed calculating the maturity and flavor indices. Wild type and transgenic fruits showed similar values for both indices. Maturity index was 5.2 for wild type and 5.3 for CMe-CPI.3.3 while flavor index was 1.49 for wild type fruits and 1.46 for CMe-CPI.3.3.

III. *Tuta absoluta* feeding trials

III.1. Enzyme histochemistry

Enzyme histochemistry assay was realized to localize the target proteinase in *Tuta absoluta* larvae and better understand how it is affected by PIs. During the third instar, *T. absoluta* larvae feed more intensively and gain more weight. Therefore, the experiment was achieved on L3 larvae. As most enzymes are heat and fixatives-sensitive, the histochemistry was realized on frozen material. The fluorescence was detected at different morphological levels. As expected, trypsin-like enzymes were localized all along the digestive system (esophagous, foregut, midgut). They were also detected in the exoskeleton and the excretory system (Malpighi tubules) (Figure 22).
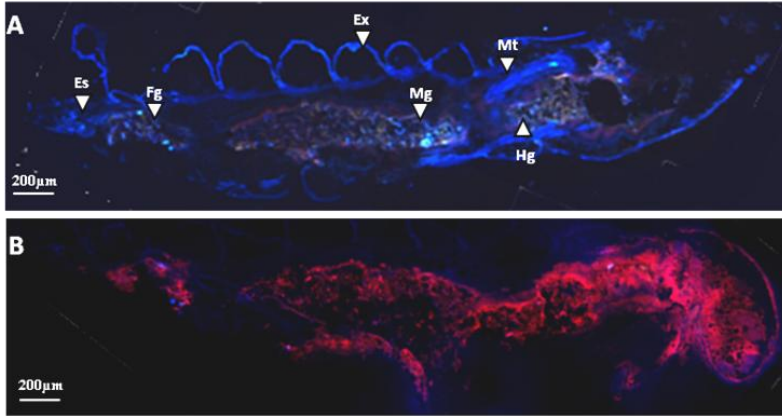
Figure 22: Enzyme histochemistry Cryocut of *Tuta absoluta* L3 larvae incubated with the serine and cysteine proteinase fluorescent substrate BAAMC. A: Larval section incubated with BAAMC florescent substrate; **B:** Negative control: larval section without BAAMC substrate; Proteases are localized along the digestive tract: Esophagus (**Es**), Foregut (**Fg**), Midgut (**Mg**), Hindgut (**Hg**), Malpighi tubules (**Mt**) and Exoskeleton (**Ex**).

III.2. Development cycle

Feeding transgenic plants affected *T. absoluta* at different levels. As can be seen in table 5, a slight delay in the first larval developmental was observed on larvae fed with leaves of the CPI2.4.5 transgenic plant, however, insects fed with the other transgenic lines showed no significant difference when compared with the wild type.

	1st instar (days)	2nd instar (days)	3rd instar (days)	4th instar (days)	Total development
CMe-CPI.3.3	3.71	3.64	2.18	3.00	12
CMe.2.1	3.61	3.33	2.08	2.44	11.75
CPI2.4.5	3.80	3.07	2.00	2.27	11.27
WT	3.07	3.21	2.57	2.83	11.25

Table 6: Larval development time of *Tuta absoluta* fed with leaves of transgenic and wild type plants.

III.3. Weight and size

The larvae were weighted 24 hours after each molting. L1 larvae were too small to be detected by the balance. In the next instars L2, L3, and L4, larval weight and size were significantly reduce when fed with each of the three transgenic plants compared with the control (Figure 23).
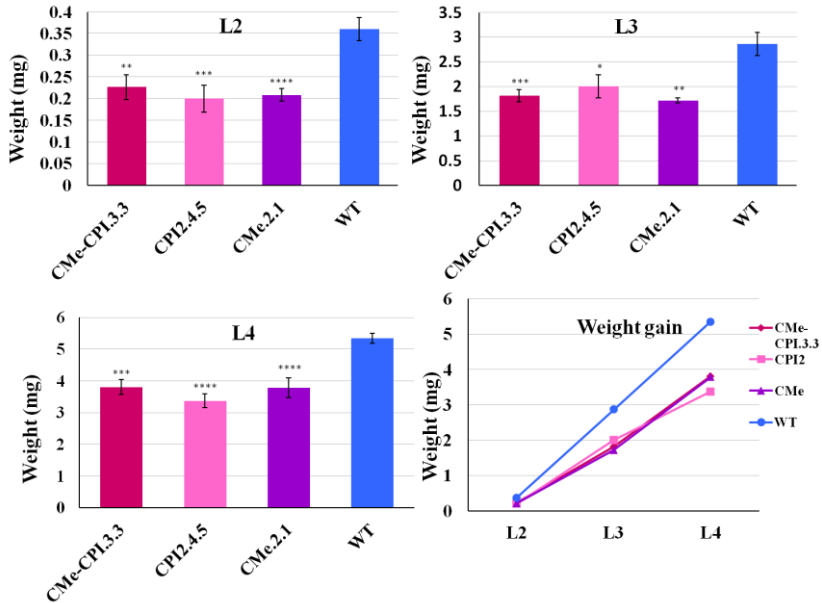
Figure 23: Mean weight of *T. absoluta* larvae fed with transgenic and wild type tomato leaves.

III.4. Survival

Along the larval development, no mortality has been registered for insects fed on control plants. However, larvae fed with CMe-CPI.3.3 suffered 43.75 % mortality. The first two instars showed the highest death rate. Larvae fed with CMe.2.1 and CPI2.4.5 showed respectively 7.14 % and 11.76 % mortality, with no significant difference with the control (Figure 24). Before death, we observed that some larvae showed inflated exoskeleton, abnormal silk secretions and

reduced mobility. Some of the adults emerged from larvae fed with the three transgenic plants showed wings deformities (Figure 25).
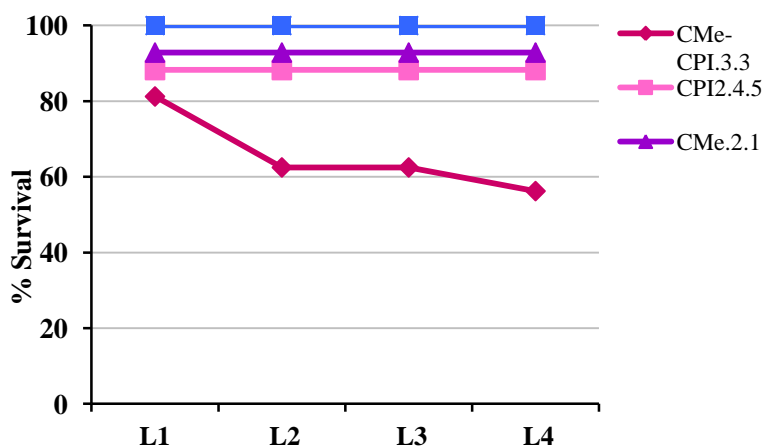
Figure 24: *Tuta absoluta* **larval survival when fed with the different transgenic and control tomato plants.**

III.5. Oviposition

The metabolic perturbations undergone during larval development affected adults' fecundity. We counted the deposited eggs of couples previously fed, during larval stages, with leaves of the different transgenic and wild type plants (Figure 25). Adults showing wing deformities were unable to copulate and consequently to lay eggs. Adults with no deformities emerged from CPI2.4.5 and CMe-CPI.3.3 fed

larvae showed a significantly reduced fecundity. The number of eggs laid by CMe-CPI.3.3 fed females was reduced by 82.2 % when compared with the control. For adults emerged from CMe.2.1 fed larvae, no significant difference was observed. They either didn't lay eggs (deformed wings) or laid a normal eggs number (Figure 25).
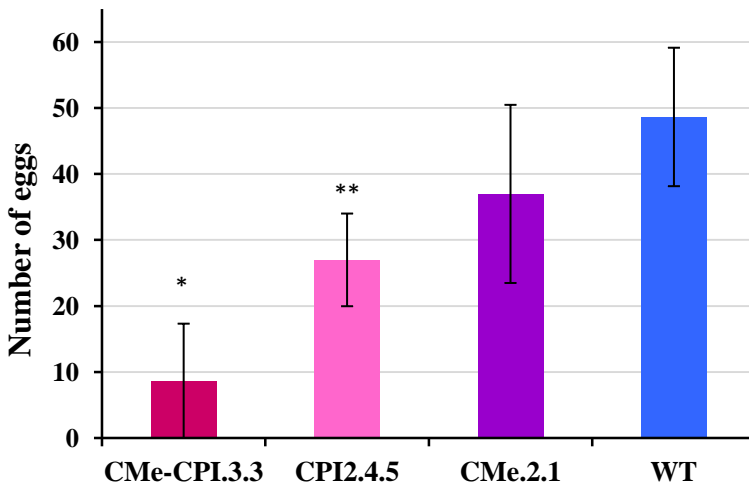
Figure 25: Number of eggs laid by *Tuta absoluta* after 48 hours.
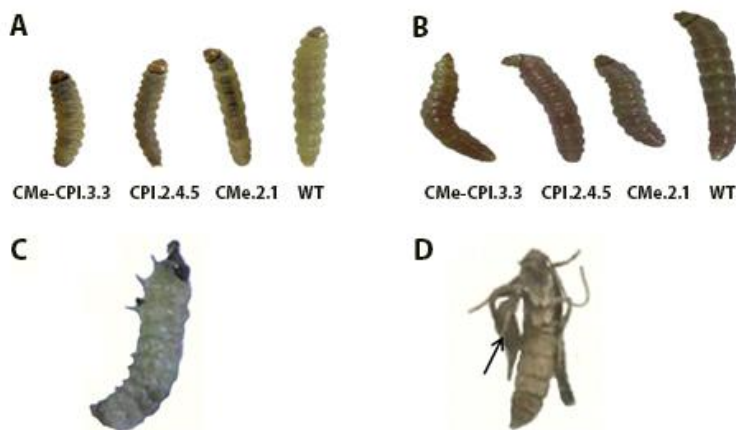
Figure 26: Morphological alterations. A: L2 larvae fed with transgenic and wild type plants; larvae fed with the three transgenic plants show reduced size; **B:** L3 larvae fed with transgenic and wild type plants; larvae fed with the three transgenic plants show reduced size; **C:** Larva fed with CMe-CPI.3.3 leaves showing exoskeleton deformities and silk secretion; **D:** Wing deformities (see arrow) observed in adults emerged from larvae fed with transgenic plants.

III.6. Overall toxicity evaluation

To estimate the combined effect of mortality and oviposition reduction on *Tuta absoluta* population, we calculated the reduction coefficient E based on the corresponding reduction values (Rv) using the Abbot formula (Abbot, 1925). The Reduction coefficient can only be calculated when there is a statistically significant difference. Therefore it was only estimated for CMe-CPI.3.3 plants.

$$RV\ survival = \frac{\%\ Control\ Survival\ - \%\ Experiment\ Survival}{\%\ Control\ Survival}$$

$$RV\ Fecundity = \frac{(Eggs\ per\ Female)\ Control - (Eggs\ per\ Female)Experiment}{(Eggs\ per\ Female)Control}$$

$$E = 100x\ [1 - (RVsurvival\ x\ RVfecundity)] = \textbf{64.03}\ \%$$

III.7. Insect enzymatic activity

Feeding on the three transgenic plants reduced significantly *T. absoluta* trypsin activity. Trypsin and papain activity were estimated by spectrophotometry in the crude extract of *Tuta absoluta* larvae fed with each transgenic plant and the control, 24 hours after each molting (Figure 27). The papain activity was very low, below 2% of the total proteins. However, trypsin-like enzymes were highly present. In L1 larvae, trypsin represented about 15 % in larvae fed with control plants and about 23 % in those fed with transgenic ones. In the stressed larvae, trypsin-like enzymes are induced to compensate the effect of PIs ingestion. Unexpectedly, this response is also observed in larvae fed with CPI2.4.5 although these plants do not express any foreign trypsin inhibitor. In the next instar, trypsin activity decreases in larvae fed with each transgenic tomato leaves, while it stays unchanged in those fed with wild type plants. In the third

instar, when larvae presents the higher weight gain and feed the most, the trypsin activity increases reaching 40.2 % of the total larval proteins in insects fed with the control. In contrast, trypsin-like enzymes remain below 20 % of the total proteins in larvae fed with the transgenic leaves. In L4, larvae prepare for pupation. They reduce their feeding and movements. The trypsin activity is reestablished to about 15 % in all larvae.
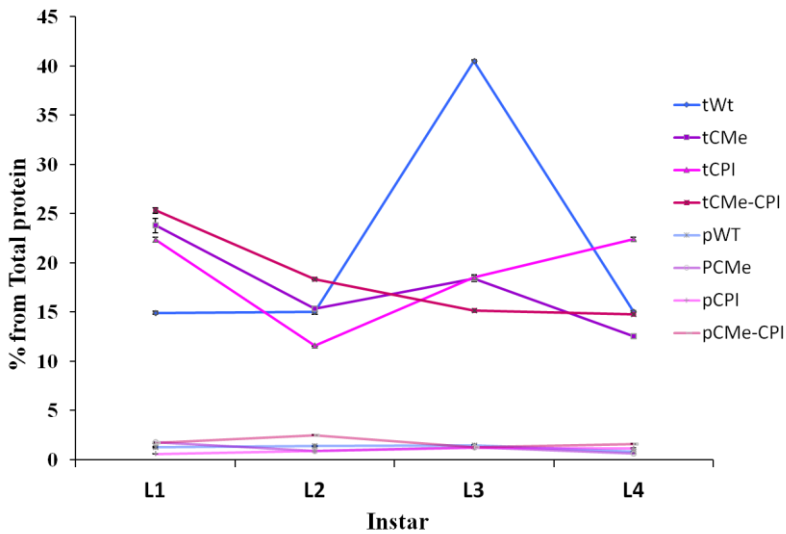
Figure 27: Trypsin and papain content in *T. absoluta* larval crude extract. "p" refers to papain and "t" to trypsin.

IV. *Nesidiocoris tenuis* feeding trials

N. tenuis development and survival was not affected by the transgenic plants. *Nesidiocoris* adults were placed with CMe-CPI.3.3 and wild type tomato plants. Their progeny development was followed until reaching the adult stage. *Nesidiocoris* principally feed on insect eggs, they were provided *Ephestia kuehniella* eggs as alternative aliment. We observed no difference in the duration of the nymphal development between insects reared on transgenic or wild type plants. In both cases, adults were observed 21 days after the beginning of the experiment. At the end of the assay, adults were collected and counted and no difference was observed. Developing on CMe-CPI.3.3 transgenic plants did not affect the survival or the fecundity of *Nesidiocoris tenuis* (Figure 28).

A

B
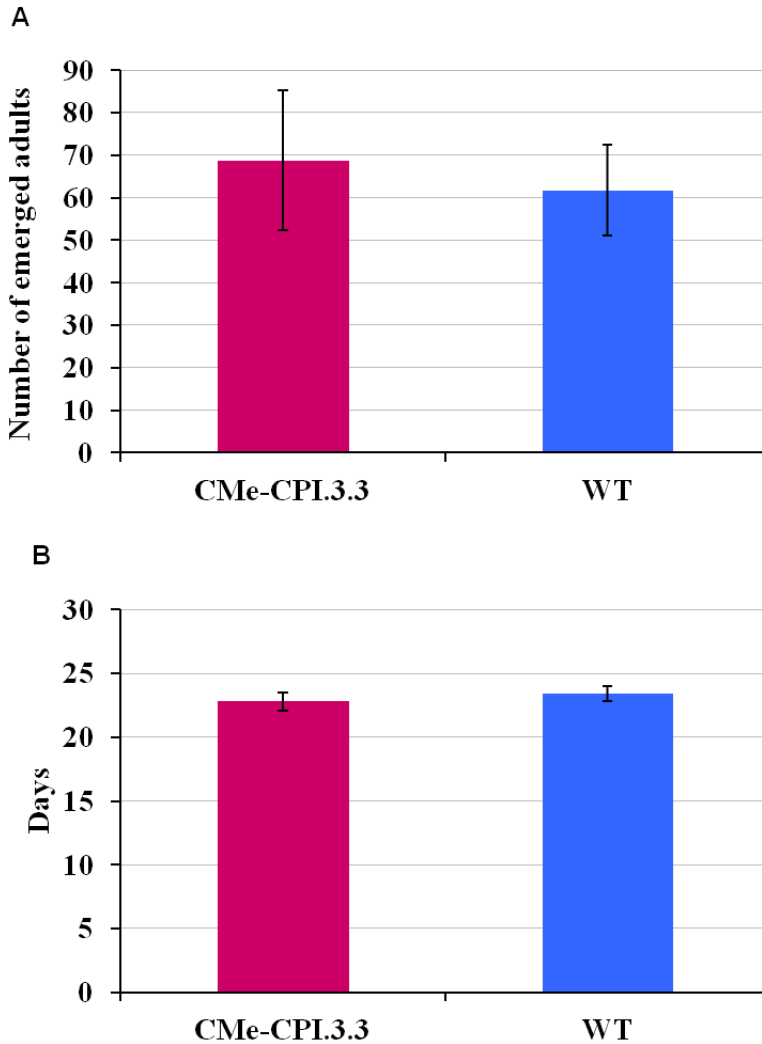
Figure 28: **Effect of PI on the development of *Nesidiocoris tenuis*. A:** Development cycle duration of *Nesidiocoris tenuis* on CMe-CPI.3.3 and wild type plants; **B:** Number of *Nesidiocoris tenuis* adults emerged after developing on CMe-CPI.3.3 and wild type plants.

V. *Pin2* expression analysis

In order to check if the expression of barley PI affects the expression of tomato endogenous PIs genes, we quantified by qRT-PCR the expression of *Pin2* gene in undamaged transgenic plants (Figure 29). As shown in figure 29, the expression of *Pin2* is induced in the transgenic plants harboring *Icy2* gene. However, no difference is observed between CMe.2.1 and the WT control. Moreover the increment in *Pin2* expression is proportional to *Icy2* expression. Indeed, CMe-CPI.3.3 plants express *Icy2* about 2.5 times more than CPI2.4.5 and *Pin2* about 2.75 times more. This suggests that Hv-CPI2 expression in tomato induces *Pin2* expression.
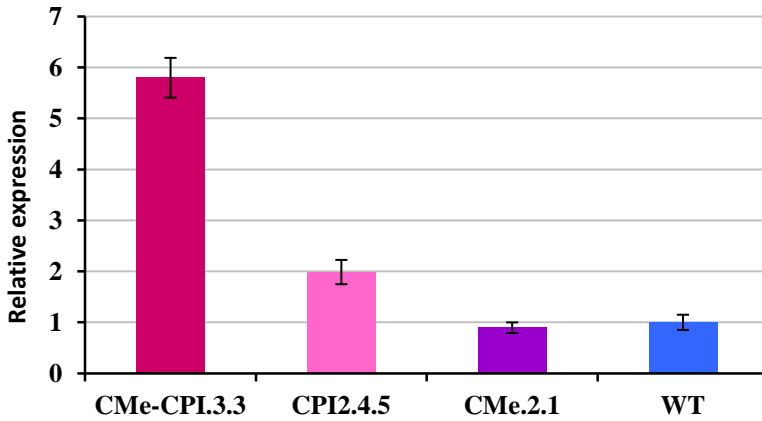
Figure 29: **Relative expression of *Pin2* gene in the different transgenic plants and the wild type.**

VI. Volatiles analysis

VI.1. Olfactory response

The effect of the transgenic tomato CMe-CPI.3.3 volatiles on *Tuta absoluta* and *Nesidiocoris tenuis* behavior was tested on Y-tube olfactometer. The insects were allowed to choose between CMe-CPI.3.3 and wild type plant volatiles.

Tuta absoluta adults were not preferentially attracted by any of the two volatile sources. However, *Nesidiocoris tenuis* showed an obvious preference (63 %) for CMe-CPI.3.3 transgenic tomato (Figure 30).
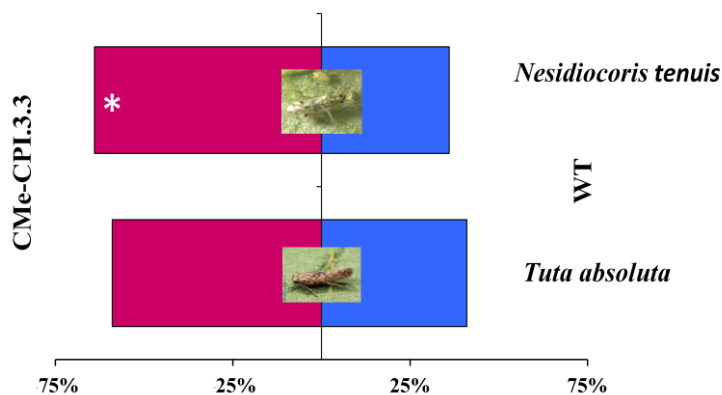
Figure 30: **Olfactory response of *Tuta absoluta* and *Nesidiocoris tenuis* adults to CMe-CPI.3.3 and wild type tomato volatiles.**

VI.2. Volatiles organic compounds (VOCs) emission profiles

VOCs emission profile of the transgenic plants differed from the wild type ones. VOCs from wild type and transgenic CMe-CPI.3.3 plants were analyzed by GC-MS. Volatile compounds from different chemical families were differentially produced in both plants. When compared with the wild type, CMe-CPI.3.3 plants showed different levels of benzenoids and terpenes. Benzaldehyde and another unknown benzenoid were secreted twice more in the transgenic plants, while monoterpenes (unknown monoterpene, α-pinene, camphene, β-myrcene, β-pinene) and

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three unknown sesquiterpenes were reduced to the third (Table 7).

Type	Compound	Kovats RI	Fold change	p value
Monoterpene	Unknown Monoterpene 1	939.2	0.311	**0.02890**
Monoterpene	α-pinene	948.1	0.278	**0.02449**
Monoterpene	Camphene	969.0	0.349	**0.04276**
Monoterpene	β-myrcene	991.3	0.315	**0.02245**
Monoterpene	β-pinene	996.5	0.307	**0.03301**
Sesquiterpene	Unknown Sesquiterpene 1	1356.4	0.290	**0.01674**
Sesquiterpene	Unknown Sesquiterpene 2	1360.4	0.280	**0.01050**
Sesquiterpene	Unknown Sesquiterpene 3	1417.1	0.423	**0.03775**
Sesquiterpene	β-caryophyllene	1464.1	0.634	0.08234
Benzenoid	Benzaldehyde	976.9	2.125	**0.00043**
Benzenoid	Unknown benzenoid 1	1058.0	1.712	**0.00304**
Benzenoid	Acetophenone	1089.2	3.071	0.09021

Table 7: Relative level of VOCs emitted by the transgenic tomato line CMe-CPI3.3 and wild type Micro-Tom plants. *p* values in bold indicate significant differences.

VII. Glandular trichomes density

The fourth leaf from wild type and CMe-CPI.3.3 transgenic tomatoes were examined under binocular loupe in both adaxial and abaxial sides. Transgenic plants leaves showed an increase in glandular trichomes density. The adaxial side of the transgenic leaves presented twice more glandular trichomes. And the abaxial side 1.6 times more (Figure 31).
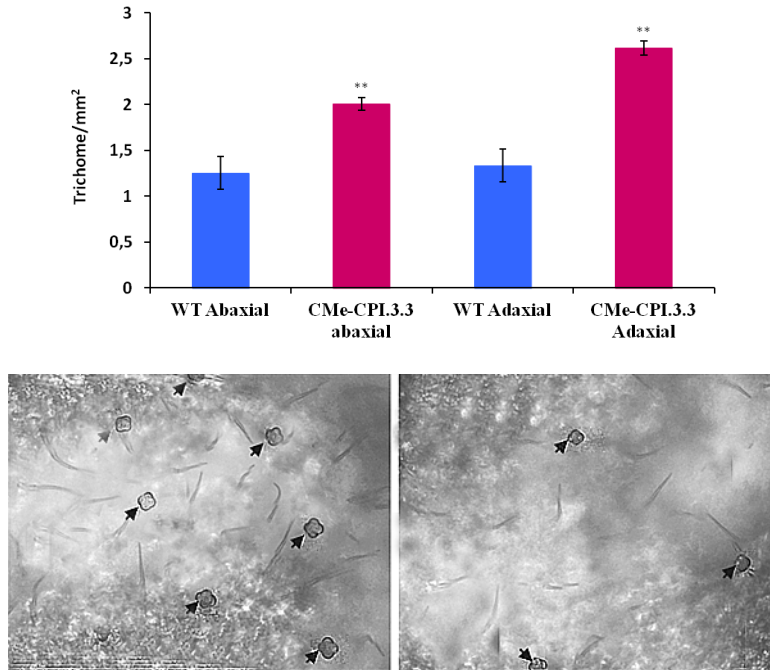
Figure 31: Trichomes density in transgenic and wild type plants. A: CMe-CPI.3.3 plants; **B:** wild type plants.

Discussion

Plant Genetic engineering for pest control

With a predicted increase of world population to reach approximately 9,000 million in 2050, food security is becoming a priority. The FAO estimates that by 2050, food production should increase by 70 % to feed additional 2300 million people. Africa should be increasing its food and feed production by 300 %. To achieve that goal, it is important to both increase production yield and prevent crop loss. Each year about 25 % of crop production is lost due to pests, 12 % due to insects. To cope with pests, 40 thousand million dollars are spent every year on 3 million metric tons of pesticides, worldwide. Despite of the contribution of those chemical treatments to crop protection, it has been demonstrated that they pose both environmental and health concerns. Other alternatives based on new technologies should be contemplated for a more sustainable pest control. In the last decades, many studies have focused on the use of recombinant DNA technologies producing genetically engineered (GE) crops. Despite of the controversy and the restrictive regulation on GE crops, they were adopted by about 17.3 million farmers, covering over 180 million hectares by 2014 (James, 2015) . Several studies have associated GE plants cultivation to different economic and environmental benefits. Use of GE crops allowed enhancing

yield, reducing insecticides spraying and subsequently increasing farmers profit (Figure 32) (Brookes & Barfoot, 2014; Klümper & Qaim, 2014).
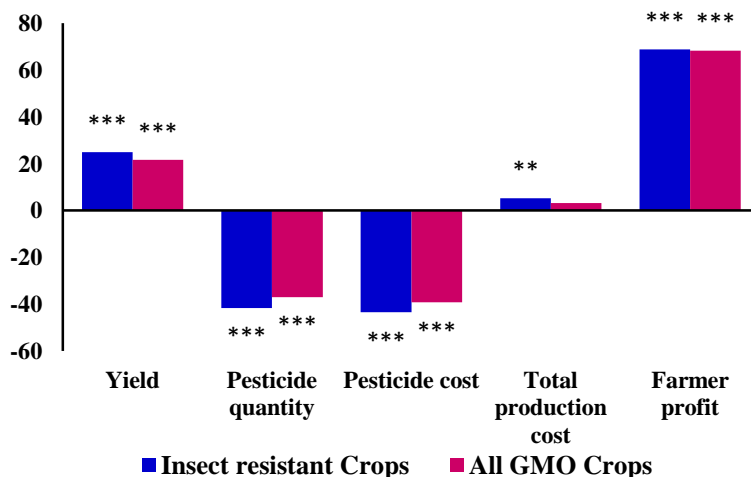
Figure 32: Economic and environmental benefits from GE crops. Adapted from Klümper and Qain, 2014.

According to Klümper and Qaim (2014), insect resistant GE crops permitted to increase yield by 24.8 %, reduce pesticide use by 41.7 % and increase farmers profit by 68.8 %. It is also worthy to mention that lately in 2016, the National Academy of Sciences, Engineering and Medicine reported that after 20 years of GMOs commercialization, no adverse

effect on human health or environment have been found (National Academies of Sciences & Medicine, 2017).

The most used approach in crop engineering for insect resistance is the expression of *Bacillus thuringiensis* endotoxins. *Bacillus thuringiensis* (Bt) is a spore forming bacteria producing insecticidal protein crystals. These proteins are produced during sporulation and are called *Bt* toxins, δ-endotoxins or crystal proteins (Cry proteins). They have been used as bio-pesticides by spore spraying since 1930's, before to be used for genetic engineering. After ingestion by a susceptible insect, the protein is recognized by receptor on the insect's midgut epithelium, inserts into the midgut membrane and leads to the disruption of the electrical K^+ and pH gradients resulting in irreversible damages to the insect midgut wall. Several bacterial strains with distinct Cry proteins have been identified. These proteins have different insecticidal spectrum. Some are toxic to Lepidoptera larvae, others to Coleoptera. Bt toxins have been expressed in several plant species with varying degrees of success. Moreover, unlike conventional broad spectrum insecticides, Bt toxins do little or no harm to non target insects, animals and humans. However, its efficacy is reduced because the rapid evolution of pest resistance (Tabashnik *et al.*, 2013).

Other alternatives have been investigated to improve plant pest-resistance. Isopentenyl-transferase gene (*ipt*) from *Agrobacterium tumefaciens* coding for an enzyme of the cytokinin-biosynthetic pathway was expressed in tomato and tobacco. Its expression decreased leaf consumption by *Manduca sexta* (Linnaeus) (Lepidoptera: Sphingidae) larvae and reduced *Myzus persicae* (Sulzer) (Homoptera: Aphididae) survival (Schuler *et al.*, 1998; Smigocki *et al.*, 2000). Cytokinins have been shown to influence plant secondary metabolism pathways whose product exhibits insecticidal properties (Li *et al.*, 2004). They are also involved in primary plant response to wounding by conditioning plants for a more rapid or higher magnitude response to subsequent insect attack (Dervinis *et al.*, 2010). They were also shown to modulate salicylic acid signaling and enhance resistance against pathogens through an increased expression of SA-related defense genes (Jameson, 2000).

Genes from higher plants have also been used. Lectins, which are carbohydrate-binding proteins, have shown toxic activity against some Homoptera, Coleoptera, Lepidoptera and Diptera. However some lectins have shown significant toxicity toward mammals (Jaffé & Vega Lette, 1968; Chrispeels & Raikhel, 1991; Powell *et al.*, 1995).

Another approach is the expression of plant anti-metabolic proteins: proteinase inhibitors (PI). The first successful expression of a foreign PI in plant was reported in 1987. The cowpea trypsin inhibitor (CpTI) was expressed in tobacco (Hilder *et al.*, 1987). Over the last two decades, several works focused on the development of transgenic plants harboring PIs genes from different sources. Transgenic plants showed higher resistance to different insects, mainly lepidopteran and coleopteran in about 90 % of cases (including field trials) (Dunaevsky *et al.*, 2005). For instance, the maize serine proteinase inhibitor gene (*mpi*) was introduced into two japonica rice varieties. The transgenic plants showed enhanced resistance to the stripped stem borer *Chilo suppressalis* Walker (Lepidoptera: Pyralidae) (Vila *et al.*, 2005). Another example is the expression of the oryzacystatin in eggplant. *Myzus persicae* and *Macrosiphum euphorbiae* (Thomas) (Hemiptera: Aphididae) fed with the transgenic eggplants suffered negative impact on population growth and an increase of mortality rate (Ribeiro *et al.*, 2006). Similarly, *Nicotiana alata* PI expresion in transgenic Royal Gala apple affected the light brown apple moth (*Epiphyas postvittiana* (Walker) (Lepidoptera, Tortricidae) larval weight and pupa size. Emerged adults also showed body shape and wings deformities (Maheswaran *et al.*, 2007).

Unfortunately, some insects are able to adapt to PIs presence in their diet. Such adaptation may be caused by the production in the insect's digestive tract of novel proteinases of a different mechanistic class, insensitive to the PI. In order to circumvent this mechanism, some researchers have co-expressed different PIs targeting multiple digestive proteinases. In this context, the sweet potato sporamin (a trypsin inhibitor) and the CeCPI (phytocystatin) from taro (*Colocasia esculenta*) were expressed in tobacco conferring resistance to *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) and the pathogens *Erwinia carotovora* and *Pythium aphanidermatum* (Senthilkumar *et al.*, 2010). Another example is the expression of the potato proteinase inhibitor II (PIN2) and the potato carboxypeptidase inhibitor (PCI) in tomato. Homozygous transgenic plants showed an increased resistance to *Heliothis obsolete* (Fabricius) (Lepidoptera: Noctuidae) and *Liriomyza trifolii* (Burgess) (Diptera: Agromyzidae) (Abdeen *et al.*, 2005).

Yet another way of obtaining highly active inhibitors against pests' proteinases, is the construction of hybrid forms of inhibitors with different active domains, capable of acting on proteases of different catalytic classes. The first protein of such kind was constructed using the soybean multicystatin (SMC) which has three active domains. The third domain of

the protein was replaced by the bitter melon (*Momordica charantia*) serine proteinase inhibitor. The obtained hybrid proteinase inhibitor had both trypsin and papain inhibitory activity and suppressed growth of *Spodoptera exigua* (Fabricius) (Lepidoptera: Noctuidae) larvae (Kouzuma *et al.*, 2000). Another hybrid inhibitor was obtained by fusing the maize proteinase inhibitor and the potato carboxypeptidase inhibitor (PCI) in a single open reading frame. The fusion protein was expressed in rice plants causing an important larval weight reduction of *Chilo suppressalis* (Quilis *et al.*, 2014).

Although significant protection against insects has been achieved by expressing PIs in transgenic plants, this approach have almost not been commercially used. The ability of insects to adapt to single PI and the lack of long term studies in field represent a limiting factor. The genetically engineered cotton which expresses a Bt toxin and CpTI (Cowpea trypsin inhibitor) is the only commercially available plant expressing a foreign PI. Many researches are dedicated to identify genes to improve different crops through genetic engineering, resulting in several publications end patents. However, these findings are not reflected in the number of biotech crops released in the market. Twenty years after the commercialization of the first GE plant, the market of

transgenic crops is still dominated by only four crop plants (soybean, cotton, maize and canola) with two improved traits (pest and herbicide resistance). The discrepancy between research and development is probably due to three main factors: high regulatory costs, restricted access to intellectual property and reluctance of consumers to GE crops. Many GE agronomic traits that showed efficiency in the field may not be valuable enough to the producer to justify their commercial application and the resulting costs. For instance commercial production of potato plants expressing the insecticidal *B. thuringiensis* protein Cry3A was profitable for companies specialized in plant-incorporated pest resistance. However, Colorado potato beetle infestation is not a major issue for american growers, who use imidacloprid-based insecticides to effectively control various pests. This resulted in the remove of the transgenic potatos from the market (Rommens, 2010). Also, Biotech products having documented agronomic, economic and environmental advantages have been removed from the market due to the concerns of processors and distributors about potential consumer rejection (Gianessi *et al.*, 2003). New products should have clear advantages for producers, marketers and consumers to be commercially viable. In order to gain consumers acceptation and support, GE food should provide

direct benefits to the client, such as lower price, enhanced flavor or health benefits.

Expression of BTI-CMe and Hv-CPI2 enhances tomato resistance to *Tuta absoluta*

In our work, we focused on the use of proteinase inhibitors (PIs) as molecular tools to improve plants insect-resistance. PIs are small ubiquitary proteins induced in plants in response to pests and pathogens. They achieve different functions in plants: PIs are involved in storage proteins mobilization, programmed cell death and plant defense. Their insecticidal potential is due to their capacity to inactivate herbivory insects' digestive enzymes, hindering their growth and reducing their survival. Proteinase inhibitors expression in different crops has enhanced their resistance to pests (Abdeen *et al.*, 2005; Smigocki *et al.*, 2013; Quilis *et al.*, 2014). However, some insects were able to develop resistance mechanisms by synthesizing different digestive proteases of distinct families. In our work, we chose to co-express two proteinase inhibitors of two different mechanistic families to avoid insect adaptation. We also selected proteinase inhibitors from a genetically distant plant source. Some researchers suggested that insects feeding on dicots are unable to adapt to proteinase inhibitors from monocots (Duan *et al.*, 1996; Pompermayer *et al.*, 2001). BTI-CMe was

previously expressed in rice and wheat and enhanced their resistance to *Sitophilus oryzae* (Linnaeus) (Coleoptera: Curculionidae) and *Sitotroga cerealella* (Olivier) (Lepidoptera: Gelechiidae) respectively (Altpeter *et al.*, 1999; Alfonso-Rubí *et al.*, 2003). Hv-CPI2 is a cystein proteinase inhibitor from barley, with an important *in vitro* activity (Martinez *et al.*, 2009). Transgenic plants co-expressing both PIs showed stronger insecticidal effect on *Tuta absoluta* larvae. This could be associated either with a synergistic effect of both inhibitors or a higher transgene expression levels in CMe-CPI.3.3 plants. When fed with transgenic CMe-CPI.3.3 leaves, *Tuta absoluta* larvae suffered weight reduction. Indeed, mean weight was reduced by 34.2 % for *Tuta absoluta* larvae fed with CMe-CPI.3.3 leaves when compared with the wild type plants. *Tuta absoluta* larvae seemed unable to digest the ingested aliment. They were not capable of degrading the nutrient and use them for their correct growth and development. Larval survival was also significantly reduced. While no larval mortality was registered for insects fed on wild type plants, 43.75 % of the larvae fed on CMe-CPI.3.3 plants did not reach pupae stage. Larvae from the first and the second instars were the more susceptible with the highest mortality rate (18.75 % each). The observed effects on larval weight and survival are explained by the inhibitory activity of the expressed PIs

against *Tuta absoluta* digestive enzymes. No previous study has identified *Tuta absoluta* digestive proteases. However, it is well documented that Lepidoptera predominantly use serine proteinases for their digestion, while Coleoptera usually rely on cystein proteinases (Saikia *et al.*, 2010; Schlüter *et al.*, 2010). For instance, trypsin-like and chymotrypsin-like enzymes represent respectively 40 % and 30 % of the tomato moth *Lacanobia oleracea* digestive enzymes (Gatehouse *et al.*, 1999). *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), another tomato Lepidoptera pest, mainly presents serine proteinases (Johnston *et al.*, 1991; Christeller *et al.*, 1992; Gatehouse *et al.*, 1997). We were able to detect the presence of the target proteinases (trypsin and papain) by enzyme histochemistry. They were localized along the whole digestive system (foregut, midgut and hindgut), in the excretory system (Malpighi tubules) and the exoskeleton. We analyzed *Tuta absoluta* larvae trypsin and papain activity at all the larval stages. While papain activity was almost inexistent, trypsin-like enzymes were highly represented. In larvae fed with control plants, trypsin-like enzymes reached 40 % of total larval proteins in L3 instar. At this instar, proteolytic activity reaches its highest levels. Larvae increase considerably in size and start to acquire their characteristic green color due to intensive feeding. However, larvae fed on the transgenic

CMe-CPI.3.3 transgenic plants does not show any increase in the proteolytic activity at this stage. Their weight gain is limited despite of feeding. The consumption of PIs hinders nutrients uptake and subsequent growth. Even if *Tuta absoluta* larvae did not show cysteine proteinase activity, larvae fed with CPI2.4.5 plants also showed the same enzymatic profile, with no increase in trypsin-like activity during the third instar. These results suggested that the effect observed on *Tuta absoluta* is not strictly due to transgene expression.

In addition to the deleterious effects observed on larvae, we studied the effects of PIs on the emerged adults. The majority of *Tuta absoluta* adults emerged from larvae previously fed with transgenic plant leaves presented deformed wings. These individuals could hardly fly and were unable to copulate and produce any egg. Oviposition assays demonstrated that CMe-CPI.3.3 fed insects laid 82.3 % fewer eggs than those fed with non transgenic plants. Similar results were reported in *Helicoverpa armigera* and *Spodoptera litura* when using non host PIs from bitter gourd and Capsicum respectively (Telang *et al.*, 2003; Tamhane *et al.*, 2005). Tomato PI also affected notably the fecundity of *Helicoverpa armigera* according to Damle et al. (2005). The fecundity of Lepidoptera adults is an important parameter for determining

the effect of larval diet on the adult stage. Also, low fecundity value means less progeny, having direct impact on the subsequent generation. In order to evaluate the global effect on transgenic leaves ingestion on *Tuta absoluta*, both survival and fecundity rates were considered. Global population reduction coefficient was 64 %. BTI-CMe and Hv-CPI2 consumption had various negative effects on *Tuta absoluta* development, survival and fecundity. Previous studies have shown that the effect of PIs on insects is dose dependent, higher resistance is acquired when PIs are expressed at high levels (De Leo *et al.*, 1998; Rahbé *et al.*, 2003). Generating genetically engineered tomato plants expressing higher levels of BTI-CMe and Hv-CPI2 could inflict stronger harm to *Tuta absoluta* and provide a better control of its population.

Expression of BTI-CMe and Hv-CPI2 in tomato had no harmful effects on *Nesidiocoris tenuis*

This strategy could also be combined with the use of *Tuta absoluta* predators like *Nesidiocoris tenuis*. This mirid is an efficient control agent of *Tuta absoluta* both in the field and greenhouses. However, to reach a reliable control of this pest, a high density of mirids is needed. The inconvenient is that being a zoophytophage, it also feeds on tomato plants. When it is present at high population density, *Nesidiocoris tenuis* inflicts harms to tomato stem and fruits, generating necrosis.

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A combined strategy using both transgenic plants and low density of *Nesidiocoris tenuis* could allow an efficient control of *Tuta absoluta*. In order to confirm the compatibility of these two approaches, *Nesidiocoris tenuis* was allowed to reproduce and develop on transgenic CMe-CPI.3.3 and wild type tomato plants. No differences in fecundity, development or survival were observed between insects fed with both tomato lines. *Nesidiocoris tenuis* feeds mainly on insect larvae and eggs. Their digestive enzymes are probably distinct from strict phytophagous insects. Moreover, when prey is available, (larvae, eggs), *Nesidiocoris tenuis* feeding on tomato plants is sporadic and therefore, low quantities of PIs are ingested. This could explain the innocuity of these PIs on the mirid.

Hv-CPI2 expression induces tomato defense

The impact of PIs expression in transgenic plants on phytophagous insects have been largely studied, however, no previous study investigated their effect on the plant endogenous defense mechanisms. Our results suggest that the expression of the barley cysteine proteinase inhibitor Hv-CPI2 in tomato activates endogenous direct and indirect defense mechanisms.

As mentioned above, larvae fed with CPI.2.4.5 plants showed a decrease in trypsin activity suggesting that the deleterious effects may not only be caused by the introduced transgenes. We analyzed the expression of the tomato wound inducible serine proteinase inhibitor PIN2 in the different transgenic plants and in the wild type. Unexpectedly, we found that in the transgenic lines expressing Hv-CPI2, the level of expression of PIN2 was increased. Increased expression level of Hv-CPI2 was correlated with an increment in PIN2 expression. However no difference in *Pin2* expression is observed in CMe.2.1 plants compared with the wild type. This suggests that *Pin2* is induced in presence of the barley cystatin Hv-CPI2. PIN2 presents trypsin and chymotrypsin inhibitory activity (Bryant *et al.*, 1976). This proteinase inhibitor has previously been expressed in plants to improve their resistance against pests. Its expression in tobacco reduced *Manduca sexta* growth (Johnson *et al.*, 1989). When PIN2 homolog from potato was expressed in rice and wheat, it enhanced their resistance respectively to *Sesamia inferens* (Walker) (Lepidoptera: Noctuidae) and *Heterodera avenae* (Wollenweber) (Nematoda: Heteroderidae) (Duan *et al.*, 1996; Vishnudasan *et al.*, 2005).

According to these finding, CMe-CPI.3.3, in fact, overexpresses three PIs of different mechanistic classes: two

trypsin (BTI-CMe and PIN2), a cystatin (Hv-CPI2) and a chymotrypsin (PIN2) inhibitors. The co-expression of these three PIs make *Tuta absoluta* adaptation to the transgenic plants harder and less probable. As suggested in previous studies, insects are, in some cases, able to adapt to a single PI. However, this response could be avoided by combining different PIs of different classes (Oppert *et al.*, 2003; Abdeen *et al.*, 2005). Oppert et al. (2003) have reported that the Colorado flour beetle, when fed with cystatin supplemented diet, produces serine proteinase digestive enzymes as a compensatory response. The same phenomenon was observed in *Helicoverpa zea*, where, the presence of the Soybean trypsin inhibitor was compensated by the production of chymotrypsins (Mazumdar-Leighton & Broadway, 2001). It would be difficult for *Tuta absoluta* larvae during their short larval development to achieve a compensatory mechanism toward three PIs of different families.

PIN2 is highly expressed in tomato trichomes both constitutively and in response to phytophagous insects attack. Trichomes are hair-like epidermal protuberances produced by most plant species (Werker, 2000). They assume different functions, such as protection against insects (Levin, 1973). Their production is usually constitutive; however, some plant species increase trichome density in new leaves upon

damage. CMe-CPI.3.3 plants showed higher glandular trichomes density when compared with wild type plants. This finding agrees with previous studies. Luo *et al*. (2009) have shown that the expression of the night shade (*Solanum americanum*) *SaPIN2* gene increased glandular trichomes density in tobacco and enhanced its resistance toward the larvae of the two lepidoptera species *Helicoverpa armigera* and *Spodoptera litura*. Tomato plants have both non glandular and glandular trichomes. While the first ones act as a mechanical barrier against pests, the second type is responsible for the secretion of a variety of metabolites and volatiles which can be harmful or repellent to insects and attractant to their predators (Duffey, 1986). Plants exposed to pest damages tend to produce new leaves with higher trichome density. It has been shown that, when fed with induced leaves, insects consumed less foliage and grow less compared to those fed with non-induced ones (Björkman *et al.*, 2008). In *Lycopersicon spp.*, The chemical removal of glandular trichomes resulted in decreased mortality and increased longevity of pests such as *Manduca sexta* (Barbour *et al.*, 1991) (Barbour *et al*., 1991), *Helicoverpa armigera* (Simmons *et al.*, 2004) and *Myzus persicae* (Simmons *et al.*, 2003). However, the increase of trichome density engendered decrease of survival and increase of entrapment for different pests such as *Helicoverpa armigera* (Simmons *et al.*, 2004),

Tetranychus urticae (Carter & Snyder, 1985), or *Myzus persicae* (Simmons *et al.*, 2003).

As trichomes are responsible for the production of some volatile organic compounds (VOCs), we investigated plants volatiles production and insects' olfactory response. *Nesidiocoris tenuis* adults were attracted by CMe-CPI.3.3 transgenic plants volatiles, while *Tuta absoluta* has no preference for either of the two plant lines. These results were supported by the VOCs analysis. CMe-CPI.3.3 transgenic plants have shown increased levels of benzenoids and reduced levels of monoterpenes and sesquiterpenes when compared with the wild type plants. Benzenoids have been described as insect attractants. They have, thus, been reported to attract natural enemies of plant pests. Octyl benzaldehyde was shown to attract *Orius tristicolor* (White) (Hemiptera: Anthocoridae) (a bug depredator of the acari *Tetranychus urticae* (Koch) (Acari: Tetranychidae) and trips) and *Sepsis punctum* (Fabricius) (Diptera: Sepsidae) (a fly predator of Lepidoptera). In addition to the attraction of natural enemies, benzenoids also act as repellents of phytophagous pests. *Sesamum indicum*, which represents a natural refuge for mirids shows a strong attraction for *Nesidiocoris tenuis* when compared with tomato. Naselli *et al*. (2017) have associated this attraction with reduced levels of hydrocarbon

monoterpenes when compared with tomato. These results agree with our findings. The fact that the CMe-CPI.3.3 plant secretes lower concentrations of hydrocarbon monoterpenes (α-pinene, β-mycene, β-pinene) and higher levels of benzenoids could explain the attraction that it has for *Nesidiocoris tenuis* adults.

VOCs are classified based on their biosynthesis origin, among them, terpenoids and benzenoids. Biosynthesis of different VOCs branch off from a common primary metabolic pathway (Figure 33).

Terpenoids constitute the largest class of volatile constituents. They are derived from two common five carbon precursors: Isopentenyl diphosphate (IPP) and its allylic isomer Dimethylallyl diphosphate (DMAPP) (McGarvey & Croteau, 1995). In plants, two pathways are responsible for their biosysnthesis: the mevalonate (MVA) and the methylerythritol phosphate (MEP) pathways. The MVA pathway consists of six enzymatic reactions. It is initiated by the condensation of three molecules of acetyl-CoA with the 3-hydroxy-3-methylglutaryl-CoA which undergoes reduction to MVA followed by two phosphorylations and a decarboxylation/elimination step with formation of IPP as the final product (Lange *et al.*, 2000). The MEP pathway which occurs in the plastid, involves seven enzymatic reactions. It

starts with the condensation of D-glycerldehyde-3-phosphate (GAP) and pyruvate (Pyr) to produce 1-deoxy-D-xylulose-5-phosphate which undergoes subsequent isomerization/ reduction leading to the formation of MEP, the pathway specific intermediate. Five subsequent reactions are then required to convert MEP to IPP and DMAPP. MEP pathway relies on primary metabolism for the supply of Pyr and GAP derived respectively from glycolysis and the pentose phosphate pathway (PPP). MEP is often insured higher carbon flux than the MVA pathway (Laule *et al.*, 2003; Dudareva *et al.*, 2005).

Benzenoids constitute the second largest class of VOCs (Knudsen & Gershenzon, 2006). They are biosynthesized from the aromatic amino acid: Phenylalanine (Phe). Seven enzymatic reactions of the shikimate pathway and three of the arogenate pathway are needed (Tzin & Galili, 2010; Maeda & Dudareva, 2012). The precursors of the shikimate pathway are phosphoenolpyruvate (PEP) and D-erythrose 4-phosphate (E4P), provided respectively from glycolysis and PPP pathways. The same metabolic routes provide precursors for the MEP pathway, therefore it has to compete with the shikimate pathway (Razal *et al.*, 1996; Dudareva *et al.*, 2013). This competition for the substrate could explain the VOCs profile observed in CMe-CPI3.3 transgenic plants.

While benzenoids synthesis is privileged, terpenoids emission is reduced. The rate of synthesis of any VOC is not only conditioned by the activity of the enzymes responsible for its formation, but is rather controlled by the amount of available substrate (Effmert *et al.*, 2005; Guterman *et al.*, 2006). Precursor availability is also known to play a key role in the regulation of rhythmic emission of VOCs (Kolosova *et al.*, 2001; Maeda *et al.*, 2010; Colquhoun *et al.*, 2011) as plants emit volatiles with different diurnal and nocturnal patterns (Lerdau & Gray, 2003; Martin *et al.*, 2003; van Doorn & Woltering, 2008). The first enzyme of the shikimate pathway is the 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHP synthase). This enzyme plays an important role in controlling carbon flux into the pathway (Tzin *et al.*, 2012). Previous studies have shown that this enzyme is induced by jasmonic acid (Hara *et al.*, 1994; Suzuki *et al.*, 1995). This phytohormone is also known to induce glandular trichomes differentiation (Li *et al.*, 2004; Boughton *et al.*, 2005; Peiffer *et al.*, 2009) and proteinase inhibitors expression (Farmer & Ryan, 1990; Howe *et al.*, 1996).
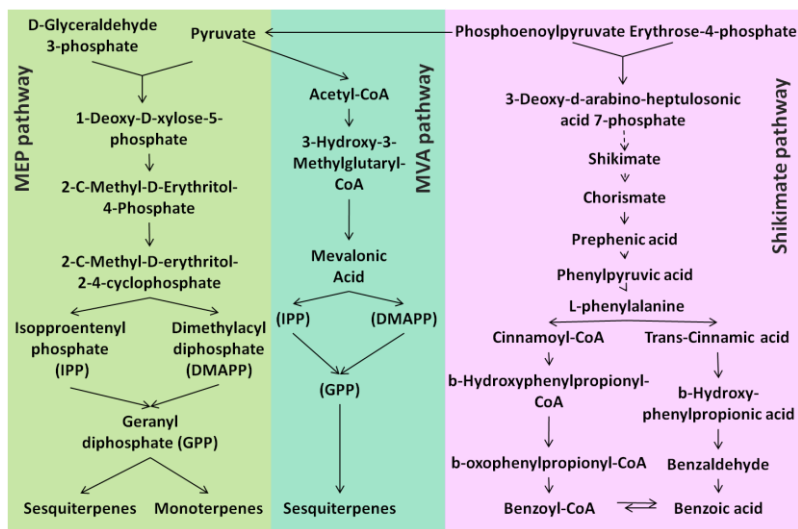
Figure 33: Metabolic pathways leading to VOCs biosynthesis. A: Terpenes synthesis through the MEP pathway; **B:** Terpenes synthesis through the MVA pathway; **C:** Benzenoids synthesis through the shikimate pathway.

Although the mechanism is still to be elucidated, we suggest that expression of the barley cysteine proteinase inhibitor Hv-CPI2 in tomato might induce jasmonic acid synthesis via the octadecanoid pathway. The increase in this hormone could be responsible for the activation of the defensive mechanism observed in the transgenic plants (Figure 34). Jasmonic acid mediated pathways are the frontline defense mechanism normally activated in response to various threats like phytophagous insects. In the transgenic CMe-CPI.3.3 plants, this mechanism seems constitutively activated in absence of

any biotic or abiotic stress. Direct and indirect defense arsenals are thus activated attracting predators (VOCs) and expressing insecticidal proteins (PIs).
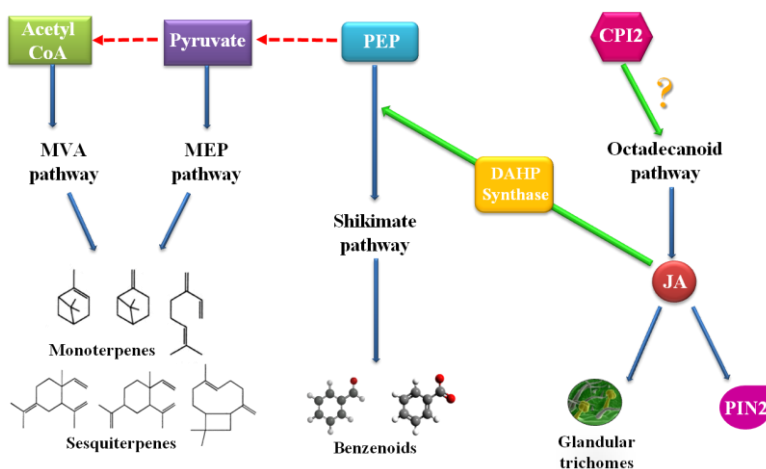
Figure 34: **Defense mechanism pathways in tomato.** Suggested activation is indicated with green arrows and inhibitions with red arrows.

Conclusions

The results found in this work allow us to make the following conclusions:

First: We succeeded to improve the *in vitro* BTI-CMc trypsin activity by introducing a single mutation in its putative reactive site.

Second: When expressed together, BTI-CMe and Hv-CPI2 had a synergistic effect. The double transgenic plants showed higher resistance against *Tuta absoluta* than plants expressing each one of the transgenes.

Third: Feeding on plants expressing proteinase inhibitors affected *Tuta absoluta* at different levels: survival, weight and physiology during larval instars, and morphology and fecundity in the adult stage.

Fourth: *Tuta absoluta* digestion relies mainly on trypsin–like enzymes that are sensitive to the barley proteinase inhibitors expressed in the tomato transgenic plants.

Fifth: *Nesidiocoris tenuis* development and survival were not affected by the presence of the proteinase inhibitors.

Sixth: Volatiles emitted by the double transgenic plants attracted *Nesidiocoris tenuis* adults. However, they did not affect *Tuta absoluta* behavior, allowing the combined use of genetic engineering and biocontrol strategies.

Seventh: Barley cystatin, Hv-CPI2, expression promoted plant defense, inducing the tomato endogenous wound inducible

proteinase inhibitor 2 (*Pin2*) gene, increasing glandular trichomes production and modifying their volatile organic compounds emission.

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