UNIVERSITÀ DEGLI STUDI DELLA Tuscia

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UNIVERSITÉ PAUL CÉZANNE AIX-MARSEILLE III

DIPARTIMENTO DI AGROBIOLOGIA E AGROCHIMICA

Dottorato di ricerca in
Biotecnologie Vegetali

XXIII Ciclo

Isolation and functional characterization of pectin methylesterase inhibitor (PMEI) genes in durum wheat (*Triticum durum* Desf)

BIO/04

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A Giuseppe e Niccolò,
amori della mia vita

ABSTRACT

One of the main goal of breeding programs is the development of crop varieties that simultaneously control different pathogens. This can be obtained by improving the pre-existing plant defence mechanisms such as the structure and composition of the plant cell wall, which is one of the first barriers encountered by the microbial pathogens during plant tissue colonization. To overcome this obstacle, most fungal pathogens produce a variety of enzymes that degrade the wall polysaccharides; among them, pectin degrading enzymes are among the first to be secreted by the pathogens to enter and spread into the plant tissue.

Pectin in the plant cell wall is secreted in a highly methylesterified form and is demethylesterified *in muro* by pectin methylesterase (PME). PME activity contributes to modify the structure of pectin, affecting also the susceptibility to hydrolytic attack of pathogen pectin degrading enzymes. The activity of PME is regulated by specific interaction with a protein inhibitors (PMEIs). Since the modification of pectin are associated with important plant physiological processes and also with plant defence response, the interaction PME-PMEI can have an important biological role.

In wheat, genes encoding for pectin methyelesterase inhibitor have not been characterized, for this reason during this work three different genes *Tdpmei2.1*, *Tdpmei2.2*, *Tdpmei7.3* were characterized from durum wheat cv. Svevo. It has been demonstrated that these genes encode for functional inhibitor able to reduce the PME activity of Orange peel PME (OpPME). Moreover, the TdPMEI2.1, TdPMEI2.2 e TdPMEI7.3 are able to inhibit the endogenous wheat PME activity, showing a specificity against PME activity from different wheat tissues. All the three inhibitors do not show inhibitory capability against microbial PME.

Tdpmei2.1 e *Tdpmei2.2* have been localized on the long arm of chromosome group 2 and their sequence is strongly conserved in wild wheat progenitors.

To shed light on the possible physiological role of these inhibitors, the transcript accumulation of *Tdpmei2.1*, *Tdpmei2.2* and *Tdpmei7.3* has been investigated. Results show that trascrip*Tdpmei2.2* and *Tdpmei7.3* undergo intron retention. The complete removal of intron of both genes is observed only in anther tissue. Differently, the *Tdpmei7.3* transcript accumulated in all tissue analyzed but strongly in stem tissue.

Transcript accumulation of *Tdpmei* genes has been investigated also following infection with the fungal pathogen *Bipolaris sorokiniana*, but no increase in the level of transcripts was observed.

To verify the capability to modulate *in planta* the wheat endogenous PME activity, wheat transgenic wheat plants overexpressing the *Tdpmei7.3* were produced. T_0 plants showed different level of PME activity reduction that varied from about 10% to 85% of the control PME activity.

RIASSUNTO

Una delle strategie più promettenti nei programmi di miglioramento genetico riguarda sicuramente la costituzione di varietà vegetali in grado di resistere all'aggressione dei patogeni, che rappresentano una delle più serie minacce per la produttività agricola. Ciò può essere ottenuto rafforzando i meccanismi di difesa pre-esistenti della pianta, come la struttura e la composizione della parete cellulare vegetale che è una delle prime barriere che i patogeni devono superare per colonizzare il tessuto ospite. Per superare questo ostacolo, la maggior parte dei patogeni fungini produce una serie di enzimi che degradano i polisaccaridi della parete cellulare. Tra questi, gli enzimi pectici sono tra i primi ad essere secreti dai patogeni per poter penetrare e colonizzare il tessuto ospite.

La pectina è secreta nella parete cellulare in forma altamente metilesterificata ed in seguito viene de-esterificata *in muro* dalle pectin metilesterasi (PME). L'attività delle PME contribuisce a modificare la struttura della pectina, influenzandone anche la suscettibilità all'azione idrolitica degli enzimi pectici prodotti dai patogeni. L'attività di questo enzima è modulata dall'interazione specifica con l'inibitore della pectin metilesterasi (PMEI). Poiché le modificazioni della pectina sono associate a importanti processi fisiologici della pianta e anche alle risposte di difesa ai patogeni, l'interazione PME-PMEI può rivestire un ruolo biologico importante.

Dal momento che in frumento geni codificanti per l'inibitore della pectin metilesterasi non sono stati caratterizzati, nel corso di questo lavoro sono stati isolati da frumento duro cv. Svevo tre geni, *Tdpmei2.1*, *Tdpmei2.2*, *Tdpmei7.3*. E' stato dimostrato che questi geni codificano per inibitori funzionali in grado di ridurre l'attività della PME di arancio (Orange peel, OpPME). E' stato anche dimostrato che gli inibitori TdPMEI2.1, TdPMEI2.2 e TdPMEI7.3 sono in grado di inibire l'attività PME endogena di frumento, mostrando una diversa specificità nei confronti dell'attività PME ottenuta da diversi tessuti. I tre inibitori non mostrano attività inibitoria nei confronti di PME microbiche, come già descritto per altri PMEI.

I geni *Tdpmei2.1* e *Tdpmei2.2* sono localizzati sul braccio lungo dei cromosomi del gruppo 2 e presentano una sequenza altamente conservata nei progenitori selvatici del frumento.

Per fare luce sul possibile ruolo fisiologico di questi inibitori, sono state eseguite analisi di espressione dei tre geni in diversi tessuti ottenuti da piante a stadi di sviluppo differenti. I risultati hanno dimostrato che l'espressione dei geni *Tdpmei2.1* e *Tdpmei2.2* è regolata da un meccanismo di splicing alternativo noto come ritenzione dell'introne. E' stato osservato che la completa rimozione dell'introne dei due geni avviene solo nelle antere. I trascritti del *Tdpmei7.3* sono presenti in tutti i tessuti analizzati ma mostrano un forte accumulo a livello del culmo.

L'espressione dei tre geni *pmei* di frumento è stata analizzata anche in piante infettate con il patogeno fungino *Bipolaris sorokiniana*. I risultati non hanno mostrato un incremento dell'accumulo dei trascritti in seguito all'infezione.

Infine, la capacità *in planta* di modulare l'attività PME endogena è stata verificata tramite la produzione di piante transgeniche di frumento sovra esprimenti il gene *Tdpmei7.3*. Analisi preliminari hanno mostrato che le piante transgeniche mostrano vari livelli di riduzione dell'attività PME rispetto al wild type.

RESUME

Un des requis pour le développement d'une agriculture durable est l'utilisation de variétés résistantes aux microorganismes pathogènes. Dans cette optique, il semblerait particulièrement efficace de produire des génotypes renforcés en des systèmes naturels de défense, comme la paroi cellulaire, qui constitue une barrière pour nombreux pathogènes. La majeure partie des pathogènes produisent un arsenal enzymatique capable d'hydrolyser les composants polysaccharidiques de la paroi. Parmi celle-ci les enzymes pectiques revêtent un rôle primaire durant le procès d'infection.

La pectine est secrétée dans la paroi cellulaire sous forme hautement méthylesterifiée et après, par l'action du pectin méthylestérase (PME), vient de-méthylesteréfiée. L'action de cet enzyme est contrôlée par l'interaction spécifique avec l'inhibiteur de pectin méthylestérase (PMEI). Puisque la modification de pectine est associée avec d'importantes phénomènes physiologiques et aussi avec les réponses de défense contre les pathogènes, l'interaction PME-PMEI peut revêtir un rôle biologique très important.

Puisque les gènes *pmei* du blé n'ont été pas caractérisés, dans ces travaux de thèses trois gènes du blé dur ont été isolés, *Tdpmei2.1*, *Tdpmei2.2*, *Tdpmei7.3*. On a démontré que les gènes codifient des inhibiteurs fonctionnels qui sont capable de réduire l'activité du PME de l' orange. Les trois inhibiteurs TdPMEI2.1, TdPMEI2.2 et TdPMEI7.3 inhibent aussi l'activité PME endogène du blé avec un différent degré de spécificité. Les trois inhibiteurs ne sont pas capable d' inhiber l'activité PME du microorganismes.

Les gènes *Tdpmei2.1* et *Tdpmei2.2* sont localisée sur le bras long du chromosome du groupe 2 et leurs séquences montrent un haut degré de conservation entre les espèces sauvages du blé.

Les analyses d'expressions ont été effectués durant les diverses phases du développement du blé. Les résultats ont montré que l'expression des gènes *Tdpmei2.1* et *Tdpmei2.2* est réglée par le mécanisme de rétention de l'intron. On a observé que les introns de ces gènes ont été complètement enlevés dans les anthères. L'accumulation de transcrits du *Tdpmei7.3* est majoritaire dans la tige.

Les analyses d'expressions ont été effectuées aussi après l'infection avec le champignon *Bipolaris sorokiniana* et les résultats n'ont pas montré, pour les trois gènes d'intérêt, un dégrée d'accumulation majoritaire dans les plantes infectés par rapport aux plantes non infectées. En fin, la capacité de moduler l'activité PME *in planta* a été vérifiée avec la production de plantes transgéniques surexprimant le gène *Tdpmei7.3*. Des analyses préliminaires ont montré que les plantes transgéniques ont un degré variable de réduction de l'activité endogène PME par rapport aux plants non transformés.

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Abreviations

HG: Homogalacturonan
GalA: galacturonic acid
PR: Pathogenesis related (protein)
PG: Polygalacturonase
PME: Pectin methylesterase
PMEI: Pectin methylesterase inhibitor protein
CWDE: cell wall degrading enzyme
PAMP: pathogen-associated molecular pattern
Amp: Ampicilline
AOX1: Alcool oxydase 1
DNA: Desoxy ribonucleic Acid
RNA: Ribonucleic Acid
dNTP: Desoxynucleotid triphosphate
DNS: 3-5 dinitrosalicylic acid
DTT: Dithiothreitol
EDTA: Ethylen diamine tetraacetic acid
kDa : kiloDalton
bp : Base pair
LB : Luria Bertani
YNB: Yeast Nitrogen Base with Ammonium Sulfate without amino acids
YPD: Yeast Extract Peptone Dextrose Medium
BMGY: Buffered Glycerol-complex Medium
BMMY: Buffered Methanol-complex Medium
MM: Minimal Methanol
MD: Minimal Dextrose
PAGE: Poly-acrylamide gel electrophoresis
PCR: Polymerisation chain reaction
PVDF: Polyvinylidene difluorure
SDS: Sodium dodecylsulfate
Taq: Polymerase from *Thermus aquaticus*
Tris: Tris-(hydroxymethyl)aminomethane
dpa: days after pollination
cv : cultivar
MS: mass spectrometry
CS: Chinese Spring
NT: nulli-tetrasomic lines of *T. aestivum* cv. Chinese Spring
DT: ditelosomic lines of *T. aestivum* cv. Chinese Spring
LDN: Langdon
hpi: hours post-infection

Figures

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Introduction

1.1 The Wheat

Wheat is among the oldest and most extensively grown of all grain crops.
It is widely accepted that wheat was first grown as a food crop about 8,000-10,000 years ago. Vavilov indicated the Middle-Eastern as the centre of origin of wheat, in the area between the Mediterranean coast and the flatlands between the Tigris and the Euphrates rivers.
From these regions, wheat cultivation spread to Egypt and then contributed to the development of its civilization. In Western Europe, wheat was probably introduced during the Aryan population migrations. In ancient times wheat then spread in the Mediterranean region and afterwards into Germany and Britain along with the conquest of Roman legions.
Wheat cultivation spread to all world (Fig. 1.1) and became one of the three most important grain species in the world. It is grown on more land area than any other commercial crop with its 225 million of hectares and FAO estimated that its production reached in 2009 about 680 million of tonnes (Mt) (FAOSTAT Data, 2009).

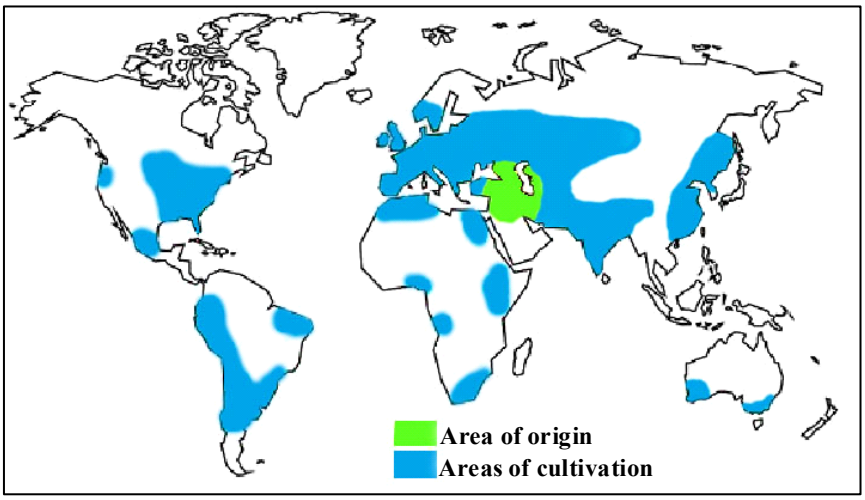


Fig. 1.1 Areas of origin and diffusion of wheat (from http://en.wikipedia.org)

Wheat is a major component of most diets of the world because of its agronomic adaptability, ease storage, nutritional goodness and the ability of its flour to produce a variety of palatable, interesting and satisfying foods (Wrigley, 2009). Doughs produced from bread wheat flour differ from those made from other cereals in their unique visco-elastic properties conferred by gluten proteins (Orth and Shellenberger, 1988). Besides being a high carbohydrate food, wheat contains valuable protein, minerals, and vitamins. Wheat is also a popular source of animal feed and it is often used by the industry to make adhesives, paper additives and

biodegradable plastic (Orth and Shellenberger, 1988; Nesbitt and Samuel, 1995). In the last few years there has been an increase in the use of wheat for biofuel and biodiesel production (www.nap.edu.).

1.2 Origin and phylogeny of cultivated wheat

Wheat belongs to the family *Graminaceae*, tribe *Triticeae* and genus *Triticum*.

Wheat genus includes several species that can be divided according to the somatic chromosome number, diploid, tetraploid and hexaploid.

In 1913 Schultz, on the basis of the plants morphology, proposed the subdivision of wheat in three groups. Such subdivision was soon validated by cytogenetic studies by Sakamura (1918) who determined the chromosome pattern of some types of wheat and came up with a classification based upon ploidy of wheat single species (diploid, tetraploid, hexaploid) with relationship to the basic chromosome number (http://www.fao.org/DOCREP/006/Y4011E/y4011e04.htm). Afterwards, analyzing the meiotic chromosome pairing in hybrids, Kihara (1924) identified several types of genomes and grouped the wheat in diploid (2n=2x=14) with genome called AA, tetraploid (2n=4x=28) with AABB genome and hexaploid (2n=6x=42) with AABBDD genome, confirming that 1x=7 is the basic chromosome number of the tribe *Triticeae*. In figure 1.2. is summarized the evolution of polyploid wheats. The species belonging to the *Triticum* genus now cultivated are T. *aestivum* L. (hexaploid 2n=6x=42), *T. turgidum* L. (tetraploid 2n=4x =28), *T. timopheevi Zhuk*. (tetraploid 2n=4x=28) and *T. monococcum* L. (diploid 2n = 2x = 14). Today, *T. aestivum (T. aestivum* ssp. *aestivum)* and *T. durum (T. turgidum* ssp. *durum)* are the most cultivated wheat species.

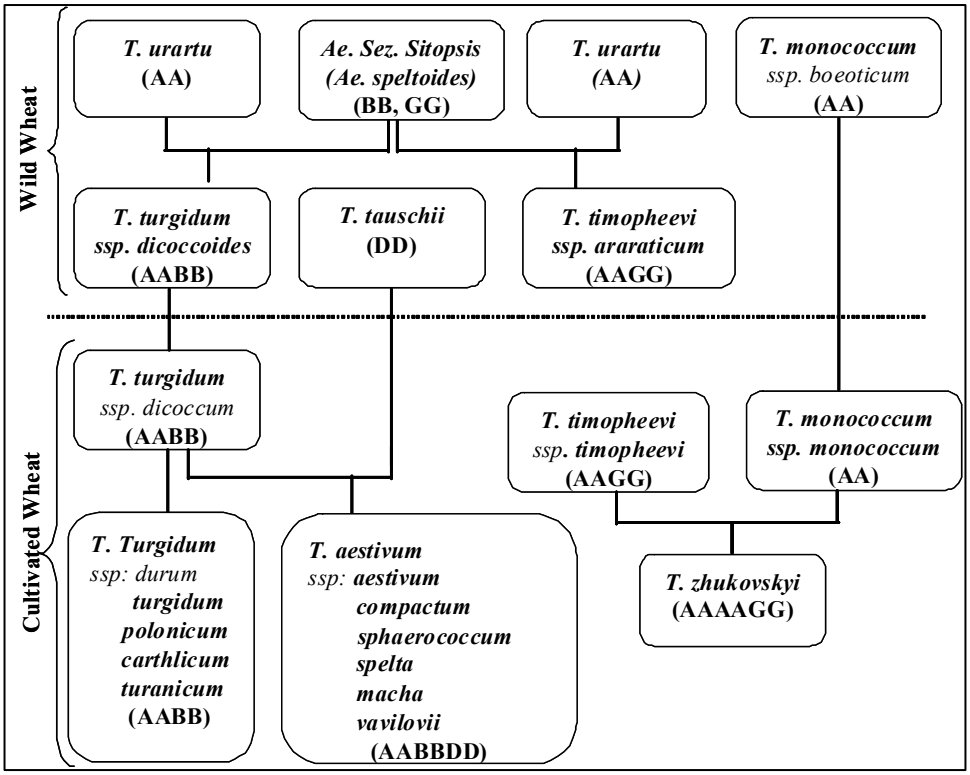


Fig. 1.2 Evolution of polyploidy wheats (adapted from Dvořák *et al.,* 1993; Feldman *et al.,* 1997).

1.3 Developmental stages systems of wheat

Much of agronomical advice and research is based on identifying specific growth stages at which to assess the crop. The main growth stages, based on the external appearance of the crop, are germination, seedling establishment and leaf production, tillering and head differentiation, stem and head growth, head emergence and flowering, and grain filling and maturity.

A number of staging systems have evolved for describing wheat development: the Zadoks system (Zadoks *et al*.,1974), the Haun (Haun, 1973) and the Feekes-Large systems (Large, 1954). This last system has been widely used, but is becoming less popular because it is not such detailed compared to the other two systems. The Haun system instead is concerned mainly with the leaf production stage of development and for this reason its application is limited in the field where decisions are made using development indicators other than leaf numbers

The Zadoks system is becoming the most universally accepted for describing wheat development because its stages are easy to identify in the field and it is more detailed than other systems, allowing for precise staging.

This system is based on two-digit code: the first digit refers to the principal stage of development beginning with germination (stage 0) and ending with kernel ripening (stage 9) and the second digit, instead, between 0 and 9 subdivides each principal growth stage. For example, in seedling growth the first digit is 1 while the second digit indicates the number of emerged leaves. To be counted, a leaf must be at least 50 percent emerged. For example, the Zadoks stage 13, indicates that the third leaf is least 50 percent emerged on the main shoot.

Regarding the tillering principal stage (stage 2), the second digit indicates the number of emerged tillers present on the plant.

Since stages may overlap, like in the previous examples regarding seedling growth and tillering, it is possible to combine Zadoks indexes to provide a more complete description of a plant's appearance. For example, a plant with one tiller and three leaves could be described by either or both of the Zadoks stages 13 and 21.

In the following table is reported the Zadoks two-digit code system with corresponding Feekes scale (Table 1.1).

Table 1.1 Condensed summary of the Zadoks two-digit code system for growth staging in wheat with corresponding Feekes scale

Zadoks code		**Description**	**Corresponding Feekes code**
Principal stage	**Secondary stage**		
0		Germination	
	0	Dry kernel	
	1	Start of imbibition (water absorption)	
	5	Radicle emerged	
	7	Coleoptile emerged	
	9	Leaf just at coleoptile tip	
1		Seeding development	1
	0	First leaf through coleoptile	
	1	First leaf at least 50% emerged	
	2	Second leaf at least 50% emerged	
	3	Third leaf at least 50% emerged	
	4	Fourth leaf at least 50% emerged	
	5	Fifth leaf at least 50% emerged	
2		Tillering	2
	0	Main shoot only	

	1	Main shoot plus 1 tiller visible	
	2	Main shoot plus 2 tillers	
	3	Main shoot plus 3 tillers	
	4	Main shoot plus 4 tillers	
	5	Main shoot plus 5 tillers	3
3		Stem elongation	
	1	First node detectable	6
	2	Second node detectable	7
	3	Third node detectable	
	7	Flag leaf just visible	8
	9	Flag leaf collar just visible	9
4		Boot	
	1	Flag leaf sheath extending	
	3	Boot just beginning to swell	
	5	Boot swollen	10
	7	Flag leaf sheath opening	
	9	First awns visible	
5		Head emergence	
	1	First spikelet of head just visible	10.1
	3	One-fourth of head emerged	10.2
	5	One-half of head emerged	10.3
	7	Three-fourths of head emerged	10.4
	9	Head emergence complete	10.5
6		Flowering (not readily visible in barley)	
	1	Beginning of flowering	10.5.1
	5	Half of florets have flowered	10.5.2
	9	Flowering complete	
7		Milk development in kernel	
	1	Kernel watery ripe	10.5.4
	3	Early milk	
	5	Medium milk	11.1
	7	Late milk	
8		Dough development in kernel	
	3	Early dough	
	5	Soft dough	11.2
	7	Hard dough, head losing green color	
	9	Approximate physiological maturity	
9		Ripening	
	1	Kernel hard (difficult to divide with thumbnail)	11.3
	2	Kernel cannot be dented by thumbnail, harvest ripe	11.4

1.4 Plant Cell Wall

Plant cell wall is a complex and dynamic system that determine plant structure and it is fundamental in plant growth and development, resistance to pathogen invasion, quality of plant-based foods and the properties of plant fibres and fuels.

Plant cell wall is a metabolically active compartment responsible for cell adhesion, is involved in cell–cell communication and is selectively permeable (Farrokhi *et al*, 2006); it changes throughout the processes of cell division, growth and differentiation and can vary its composition and organization in relation to environmental cues.

Plant cell wall is involved also in plant defence: upon a pathogen attack, plants often deposit callose at sites of pathogen penetration, accumulate phenolic compounds and toxins in the wall and synthesize lignin-like polymers to reinforce the wall. The plant cell wall represents also an important defensive structure that many pathogens encounter first before facing intracellular plant defences (Hématy *et al*, 2009). Pathogens use mechanical force or release cell wall degrading enzymes (CWDEs) to break down this barrier. At the cell wall, pathogens also release pathogen-associated molecular patterns (PAMPs). Plants, in turn, are able to sense these PAMPs and damage to their cell walls, which activate a variety of defences, including the production of reactive oxygen species (ROS), the production and export of anti-microbial compounds and fortification of the cell walls.

Two types of cell walls can be distinguished. Primary walls are deposited during cell growth, and need to be both mechanically stable and sufficiently extensible to permit cell expansion while avoiding the rupture of cells under their turgor pressure (Reiter, 2002). The main components of the primary plant cell wall include cellulose, in the form of microfibrils, and two groups of branched polysaccharides, pectins and hemicelluloses or cross-linking glycans (Fig. 1.3) The latter two classes of cell wall components, often referred to as matrix polysaccharides, are synthesized within Golgi cisternae, whereas cellulose is generated at the plasma membrane in the form of paracrystalline microfibrils.

Secondary cell walls are deposited after the cessation of cell growth and confer mechanical stability upon specialized cell types such as xylem elements and sclerenchyma cells. These walls are often impregnated with lignins.

In addition to polysaccharides, proteins can be found in all plant cell wall. They include proteins rich in the amino acids hydroxyproline/proline (Hyp/Pro), serine/threonine (Ser/Thr)

and glycine (Gly) and particularly hydroxyproline-rich glycoproteins (HRGPs), such as extensin, and glycine-rich proteins (GRPs) (Jonhson *et al.*, 2003).

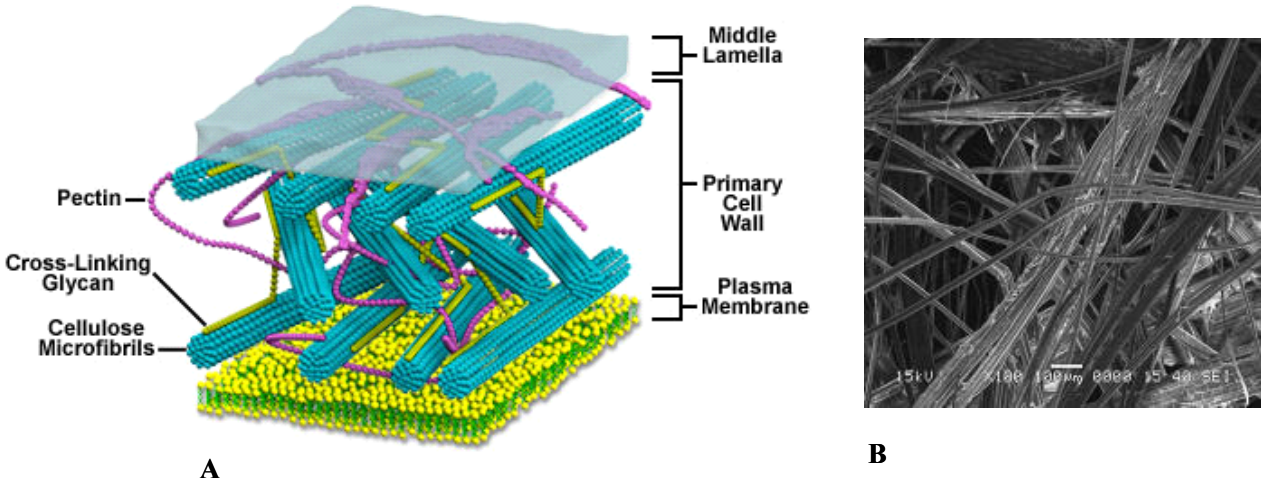


Fig. 1.3 A) Plant cell wall model (http://www.molecularexpressions.com). **B)** Electronic microscopy of plant cell wall (x100) (http://plantes-a-fibres.goum.info).

Dicots and grasses cell walls have significant compositional differences (Carpita and Gibeaut, 1993) (Tab.1.2). Whereas the overall architectures are similar, they differ in the types and relative abundance of non-cellulosic polysaccharides, cross-linking of polysaccharides, and abundance of proteins and phenolic compounds. Primary cell walls of flowering plants can be divided into two main categories (Carpita and Gibeaut, 1993, Carpita, 1996). Type I cell walls, found in dicots, noncommelinoid monocots and gymnosperms, consist mainly of cellulose fibres (30%) encased in a network of xyloglucan (XyG), pectin (35%) and structural proteins. By contrast, type II cell walls, found only in the commelinoid monocots, are composed of cellulose fibres encased in glucuronoarabinoxylans (GAX), high levels of

hydroxycinnamates, and very low levels of pectin (2-10%) and structural proteins. In addition, the cell walls of grasses (family Poaceae) and some related families in the order Poales contain significant quantities of mixed linkage glucans (MLG), known as β-glucans (Vogel, 2008).

Table 1.2 Approximate composition (% dry weight) of typical dicot and grass primary and secondary cell walls (from Vogel, 2008)

	Primary wall		**Secondary wall**	
	Grass	**Dicot**	**Grass**	**Dicot**
Cellulose	20–30	15–30	35–45	45–50
Hemicelluloses				
Xylans	20–40	5	40–50	20–30
MLG	10–30	Absent	Minor	Absent
XyG	1–5	20–25	Minor	Minor
Mannans and glucomannans	Minor	5-10	Minor	3-5
Pectins	5	20-35	0.1	0.1
Structural proteins	1	10	Minor	Minor
Phenolics				
Ferulic acid and r-coumaric acid	1-5	Minor (except order Caryophyllales)	0.5-1.5	Minor
Lignin	Minor	Minor	20	7-10
Silica			5-15	Variable

1.5 Pectins

Pectin is one of the main component of plant cell wall and it is considered one of the most complex macromolecules in nature (Jolie *et al*, 2010).

Pectin is present in the middle lamella, primary and secondary cell walls and is deposited in the early stages of growth during cell expansion, contributing to physical integrity and physiologal status of cell wall. It also influences various cell wall properties such as porosity, surface charge, pH and ion balance (Vorangen *et al*, 2009). Furthermore, pectic oligosaccharides may activate plant defence responses: they elicit the accumulation of

phytoalexins with anti-microbial activity and induce lignification and accumulation of protease inhibitors in plant tissues (Vorangen *et al*, 2009).

Pectin is composed of heterogeneous polysaccharides rich in galacturonic acid (GalA) and other monosaccharides. The main pectic polymer are homogalactoronan (HG), rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II). HG is a linear homopolymer of (1→4)-linked α-D-GalA and it is the most abundant pectic polysaccharides in plant cell walls. GalA residues of HG can be partially methyl-esterified at the C-6 carboxyl and can be also O-acetylated at C-2 or C-3 (Fig. 1.4). The degree and pattern of methyl-esterification of HG may influence plant cell wall properties that are involved in cell expansion and development, intracellular adhesion and defence mechanism (Willats *et al*, 2001).

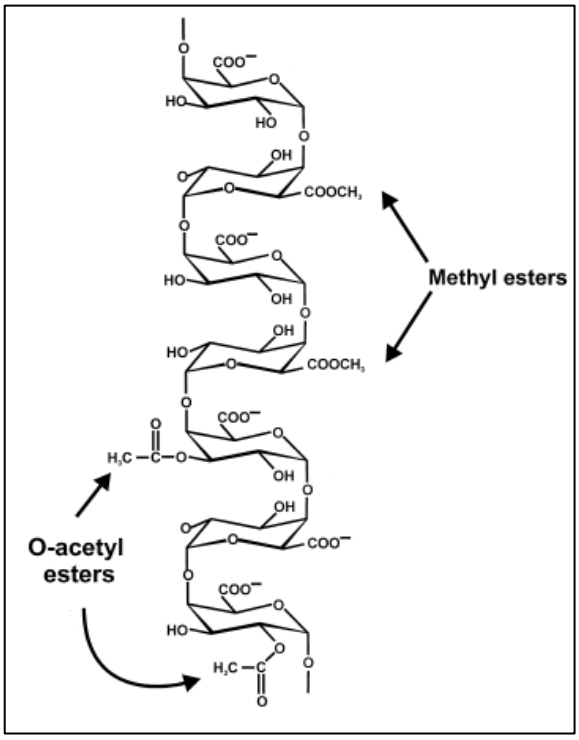


Fig. 1.4 The primary structure of homogalacturonan. (from Rydley *et al*, 2001).

In particular, a stretch of HG with un-esterified galacturonic acid residues can form Ca^{2+} linkages promoting the formation of the so-called 'egg-box'. Such association promotes the formation of supramolecular pectic gels, which can affect mechanical properties of cell walls (Willats *et al*, 2001) (Fig. 1.5).

Rhamnogalacturonan II (RGII) has a backbone of about 9 GalA residues that are (1→4)-α-linked and is substituted by 4 different side chains consisting of a variety of different sugars residues, among which sugars such as apiose, aceric acid, 3-deoxy-D-lyxo-heptulorasic acid and 2-keto-3-deoxy-D-manno-octulosonic acid (Perez *et al*., 2003).

It is widely accepted that pectins are synthesized in the *cis* Golgi, methylesterified in the medial Golgi and substituted with side chains in the *trans* Golgi cisternae before being secreted into the wall as highly methylesterified (70-80%) forms (Micheli, 2001). Subsequently, they can then be modified by pectinases such as pectin methylesterases (PMEs), which catalyse the demethylesterification of homogalacturonans with the release of acidic pectins and methanol.

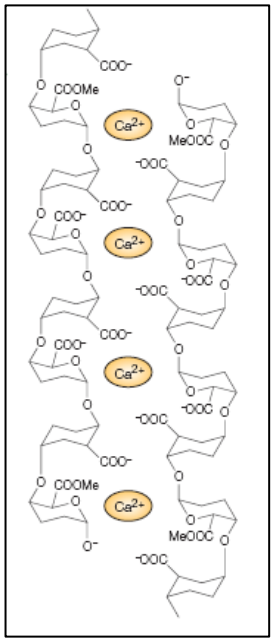


Fig. 1.5 Cross-link between carboxylic groups of HG mediated by Ca^{2+} (from Cosgrove, 2005).

1.6 Carbohydrate active enzymes and their plant proteinaceous inhibitors.

Carbohydrate active enzymes, such as glycosidases, transglycosidases, glycosyltransferases, polysaccharide lyases and carbohydrate esterases, are involved in enzymatic processing of plant carbohydates. Some of them are also produced by microbial pathogens and insects to overcame the physical barrier represented by the cell wall and to invade the plant tissue. Cell wall-degrading enzymes of microbial origin (CWDEs) are produced by pathogens at the early

stages of infection and are important factors of microbial pathogenicity (Collmer and Keen 1986; Alghisi and Favaron 1995; D'Ovidio *et al*., 2004a).

In turn, plants synthesize proteinaceous inhibitors against the endogenous carbohydrate active enzymes as well as against microbial enzymes (Juge et al, 2006; Bellincampi *et al*, 2004) (Table 1.3). For example invertase inhibitor, pectin methylesterase inhibitor and limit dextrinase inhibitors are active against plant carbohydrate-active enzymes; on the other hand, polygalacturonase inhibiting proteins (PGIPs), Triticum aestivum xylanase inhibitors (TAXI) and xylanase inhibitor protein I (XIP-I), pectin lyase inhibitor, xyloglucan-specific endoglucanase inhibitor (XEGIP) inhibit carbohydrate-active enzymes produced by fungal pathogens or insects (Bellincampi *et al*, 2004).

Table 1.3 Different classes of inhibitors directed against carbohydrate-active enzymes (from Juge, 2006)

Name	Source	Enzyme specificity CAZy[a]	Molecular mass (kDa)	Structural class SCOP fold[b]	Pfam[c]	PDB code
PGIP	Dicots, *Allium porrum*, wheat, rice	Fungal PGs (GH28)	34.0–54.0	LRR	PF00560	1OGQ
PMEI	Kiwi, *Arabidopsis*	Plant PMEs (CE8)	16.0–22.0	Bromodomain-like	PF04043	1XG2
PNLIP	Sugar beet	Fungal PNL (PL1)	57.5	ND	ND	ND
XIP	Graminaceous monocots	Fungal GH10 and GH11 endoxylanases	29.0	TIM-barrel	PF00704	1OMO, 1TA3, 1TE1
TAXI	Graminaceous monocots	Fungal and bacterial GH11 endoxylanases	40.0	Pepsin-like	PF00026	1T6E
TL-XI	Wheat	Fungal and bacterial GH11 endoxylanases	18.0	Thaumatin-like[d]	PF00314[d]	ND
XEGIP	Tomato, carrot, tobacco	Fungal XEG (GH12)	51.0	Pepsin-like[d]	ND	ND

Abbreviations: ND, not determined

[a] Carbohydrate Active Enzyme database (http://www.cazy.org/CAZY)

[b] Structural Classification of Proteins (http://www.scop.berkeleiy.edu)

[c] Pfam protein families database (http://www.sanger.ac.uk/Sotftware.Pfam/)

[d] Based on sequence and homology modelling

1.7 Pectinolytic enzymes and its inhibitors

Pectin is a dynamic entity and its structure is continuously modified by pectic enzymes such as polygalacturonases (PGs), pectin methylesterases (PME), rhamnogalacturonases (RG) and pectin lyases (PL).

Here we report a close-up view on pectin methyesterase and its proteinaceous inhibitor (PMEI)

1.8 Pectin methylesterases (PMEs)

Pectin methylesterase (PME, E.C. 3.1.1.11, CAZy class 8 of carbohydrate esterase) catalyses the specific hydrolysis of the methyl ester bond at C6 of a GalA residue in a linear HG domain (Jolie *et al*, 2010), contributing to alters the degree and pattern of methylesterification. As previously described , HG is synthesized in the Golgi apparatus, methylesterified in the *medial* Golgi by pectin methyltransferases and deposited in the cell wall in a highly (70-80%) methylesterified form. It is subsequently demethylesterified *in muro* by the action of PMEs, which release methanol and protons and create negatively charged carboxyl groups (Moustacas *et al*, 1991)(Fig. 1.6).

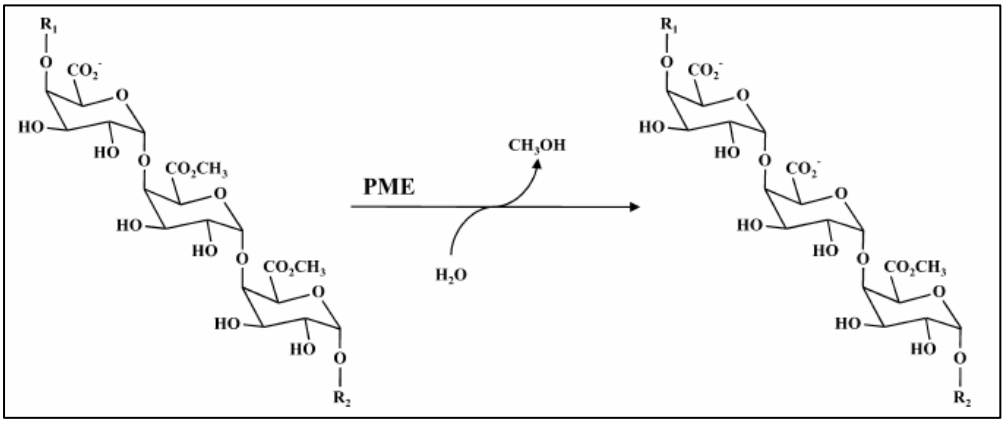


Fig. 1.6 Demethoxylation reaction of the homogalacturonan chain of pectin catalised by PME (R1/R2 : initial and terminal fragment of the epctic polymer) (Jolie *et al*, 2010).

PMEs are ubiquitous enzymes (Pelloux *et al*, 2007) and have been found in fruits, leaves, flowers, stems and in roots of all higher plants examined. PMEs are also produced by phytopathogenic bacteria and fungi and by symbiotic micro-organisms during their interactions

with plants. They have been also found in a few yeasts and insects (Jolie *et al*, 2010).

In many plants, several PME isoforms have been detected, differing in p*I* and biochemical activity. PME isoforms are encoded by a large multigene family. Analysis of the *Arabidopsis thaliana* genome has revealed 66 ORFs annotated as putative full-length PMEs (Wolf *et al*, 2009a). The PME ORFs of *Populus trichocarpa* are 89 (http://genome.jgi-

psf.org/Poptr1_1/Poptr1_1.home.html) (Geisler-Lee *et al*, 2006) whereas those numbers is substantially lower (35 ORFs) in *Oryza sativa* (http://www.tigr.org/tdb/e2k1/osa1/) and *Brachypodium distachyon* (29 ORFs) (www.brachybase.org), probably due to the low level of pectins in their cell wall.

Relative proportions of PME isoforms may vary and can have organ or stress-specific expression patterns (Pelloux, 2007).

Differential expression of PME isoforms, both spatially and temporarily, is believed to be a major mechanism in plants to regulate the endogenous PME activity (Bosch *et al*, 2005).

A high number of plant and microbial PMEs have already been sequenced. Comparison of amino-acid sequences show that plant and microbial PME share five segments of high sequence similarity, representing the 'signature patterns' characteristic for PME, six strictly conserved residues (three Gly residues, one Asp, one Arg and one Trp) and several highly conserved and functionally important aromatic residues (Pelloux *et al*, 2007; Markovic and Janecek , 2004).

In higher plant *pme* genes encoded the so-called pre-pro-proteins, in which the mature, active part of the protein is preceded by an N-terminal extension of variable length and a rather low level of amino-acid identity between isoforms. This pre-pro-extension is presumed to be cleaved off before secretion to the apoplasm, where only the catalytic part is found (Micheli, 2001). The pre-region (or signal peptide), formed by a common type signal peptide (SP) and by a transmembrane domain (TM or signal anchor), is required for protein targeting to the endoplasmic reticulum. Different PMEs possess one, both, or neither of these motifs. Those with neither motif are classed as putatively soluble isoforms. The mature active part of the protein (PME domain, Pfam01095, IPR000070) is preceded by an N-terminal extension (PRO region Pfam04043, IPR006501) (Markovic and Janecek, 2004), followed by a processing motif (PM) that might be a putative target for subtilisin-like proteases (Rautengarten, 2005). The PRO region shares similarities with the pectin methylesterase inhibitors (PMEI); it can vary in length and shows a relatively low level of amino acids identity between isoforms (Markovic and Janecek, 2004).

PME can be classified on the basis of the presence or absence of the PRO region in group 1/type II PMEs (250 to 400 amino acids; 27–45 kDa) which contain five or six introns and lack of PRO region, and group 2/type I PMEs (500–900 amino acids; 52–105 kDa) which instead contain only two or three introns and 1-3 PRO regions (Fig. 1.7) (Micheli, 2001; Tian *et al*., 2006). The group 1 PMEs have a structure close to that of the PMEs identified in phytopatogenic organisms (bacteria and fungi).

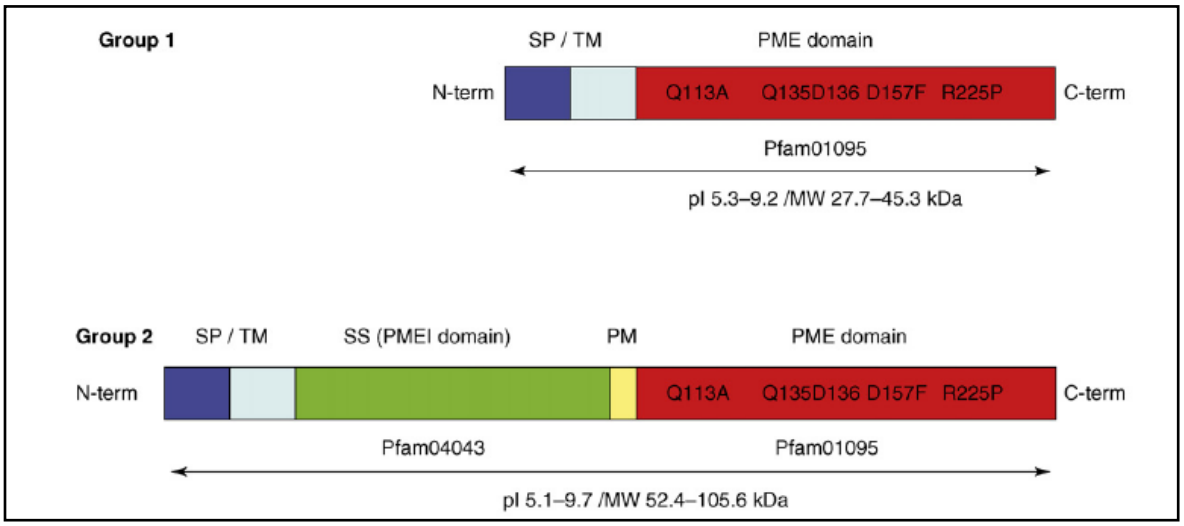


Fig. 1.7 Pectin methylesterase (PME) structural motifs. Group 1 and group 2 PMEs possess a conserved PME domain (Pfam01095) with characteristic highly conserved amino acid fragments. Amino acids involved in the catalytic site are highlighted (Q113, Q135, D136, D157, R225; according to the carrot PME numbering). Group 2 PMEs possess a PRO region, which shares similarities with the PMEI domain (Pfam04043), and a processing motif (PM) that might be a putative target for subtilisin-like proteases. The targeting to the endomembrane system leading to the export of PMEs to the cell wall is mediated either by a common type signal peptide (SP) or a transmembrane domain (TM or signal anchor) (from Pelloux *et al*., 2007).

SP/TM and PRO region are processed to produce the mature active PMEs. The PRO-PME processing event might occur inside of the cell or immediately after release into the apoplast (Dorokhov *et al*., 2006b). Recently, it was demonstrated that proteolytic release of the PRO region at two basic motifs located between PRO region and catalytic domain of group II PMEs occurs in the Golgi apparatus and is a prerequisite for apoplastic targeting of the PME domain (Wolf *et al*., 2009b); a subtilisin-like protease is likely involved in PME maturation. Potentially, environmental and developmental cues could activate or inactivate the processing, such as to prevent the transport of PME during homogalacturonan synthesis and methylesterification which also take place in the Golgi apparatus (Fig. 1.8) (Wolf *et al.*, 2009b).

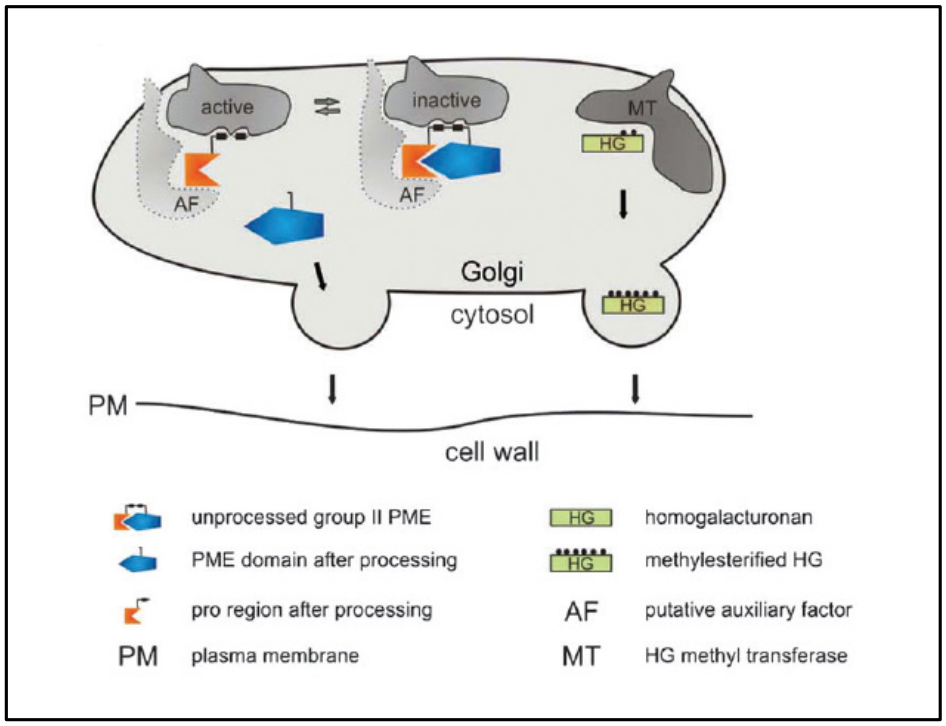


Fig. 1.8 Hypothetical model of Group II PME processing. Unprocessed group II PMEs are retained in the Golgi apparatus, possibly by either the inactive protease or auxiliary factors. Upon processing, the liberated PME domain is free to travel to the cell wall with the secretory flow (from Wolf *et al.*, 2009b).

Although the role of the PRO region is not known, several hypotheses have been proposed. It might play a role (1) in targeting PMEs towards the cell wall; (2) as an intramolecular chaperone, allowing conformational folding of the mature part of the PME or (3) as an inhibitor of the mature part of the enzyme to prevent premature demethylesterification of pectins before their secretion into the cell wall (Micheli, 2001).

After the secretion to the cell wall, mature PMEs could have three different type of action pattern: I) a single chain mechanism, where PME removes all continuous methyl-ester from a single pectin chain before dissociating from the substrate, II) a multiple-chain mechanism, where PME catalyses only one reaction before dissociating and III) a multiple-attack mechanism, where PME removes a limited average number of methyl-esters before the enzyme-pectin complex dissociates (Jolie *et al*, 2010). For plant and bacterial PMEs both a single chain and multiple-attack mechanism have been proposed (Dongowski and Bock, 1984; Kohn *et al*,1985; Christensen *et al*, 1998). In contrast, fungal PMEs attack more

randomly and a multiple-chain mechanism has been proposed for those enzymes (Limberg *et al*, 2000; van Alebeek *et al*, 2003; Duvetter *et al*, 2006)

The mode of action of PMEs on HG remains controversial. They can act randomly or linearly. When they act randomly, the demethylesterification releases protons that promote the action of endopolygalacturonases (Moustacas *et al*., 1991) and contribute to cell wall loosening. When PMEs act linearly on methylated pectin, PMEs give rise to blocks of free carboxyl groups that could interact with Ca^{2+}, forming the so called junction zones. Because the action of endopolygalacturonases in such a gel is limited, this action pattern of PMEs contributes to cell wall rigidification (Fig. 1.9).

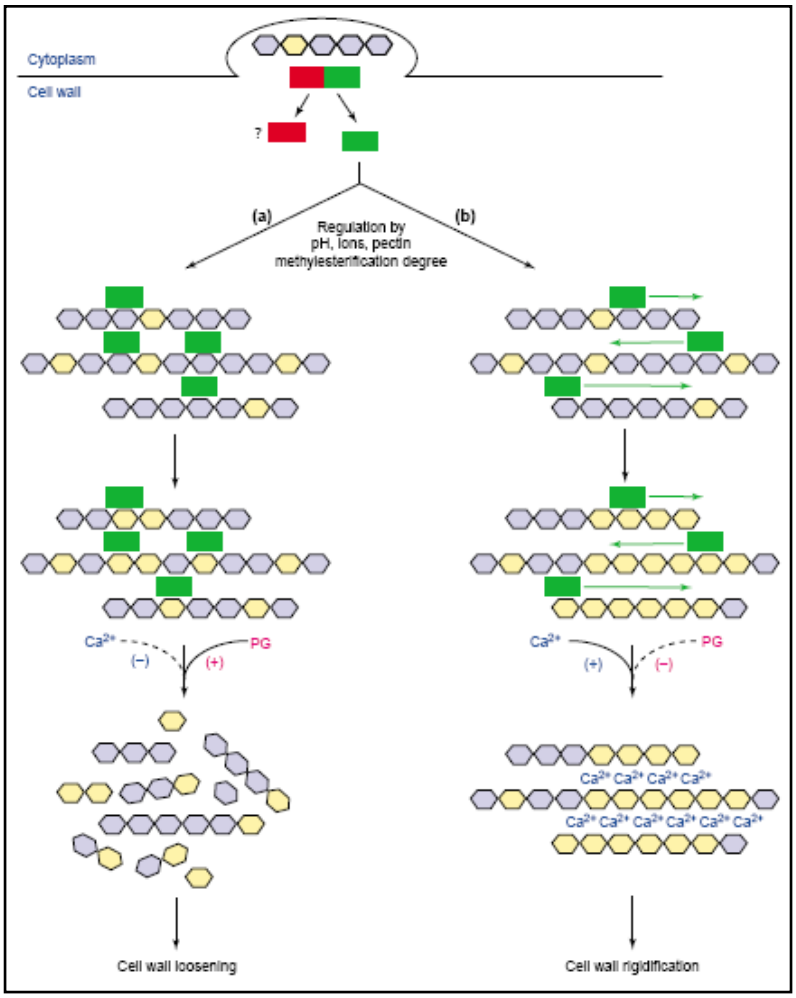


Fig. 1.9 Mode of action of pectin methylesterases (PMEs). Mature PMEs (green) can act randomly (a), promoting the action of pH-dependent cell wall hydrolases such as Endopolygalacturonases (PG) and contributing to cell wall loosening, or can act linearly (b), giving rise to blocks of free carboxyl groups that interact with bivalent ions (Ca^{2+}), so rigidifying the cell wall. Methylesterified galacturonic acids are represented in blue and demethylesterified galacturonic acids in yellow (from Micheli, 2001).

Because acidic PMEs were thought to be essentially confined to fungi, the simplest hypothesis was that random demethylesterification is produced by acidic fungal PMEs, whereas linear demethylesterification was determined by alkaline plant and bacterial PMEs. However, other studies provided evidences that the mode of action of PMEs on HG is influenced by others factors including cell wall pH and the initial pattern/degree of methylesterification of the pectins. Some isoforms can act randomly at acidic pH but linearly at alkaline pH. At a given pH, some isoforms are more effective than others on highly methylesterified pectins (Catoire *et al.*, 1998; Denes *et al*., 2000).

The attempts to elucidate the mechanisms of action and substrate specificity of PMEs were facilitated by solving 3D crystallographic structures of three PMEs: a bacterial PME, from *Erwinia chrysantemi* (PDB code 1QJV) and the same PME in complex with pectin (Jenkins *et al*, 2001; Fries *et al*, 2007) and two plant PMEs, from *Daucus carota* (Johansson *et al*., 2002) (PDB code 1GQ8) and from *Solanun Lycopersico*n (Fig. 1.12) (Di Matteo *et al*, 2005). The enzyme folds into a right handed parallel β-helix, first observed in pectate lyase C (Yoder *et al*, 1993) and typical of pectic enzymes (Jenkins and Pickersgill, 2001).
The 3D structures of the carrot and tomato PMEs show striking similarities, and are almost entirely superimposable (Fig. 1.10) (Di Matteo *et al*, 2005) In addition, both of these plant PMEs show similar folding topology to PME from *Erwinia chrysanthemi*, although one major difference lies in the length of turns protruding from the β-helix in proximity of the substrate cleft (Fig. 1.10), in particular, turns that protrude out of the β-helix are much longer in the bacterial enzyme, making its putative active site cleft deeper and narrower than that of plant PMEs (Fig. 1.10).

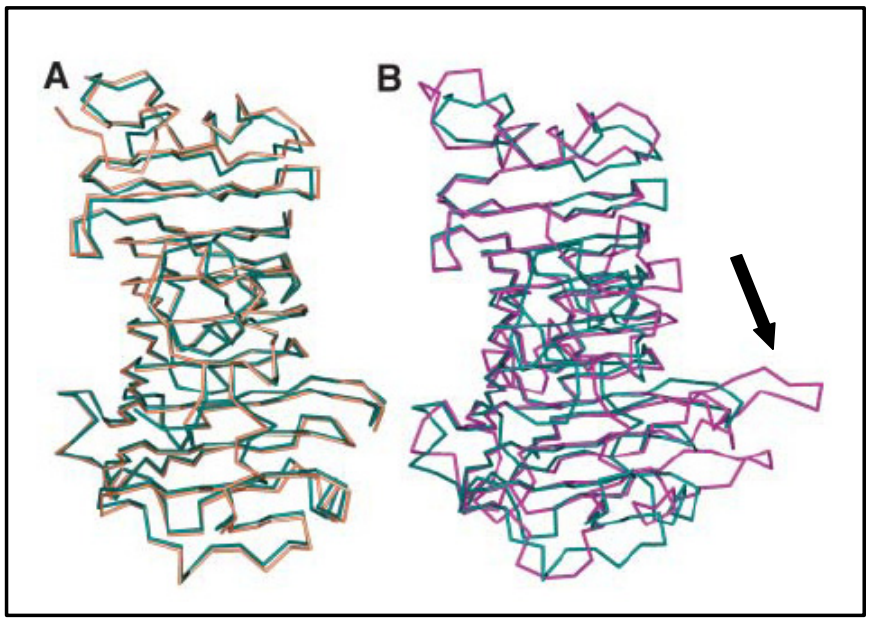


Fig. 1.10 Comparison of the known structures of PMEs. **A)** Overlay of the Cα trace of PME from tomato (green) and PME from carrot (orange). Structures are almost completely superimposable. **B)** Superimposition of PME from tomato (green) and PME from *E. chrysanthemi* (violet). Although the β-helices are completely superimposable, main differences (indicated by the arrow) are located in the length of the turns protruding out from the β-helix in proximity of the putative active site cleft (from Di Matteo *et al*., 2005).

1.8.1 The multiple roles of PMEs in plants

The multiple roles of PMEs in plants can be illustrated by their expression pattern in different organs and tissues. Various experiments have demonstrated that PMEs participate, directly and indirectly, in diverse physiological processes associated with both vegetative and reproductive plant development. PMEs are involved in cell wall extension and stiffening (Moustacas *et al*., 1991; Jenkins *et al*., 2001), cellular separation (Wen *et al*., 1999), seed germination (Ren and Kermode, 2000), root tip elongation (Pilling *et al*., 2004), leaf growth polarity (Hasunuma *et al*., 2004), microsporogenesis and pollen tube growth (Wakeley *et al.,* 1998; Futamura *et al.*, 2000; Bosch and Hepler, 2005), stem elongation (Bordenave and Goldberg, 1993), fruit ripening and loss of tissue integrity (Brummell and Harpster, 2001).

Cell adhesion and tissue elongation: it has been demonstrated that in antisense pea transgenic plant transformed with a PME gene (*rcpme*1), the PME activity increases during separation of the border cells of the root cap of pea and is correlated with an increase of acidic

pectin and a decrease in cell wall pH (Stephenson and Hawes, 1994). Analysis of transgenic plants also showed that *rcpme*1 expression is required for the maintenance of extracellular pH, elongation of the cells within the root tip and for cell wall degradation leading to border cell separation (Wen *et al*., 1999). Moreover, transgenic potato plants constitutively expressing a *Petunia inflate* PME showed more rapid elongation of the stem during the early stages of development (Pilling *et al*., 2000). The same authors observed an opposite growth phenotype after silencing of PME (Pest2) gene in potato plants (Pilling *et al*., 2004).

Microsporogenesis and pollen tube growth: many experimental evidences shed light on the PME roles in reproductive development and above all on their roles in pollen formation and pollen tube growth. It has been demonstrated that the silencing of a pollen-specific PME isoform VGD1 (At2g47040) in Arabidopsis caused only 18% reduction in total PME activity (Jiang *et al*., 2005), but although this relatively small reduction in activity, it was observed that pollen tubes were unstable when germinated *in vitro* and have a decreased fertilization *in vivo*. The mutant showed also smaller siliques with fewer seeds. Similar, albeit weaker, effects on pollen tube growth have been observed with the AtPPME1 mutants in Arabidopsis (Tian *et al*., 2006). Mutation of QUARTET1 (QRT1), a PME expressed in Arabidopsis pollen and anthers leads to impaired pollen tetrade separation during flower development. QRT1 might act in tandem with QRT3, a polygalacturonase, to degrade de-methyl-esterified homogalacturonan in pollen mother cell primary walls (Rhee *et al*., 2003; Francis *et al*., 2006), as PME-mediated de-methylesterification is thought to be required to render homogalacturonan susceptible to polygalacturonase-mediated degradation (Wakabayashi *et al*., 2000, 2003).

Fruit ripening: the role of PME in fruit ripening has been intensively studied in tomato in order to relate changes in PME activity to modifications in the cell wall structure of the pericarp (Brummell and Harpster, 2001). Transgenic tomato plants, exhibiting a 10 times lower PME activity were produced by the insertion of antisense cDNA of a fruit-specific *PME*2, (Tieman *et al*., 1992; Hall *et al*., 1999). These plants displayed an increase of about 20-40% of pectin methylesterification but relative subtle changes in fruit softening during ripening respect to untransformed plants. However, in the same transformants, almost complete loss of tissue integrity was observed during fruit senescence (Tieman and Handa, 1994). During juice processing using the antisense PME fruit, a reduced polyuronide depolymerization in the red ripe fruit was noticed together with almost 20% increase in soluble solids content, probably due to the high degree of pectin methylesterification protecting pectin from PG-mediated hydrolysis during fruit homogenization (Thakur *et al*.,

1996). Thus, PME activity plays little role in fruit softening during ripening, but substantially affects tissue integrity during senescence and fruit processing characteristics.

Wood development: Emerging roles for PMEs in vegetative development include their putative involvement in wood development. In Arabidopsis, five different PME encoding genes are highly expressed in the xylem. In poplar wood-forming tissues, transcripts of PME encoding genes, showing to be tightly regulated within the cambial meristem and during xylogenesis, have been found (Geisler-Lee *et al*., 2006). Several PME isoforms have been observed at specific stages of xylogenesis, implying that specific isoforms might have different functions at different stages during the course of xylem cell differentiation (Micheli *et al*., 2000) In addition, it has been recently found in aspen trees that PttPME1 is involved in the regulation of fiber length in the wood by strengthening cellular adhesion between developing fibers, and thus inhibiting their intrusive apical elongation (Siedlecka *et al*., 2008).

PME and phyllotaxis: plant organs are produced from meristems in a characteristic pattern. This pattern, referred to as phyllotaxis, is thought to be generated by local gradients of auxin (Jonsson *et al*., 2006), but a key role for the mechanical properties of the cell walls in the control of organ outgrowth has been proposed (Fleming *et al*., 1997). A recent study demonstrated that PME activity could induce primordia formation and override primordia patterning in Arabidopsis inflorescence meristems. On the contrary, overexpression of a pectin methylesterase inhibitor completely prevented formation of lateral organs, highlighting the importance of regulated pectin demethyl- esterification for plant development (Peaucelle *et al*., 2008).

1.8.2 PMEs and plant defence

Arabidopsis microarray database revealed that the expression levels of about 75% of predicted PME sequences vary in response to biotic and abiotic stresses. For example some *pme* transcripts are regulated by cold, wounding, ethylene, oligogalacturonides (OGs) and phloem-feeding insects (Lee and Lee, 2003; De Paepe *et al*., 2004; Moscatiello *et al*., 2006; Thompson and Goggin, 2006).

PME activity is necessary for complete degradation of pectin by PGs and PLs, since these enzymes have reduced activity on highly methyl-esterified pectin (Limberg et al. 2000; Bonnin et al. 2002; Kars et al. 2005). The disruption of *pmea* gene in the bacterium *Erwinia chrysantemi* reduces strongly its virulence on *Saintpaulia ionanta* (Boccara and Chatain, 1989; Beaulieu *et al*., 1993). Similarly, it has been shown that BCPME is an important

determinant of pathogenicity for the necrotrophic *Botrytis cinerea* (Valette-Collet *et al*., 2003).

In antisense or overexpressing plants, decreases and increases in PME activities and associated changes in the degree of methylesterification (DM) of cell wall pectins, have been correlated with changes in the susceptibility of these plants to pathogens and abiotic stresses (Marty *et al*, 1997; Dorokhov *et al*, 2006a; Lionetti *et al*, 2007). The DM can also influence the release of elicitor active oligogalacturonides (OGs) and consequently the induction of plant defence responses (Vorwerk et al., 2004; Moscatiello et al., 2006). The distribution of pectin methylesterification might modulate the susceptibility of a plant to pathogens. Significant differences in HG methylesterification patterns have been found between stem rust fungus-resistant (random distribution of methylesters) and susceptible (block-wise distribution) lines of wheat (Wietholter *et al*, 2003).

It has been reported that the binding of functional plant PMEs to the movement proteins (MPs) of some viruses is required for dispersal of the viruses to new cells via plasmodesmata (Chen *et al*., 2000). Although it is known that (i) PMEs are associated with cell wall-embedded plasmodesmata, (ii) MPs co-localize with plasmodesmata and (iii) PME-like proteins bind to MPs in cell wall extracts, the mechanism allowing PMEs to facilitate viral movement remains unclear.

In several plant species, PME mRNA and protein levels are up-regulated after herbivore attack (Divol *et al*., 2005; von Dahl *et al*., 2006). Moreover, herbivore attack causes also an increase in methanol (MeOH) emissions when compared with non-induced plants (Penŭelas *et al*., 2005; von Dahl *et al*., 2006). For example, *Nicotiana attenuata* increases MeOH emissions when attacked by *Manduca sexta* larvae or elicited with *M. sexta* oral secretion (OS). It was previously postulated that this enhanced MeOH emission originates from the demethylation of pectin by the action of PMEs (von Dahl *et al*., 2006). By silencing NaPME1, an OS-inducible PME in *N. attenuata*, it has been demonstrated that this enzyme participates in the enhanced production of MeOH after *M. sexta* OS elicitation. In order to test the hypothesis that PME may participate in the induction of defence responses during insect herbivory either by generating MeOH as a signal molecule or via changes in the structural properties of the cell wall, diverse plant responses to insect herbivores were studied and the results suggested that PME contributes, probably indirectly by affecting cell wall properties, to the induction of anti-herbivore responses (Korner *et al*., 2009).

1.8.3 Regulation of PME activity

In addition to the translational control, PME activity is regulated in part by post-translational mechanisms. It was postulated that PME activity in the cell wall is regulated by H^+ concentration in a cyclic manner during cell growth (Ricard and Noat, 1986). The enzyme optimal activity occurs at pH close to neutrality and is reduced by the local decrease of pH generated by protons released as a consequence of the methylesterification reaction. In turn, the pH decrease activates glycosidases and glycosyl-transferases, involved in cell wall extension and building up. The dilution of negative charges and increase of local pH result in re-activation of PME, starting a new cycle (Giovane *et al*., 2004).
PME activity is also regulated by hormones (Lohani S. *et al*., 2004). An important role in the regulation of PME activity is played by specific pectin methylesterase inhibitors (PMEI).

1.9 Pectin methylesterase inhibitors (PMEIs)

Over the years, several compounds with inhibitory activity on PME have been identified. The best described PME inhibitor (PMEI) is the proteinaceous inhibitor found in kiwi fruit (*Actinidia chinensis* AcPMEI) (Balestrieri *et al*, 1990). The active inhibitor was purified from fully ripe kiwi fruit, whereas it was undetectable in the unripe fruits (Balestrieri *et al*, 1990). The complete amino acids sequence comprises 152 amino acid residues with a molecular mass of 16.277 Da. Five Cys residues are included in the sequence, four of which are connected by two disulfide bridges (first to second and third to fourth). The fifth Cys residue (position 139) has a free thiol group. Protein microheterogeneities were detected at five positions of the amino acid sequence (Ala/Ser56, Tyr/Phe78, Ser/Asn117, Asn/ Asp123, and Val/Ile142), indicating that several isoforms of the protein are present in the fruit (Camardella *et al*, 2000). The presence of three cDNA sequences in *Actinidia deliciosa* (AB091088, AB091089 and AB091090) confirmed this hypothesis (Irifune *et al*, 2004). The complete coding sequences of the entire cDNAs show an N-terminal extension of 32 amino-acid residues, due to the presence of a signal peptide required for the extracellular targeting of the protein (Irifune *et al*, 2004)
The PMEI from kiwi fruit (AcPMEI) is specific for PME and possesses a wide range of inhibition properties against plant PMEs since it inhibits the PME from orange, apple, tomato, kiwi, kaki, potato, apricot, banana, cucumber, strawberry and flax (Ciardello *et al*, 2004;

Balestrieri *et al*, 1990; Giovane *et al*, 1995; Ly-Nguyen *et al*, 2004; Dedeurwaeder S. *et al*, 2009) On the contrary, it is ineffective against microbial PMEs (D'Avino *et al*, 2003: Giovane *et al*, 2004; Duvetter *et al*, 2005) and other plant polysaccharide-degrading enzymes, such as PG, amylase and invertase (Balestrieri et al, 1990; Camardella et al, 2000).

The inhibition of PME by PMEI occurs through the formation of a reversible 1:1 complex (Di Matteo *et al*., 2005). The interaction is noncovalent and reversible and the stability of the complex AcPMEI/PME-1 from tomato is strongly influenced by pH (Di Matteo *et al*., 2005), indicating that PME activity can be modulated by pH either directly or by modulation of the affinity between the enzyme and its inhibitor. Kinetic parameters of PMEIs/PME interaction have been investigated through the analysis of Surface Plasmon Resonance (SPR). For the interaction between AcPMEI and PME-1 from tomato was calculated a dissociation constant value of 53 nM at pH 7 and this value decreases 10 times at pH 6 (Mattei *et al*, 2002). Therefore, the affinity of the two proteins is higher at a pH close to that of the apoplastic environment. Regarding the kiwi PME/PMEI interaction, the dissociation of this complex at pH values ranging from 3.5 to 8.0 is undetectable, and the complete dissociation occurs only at pH 10.0. The dissociation kinetics observed at pH 9.5 is similar to that observed for tomato PME at pH 7.0. Size exclusion chromatography confirmed these results indicated that both free and immobilised kiwi PME are able to form a complex with PMEI, which is stable in a large pH range than that reported for tomato PME-1 (Ciardiello *et al*., 2008). The inhibition mode of AcPMEI on tomato and carrot PMEs was competitive, indicating that the inhibitor interact on substrate binding site preventing substrate to enter in the active cleft (D'Avino *et al*, 2003; Ly-Nguyen *et al*, 2004; Di Matteo *et al*, 2005). However, AcPMEI is able to inhibit banana or strawberry PME with non competitive mode (Ly-Nguyen et al., 2004).

Database search identified a large family of *A. thaliana* sequences closely related to the kiwi PMEI sequences. Two of these have been shown to encode functional PMEIs, named *Atpmei*-1 (At1g48020) and *Atpmei*-2 (At3g17220) (Wolf *et al*, 2003; Raiola *et al*, 2004).

The intronless *Atpmei*-1 and *Atpmei*-2 share 64% identity at the nucleotide level. The encoded AtPMEI-1 and AtPMEI-2 share 45% amino acid identity and about 38% identity with AcPMEI, Removal of the predicted N-terminal signal peptide generates mature AtPMEI-1 and AtPME2 proteins of 151 amino acids (16.266 Da, predicted pI =7.7) and 148 amino acids (15.615 Da, pI = 9.0) respectively. Like AcPMEI, both Arabidopsis proteins show a conserved C-terminal hydrophobic region (CxIxLVISN) and five conserved Cys residues, the first four of which have been shown to be engaged in disulfide bridges in AcPMEI (Camardella *et al*., 2000).

AtPMEI-1 and AtPMEI-2 proteins are active against the main PME isoform purified from tomato fruit and PMEs from kiwi and apricot fruits, carrot roots, tobacco leaves, Arabidopsis leaves and flowers, whereas they were inactive against PMEs of *Erwinia chrysanthemi*, *Aspergillus niger* and *Aspergillus aculeatus* (Raiola *et al*., 2004). Kinetic parameters of interaction between the inhibitors from Arabidopsis and PME-1 from tomato have been investigated. AtPMEI-2 shows stronger affinity for tomato PME respect to AtPMEI-1. Both inhibitors showed a better thermal stability in a wider pH range than the kiwi inhibitor (Raiola *et al*., 2004).

The analysis of the transcript level of *Atpmei*-1 and *Atpmei*-2 in different tissues revealed very low expression of both isoforms in most tissues except in flowers (Wolf *et al*, 2003).

The analysis of Arabidopsis transformants containing the GUS gene under the control of AtPMEI-1 or AtPMEI-2 promoter showed that the high expression in flowers could be largely attributed to the anthers and pollen and that the expression of AtPMEI-1 was exclusively confined to anthers and pollen, while AtPMEI-2 was also expressed at the base and the conducting tissues of the sepals. This particular localization of both inhibitors suggests that pollen and flower specific PMEs may be under post-translational control of inhibitor proteins.

Recently, a PME inhibitor from pepper (*Capsicum annuum*), named CaPMEI1 (AF477956), has been isolated. The predicted open reading frame encodes a full-length protein of 200 amino acid residues, with an estimated molecular mass of 21.39 kDa and an p*I* of 6.51 (An *et al.*, 2008).This sequences reveals a conserved pectin methylesterase inhibitor domain of 156 amino acids and the four Cys residues typical of PMEIs are also conserved. RNA analysis showed high levels of CaPMEI1 transcripts in stem tissues and relatively low levels of transcripts in leaf, root, flower and green fruit. CaPMEI1 transcripts were not detected in red fruit (An *et al.*, 2008). CaPMEI1 expression was also induced in pepper leaves by infection with bacterial pathogens and treatment with plant hormones such as SA, ethylene, MeJA and ABA. In particular, these hormone treatments strongly induced CaPMEI1 transcription, suggesting that this gene may be involved in the early stages of the active defence responses to bacterial pathogen infection and exogenous treatment with plant hormones. In enzymatic assays, purifed CaPMEI1 inhibited the activity of plant pectin methylesterase (PME). Moreover CaPMEI1 exhibited antifungal activity against a broad range of plant pathogenic fungi, including *F. oxysporum* f.sp. *matthiolae*, *A. brassicicola* and *B. cinerea*.

Very recently a novel pectin methylesterase inhibitor was isolated from *Brassica oleracea* and named BoPMEI1 (DQ116449).The ORF of 507 bp encodes 168 amino acids with a molecular mass of 18.3 kDa. Although the predicted protein reveals a PMEI domain, it shows very low

similarity to other previously described PMEIs or invertase inhibitors, but it possesses the typical features of the PMEI family, including an N-terminus signal peptide with a transmembrane region, four position-conserved cysteine residues, and a predicted secondary structure similarity, which includes the predominance of α-helice (Zhang *et al*, 2010).

The transcript patterns of BoPMEI1showed that it was expressed specifically in mature pollen grains and pollen tubes. Moreover, ectopic expression of a BoPMEI1 antisense gene in Arabidopsis suppresses the expression of its orthologous gene, At1g10770, and results in the retardation of pollen tube growth, in partial male sterility and lower seed set. These results suggest that BoPMEI1 plays an essential role in pollen tube growth, possibly by interacting with PMEs and thereby modifying the cell wall of the pollen tube (Zhang *et al*, 2010).

1.9.1 Three-dimensional structure of PMEI and PME-PMEI complex

The three-dimensional structures of AcPMEI and AtPMEI-1 have been determined (Hothorn *et al*., 2004; Di Matteo *et al*., 2005). Both inhibitors are almost all helical, with four long helices (α1, α2, α3 and α4) aligned in an antiparallel manner in a classical up-and-down four-helical bundle (Fig. 1.11). The interior of the bundle is stabilized by hydrophobic interactions and by a disulphide bridge which connects helices α2 and α3. The N-terminal region is composed of three short and distorted helices (αa, αb and αc) and extends outside the central domain. A disulfide bridge connects αa and αb (Di Matteo *et al*, 2005).

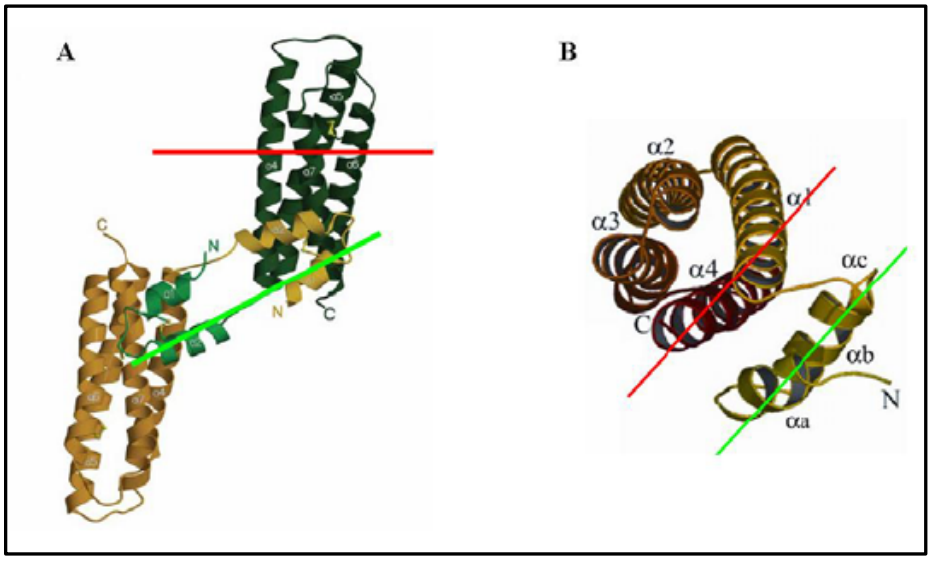


Fig. 1.11 A) Ribbon representation of the AtPMEI-1 dimer with the respective molecules shown in green and yellow. **B)** Ribbon representation of the AcPMEI monomer. Red and green lines show core plan orientation and N-terminal extension orientation, respectively (from Hothorn *et al*., 2004).

Although both inhibitors are quite similar in the bundle, significant differences are located in the N-terminal extension and in loops connecting the helices of the bundle: the N-terminal region of AcPMEI folds back and packs with the bundle through hydrophobic interaction, whereas the N-terminal extension of At-PMEI1, which crystallizes in a dimeric form (Fig 1.11), packs against the bundle of another molecule (Hothorn *et al*, 2004). Interestingly Pro28 residue of AtPMEI1, which is located in the linker between the N-terminal region and the four-helix bundle and is responsible for the orientation of the N-terminal region (Hothorn et al., 2004), is replaced by a Lys residue in AcPMEI, suggesting that the different topology of the two inhibitors is due to the presence of different residues at the same position (Di Matteo *et al.,* 2005).

As previoulsy mentioned, the three dimension structure of the complex PME-PMEI has been solved (Di Matteo *et al*, 2005). Tomato PME-1 and AcPMEI form a stoichiometric 1:1 complex in which the inhibitor covers the shallow cleft of the enzyme where the putative active site is located. The four-helix bundle of PMEI packs roughly perpendicular to the parallel β-helix of PME, and the three helices α2, α3, and α4, but not α1, interact with the enzyme in proximity of the putative active site (Fig. 1.12). The relative position of α2 and α3 helices is maintained by a disulphide bridge between Cys74 and Cys114. The N-terminal region of PMEI is poorly involved in the formation of the complex and may play a role in the stability of the inhibitors.

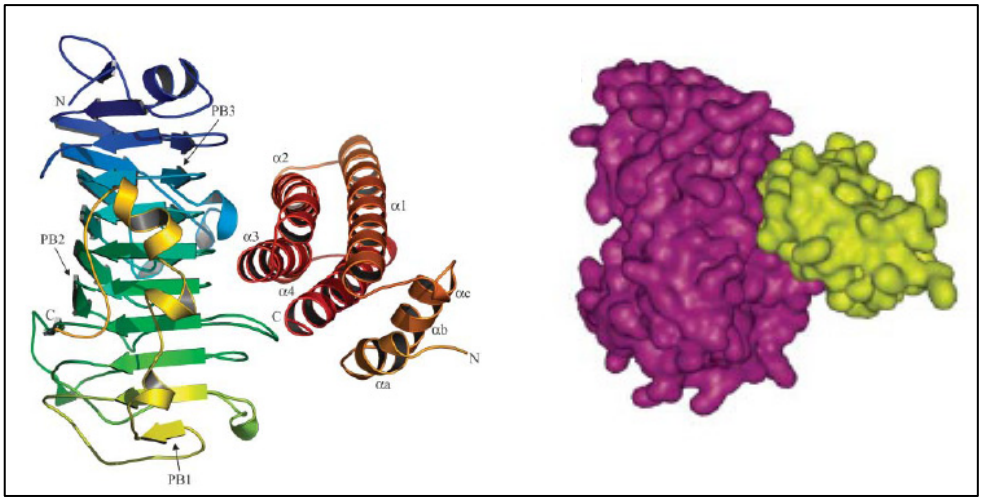


Fig. 1.12 Structure of the PME-PMEI complex. Ribbon representation illustrating the relative positions of PMEI and PME in the complex. The enzyme is shown in green–blue on the left side. The inhibitor is represented in yellow–red on the right side; the α-helices of the four-helix bundle are indicated as α1 to α4, whereas helices of the N-terminal region are named αa, αb, and αc (Di Matteo *et al*, 2005).

Detailed analysis of residues involved in forming the complex reveals that the putative catalytic residues (Asp132, Asp153, and Arg221) do not establish contacts with the inhibitor, neither do Gln109 and Gln131, which are thought to stabilize the anionic intermediate formed after the first nucleophilic attack. Instead, three aromatic residues (Phe80, Tyr135, and Trp223), likely responsible for substrate binding, interact with the inhibitor.

In summary, the inhibitor covers the active site cleft preventing the access of the substrate, and prevents the interactions of Phe80, Tyr135, and Trp223 with the substrate. These observations are consistent with the observed competitive mode of inhibition.

The structure of the PME-PMEI complex provides a possible explanation for the lack of inhibition of PMEIs on PMEs from the bacterium *E. crysanthemi* and the fungus *Aspergillus aculeatus* and also why there is no interaction between invertase and PMEs (Raiola *et al*., 2004; Giovane *et al*., 2004). In *E. crysanthemi*, the putative binding site cleft is much deeper than in plant-derived PMEs. It is conceivable that the external loops of the bacterial enzyme create a steric hindrance that prevents the interaction with the inhibitor. On the other hand, the amino acid sequence alignment among PME from tomato, carrot, and *A. aculeatus* (Swiss-Prot code Q12535) reveals that almost all of the residues important for the interaction of tomato PME with the inhibitor are conserved in plant PMEs but not in the fungal enzyme, thus providing a reason for the observed lack of interaction (Di Matteo *et al*, 2005).

1.9.2 PMEI role in plants

Similar to PME, PMEI appears to be ubiquitously express in higher plants, but its role in plants is not yet full clarified. In contrast to other inhibitors of cell wall degrading enzymes that inhibit microbial enzyme, PMEI is specific to plant PMEs. It is likely that its physiological function relies in the modulation of the endogenous PME activity during plant growth and development.

Experimental evidences support the involvement of this inhibitor in the pectin metabolism of pollen and pollen tubes cells (Pina *et al*, 2005). It was demonstrated that the PME isoform AtPPME1 and the PMEI isoform AtPMEI-2, which are both specifically expressed in Arabidopsis pollen, physically interact, and that AtPMEI-2 inactivates AtPPME1 *in vitro*. Moreover, transient expression in tobacco pollen tubes revealed a growth-promoting activity of AtPMEI-2, and a growth-inhibiting effect of AtPPME1. Confocal microscopy analysis of living pollen tube revealed that AtPMEI-2 localizes exclusively to the pollen tube tip, whereas the enzyme AtPPME1 is present both at the tip and the flanks of the tube. The tip-restricted

localization of the PMEI protein seems to be maintained by selective endocytosis of the inhibitor at the flank of the pollen tube. The authors suggested that the polarized accumulation of PMEI isoforms at the pollen tube apex by selective endocytosis could regulate cell wall stability by locally inhibiting PME activity, thus maintaining the unique pectin distribution in pollen tubes (Röckel *et al*., 2008).

Recently it was shown the involvement of PMEI in Arabidopsis phyllotaxis (Peaucelle *et al*., 2008). In fact, after demonstrating that the formation of flower primordia in the Arabidopsis shoot apical meristem is accompanied by the demethylesterification of pectic polysaccharides in the cell walls, it was reported that in Arabidopsis transgenic lines overexpressing a PMEI (referred as PMEI3), which is normally expressed in the apical meristems, the formation of lateral organs (sepals, petals and stamens) was completely suppressed in the inflorescence meristem. These results highlight the importance of regulated pectin demethylesterification for the phyllotactic pattern.

There are some evidences that also indicate a role of PMEI in plant defences.

Since PME are involved in the systemic spread of some viruses through the plant, acting as a cell-wall receptor of virus movement protein, PMEI may hamper virus propagation by forming PME-PMEI complex (Jolie *et al*, 2010).

In Arabidopsis, the overexpression of endogenous PMEI (AtPMEI-1 and AtPMEI-2) caused a reduced PME activity and an increased content in methylated pectin. Moreover transgenic plants were found to be more resistant to the necrotrophic fungus *Botrytis cinerea* (Lionetti *et al*., 2007). The resistance of these transgenic plants was related to the impaired ability of the fungus to grow on methylesterified pectins (Lionetti *et al*., 2007).

The transcript accumulation of CaPMEI in pepper leaves following various biotic and abiotic stimuli also support the role of PMEI in the defence response (An *et al*, 2008; An *et al*, 2009). Moreover, transgenic Arabidopsis lines overexpressing CaPMEI1 exhibited enhanced resistance to *Pseudomonas syringae* pv. *tomato* (An *et al*., 2008).

It is widely accepted that highly methyl esterified pectins are less susceptible to the action of PG and PL. Consequently, PMEI might indirectly play a role in plant defence against pathogen attack by increasing the degree of methyl esterification of cell wall pectin (Juge, 2006).

The importance of the pectin composition in plant resistance has been demonstrated in some plant-pathogen systems where not only the degree of methylesterification but also the distribution of the methyl esters in the pectin were found to be correlated to the susceptibility or the resistance against pathogens. For example, in tomato, a difference in the degree of

pectin methylation was related to the reaction to *Pseudomonas syringae* pv. *tomato* (Venkatesh B., 2002). In potato stem tissues, a higher percentage of methylated and branched pectins has been reported to correlate with resistance against the bacterium *Erwinia carotovora* subsp. *atroseptica* (Marty *et al*., 1997). A higher degree of esterification has been found in bean cultivars resistant to the fungus *Colletotrichum lindemuthianum* compared with that found in susceptible cultivars (Boudart *et al*., 1998).

1.10 Aim of work

Since a number of findings highlighted that pectin methyl esterification of plant cell wall play an important role both in plant development and plant defence, the aim of this work was to gain information on the components involved in regulating this feature in wheat tissues. In particular, the work was focussed on the characterization of PME activity on wheat tissues and on the structural and functional features of wheat *pmei* genes and their encoding products. The gaining of these basic information are essential to understand the role of PMEI in controlling pectin methyl esterification in wheat and to verify the possibility to manipulate this feature for wheat improvement.

Materials and Methods

2.1 Microbiological techniques

2.1.1 *Escherichia coli* and *Pichia pastoris* strains

For cloning procedures the *E. coli* strain DH5α was used. For the expression of recombinant proteins the strain Rosetta-gami DE3 (Novagen) was used. This strain carries an additional plasmid (pRARE, Chloramphenicol resistance), coding for six tRNAs seldom used in *E. coli*, and therefore supports the expression of eukaryotic genes. Mutations in the thioredoxin (trxB) and glutathione (gor) reductase enzymes promote the formation of disulfide bonds in the *E. coli* cytoplasm.

For the expression of recombinant proteins *P. pastoris* X33 (Invitrogen) was used.

2.1.2 Media and antibiotics

E. coli strains were grown in LB medium prepared according to Sambrook 1989 (Sambrook et al., 1989) or in low salt LB medium (5g/L NaCl), in the presence of Zeocin antibiotic.

The selection of DH5α strain was carried out with the Ampicillin 100µg/ml or Zeocin 25µg/ml.

The selection of Rosetta-gami strain was performed by adding to the medium the following antibiotics:, Chloramphenicol 34µg/ml, Kanamycin 15µg/ml, Tetracyclin 12.5µg/ml.

P. pastoris media and plates were prepared according to the Pichia Expression Kit Manual provided by Invitrogen (Invitrogen, 2002).

For the expression of recombinant proteins in *P. pastoris*, several different media were used.

YPD: 1% yeast extract, 1%, peptone, 2% glucose

YPDS: 1% yeast extract, 1%, peptone, 2% sorbitol 1M, 2% glucose

BMGY: 1% yeast extract, 2%, peptone, 100mM potassium phosphate pH 6.0, 1,34% YNB (Yeast Nitrogen Base), 4.10^{-5}% D-Biotine, 1% glycerol

BMMY: similar composition of BMGY, but 0,5% methanol replace glycerol

MM: 1,34%YNB; 4.10^{-5}%D-biotine and 0,5%methanol

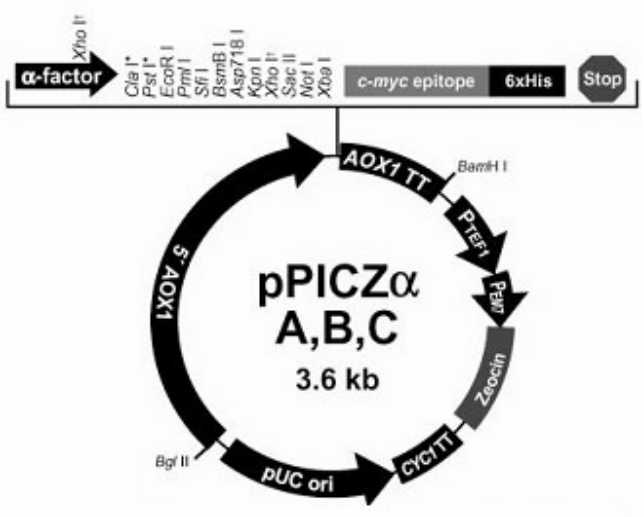
MD: 1,34%YNB; 4.10^{-5}%D-biotine and 2% glucose

The selective media for *P. pastoris* were prepared adding the antibiotic Zeocin.

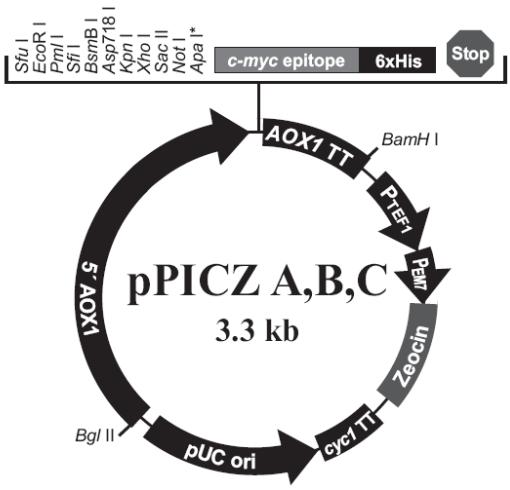
2.2 Heterologous expression of *Tdpmei* genes in *Pichia pastoris*

2.2.1Cloning of *Tdpmei* genes in pPICZ αA and pPICZ B vectors

To perform heterologous expression in *P. pastoris* of TdPMEI, we used two different types of vector: pPICZαA® and pPIZB® (Fig.2.1).



A)



B)

Fig. 2.1 Maps of vectors used for the heterologous expression in *P. pastoris*. **A)** pPICZαA®; **B)** pPICZB®

The vector pPICZαA® contains the promoter sequence of AOX1 gene for methanol induced expression of the gene of interest, the α-factor secretion signal for directing secreted expression of the recombinant protein and the Zeocin™ resistance gene for selection in both *E. coli* and *Pichia*. The pPICZB® vector has similar characteristic except for the α-factor secretion signal.

For the heterologous expression in *P. pastoris*, the region of *Tdpmei2.1* and *Tdpmei2.2* coding for predicted mature proteins, was inserted into the *Eco*RI site of the pPICZαA®.

The cloning of *Tdpmei2.1* and *Tdpmei2.2* into the vectors required the use of SOE-PCR to remove the intron contained into the coding region of each gene.

SOE-PCR involves three separate PCRs: the two DNA fragments produced in the first stage reactions are mixed to form the template for the second stage (Warren *et al*,1997). Four primers are required for each construct: two flanking primers, and two hybrid primers, one of each for each of the two first stage reaction. The hybrid oligonucleotides are designed from the known nucleotide sequences to generate fragments that will have overlapping sequence. One of the first PCRs produces a DNA fragment with the sequence 5' to splice point, and the other a DNA fragment with the sequence 3' to the splice point (Fig. 2.2). However, since the hybrid oligonucelotide span the splice point, each of the first stage products is tipped with a short sequence derived from the other. For this reason, when the two products are mixed, they can partially anneal, and, using the original flankig primers, participate in the second-stage PCR to produce the final product.

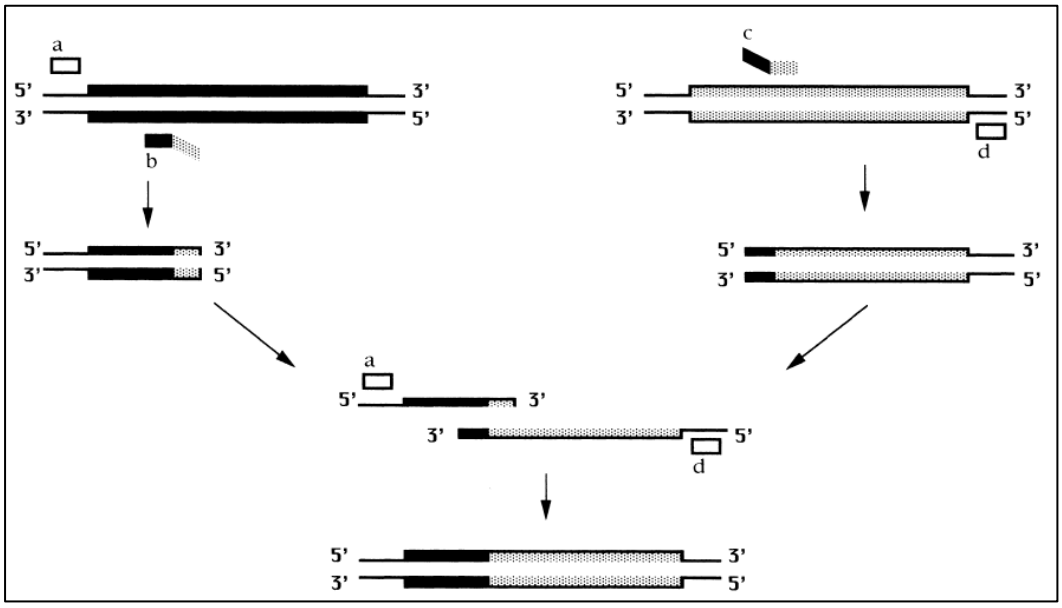


Fig. 2.2 Schematic representation of SOE-PCR (form Warren *et al*, 1997).

We used p*Tdpmei2.1* and p*Tdpmei2.2* as template for the two first stage reactions. PCR reactions were performed in a reaction volume of 25µl with 10ng of plasmid DNA (p*Tdpmei2.1* or p*Tdpmei2.2*), 2,5 units of FastStart High Fidelity PCR system (Roche Diagnostics, Monza, Italy), 1x Taq PCR buffer, 0,5µM of each of the two primers, and 100µM each deoxyribonucleotide. Amplification conditions were 1 cycle at 94°C for 2 min; 30 cycles at 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec; and a final step at 72°C for 5 min.

The two DNA fragments produced, corresponding to the exons of both *Tdpmei2.1 Tdpmei2.2*, were recovered, purified and then used as templates in a final PCR reaction with the original flankig primers. Since the two DNA fragments have overlapping sequence, they can partially anneal in this last PCR step and produce the final product in which the two exons are spiced together. For SOE-PCR we followed the same condition described above.

SOE-PCR products recovered were digested with *Eco*RI and inserted into the pPICZαA vector. For the ligation reaction, T4-DNA Ligase (Promega) was used, according to the manufacturer's instructions and then, 10µl of the ligation mix were used to transform DH5α bacterial strains. The correct coding sequence of *Tdpmei2.1* and *Tdpmei2.2* and the insertion sites were confirmed by nucleic acid sequencing.

For cloning *Tdpmei2.1* in pPICZB® vector, we used the same strategy, but the gene was inserted into the *Eco*RI and *Xba*I sites of pPICZ B® vector.

2.2.2 *Pichia pastoris* transformation and selection of recombinant clones

For the transformation of *P. pastoris*, we followed the protocol described in the Pichia Expression Kit Manual provided by Invitrogen (Invitrogen, 2002).

P. pastoris strain X-33was grown overnight in 5ml of YPD at 30°C. The overnight culture (0,5-1 ml) was used to inoculate 500 ml of fresh medium again to an OD600 = 1.3–1.5. Cells were centrifuged at 1500 g for for 5 minutes at 4°C. pellet was resuspend with 500 ml of ice-cold, sterile water. Cells were centrifuged again as in the previous step and then resuspended again with 250 ml of ice-cold, sterile water. After a similar centrifugation step, the pellet was resuspend in 20 ml of ice-cold 1 M sorbitol. Finally cells were centrifuged again and resuspended in ice-cold 1 M sorbitol in a final volume of approximately 1.5 ml. Cells were kept the on ice and use for the transformation by electroporation.

Eighty microliters of these cells were mixed with 5-10 μg of plasmid DNA (pPICZαA::*Tdpmei2.1*, pPICZαA::*Tdpmei2.2* and pPICZB::*Tdpmei2.1)*, previously linearized with *Pme*I enzyme (New England Biolabs®) for the correct integration into *P. pastoris* genome.

The cells were transferred to an ice-cold 0.2 cm electroporation cuvette and incubated on ice for 5 minutes. Then the cells were transformed by electroporation with a GenePulserII (BioRad) set to 200 W, 1.8 kV, 25 μF. Immediately 1 ml of ice-cold 1 M sorbitol was added to the cuvette. The cuvette contents was transferred to a sterile 15-ml tube and incubate at 30°C without shaking for 1 to 2 hours.

Transformed cells were spread on YPDS plates containing 100, 500 and 1000 μg/ml Zeocin. Plates were incubated from 3–10 days at 30°C until colonies form.

Transformed yeasts were selected on increasing concentration of Zeocin and only the colonies grown on the highest concentration of Zeocin were chosen since indicative of multi-copy recombinants. After this first screening, we selected the Mut^S phenotype colonies; growing these colonies on MM medium, that contains methanol as the only carbon source, and MD medium, containing sucrose as carbon source, we were able to select only the colonies where the recombination occurred at the right locus. Since the DNA insertion, downstream the *AOX1* promoter, determines a deletion of *AOX1* gene, the Mut^s colonies are not able to grow on a medium containing only methanol as carbon source.

2.1.3 Expression and purification of recombinant TdPMEI proteins

The expression of recombinant TdPMEI2.1 and TdPMEI2.2 was firstly achieved in a small-scale system in order to select the recombinant colonies able to produce and accumulate the proteins of interest. Unfortunately, only TdPMEI2.1 was accumulate.

For the purification of the recombinant TdPMEI2.1 protein, a single recombinant colony of *P. pastoris* transformed with pPICZαA::*Tdpmei2.1* or pPICZB::*Tdpmei2.1*, was used to inoculate 5 ml of BMGY and was grown at 30°C for 16-18 h. This starter culture was used to inoculate 1L of BMGY and was grown at 30°C until the OD at 600nm was between 2 and 6.

The culture was centrifuged at 3000rpm for 5 minutes. The cell pellet was resuspended in 100ml of BMMY and let grow at 30°C for 4 days under vigorous shaking. Every 24h, 1% (v/v) of methanol was added to induce expression from the alcohol oxidase promoter.

To harvest the TdPMEI2.1 proteins secreted into the medium, the culture was centrifuged (30 min, 4,000 g, 4°C.) and the medium precipitated by adding ammonium sulfate (80% saturation) under constant stirring at 4°C. The precipitated protein was collected by centrifugation (30 min, 9,800 g, 4.), resuspended in 30mM of Tris-HCl buffer pH 7 and residual ammonium sulfate was removed by dialysis against 10 l of the same buffer.

The dialysed proteins were that applied to a DEAE-Sepharose column connected to an FPLC Akta® Purifier 10 (GE Healthcare) and eluted with a linear gradient ranging from 0 to 1 M NaCl in the equilibration buffer.

2.3 Heterologous expression of *Tdpmei* genes in *Escherichia coli*

2.3.1 Cloning of *Tdpmei* gens in pOPIN F vector

To perform the heterologous expression of TdPMEIs in *E. coli*, we inserted the region encoding for the mature protein of each wheat *pmei* gene into the pOPIN F vector. This vector was kindly provided by the Oxford Protein Production Facility and allow the production of recombinant protein as fusion to a N-terminal polyhistidine tag (6x His). This vector confers Ampicilline resistance.

For this purpose we used the In-Fusion™ Advantage Cloning method (Clontech Laboratories). This cloning system requires a PCR product containing a short primer extensions to the gene that has to be express and linearize vector (Fig. 2.3).

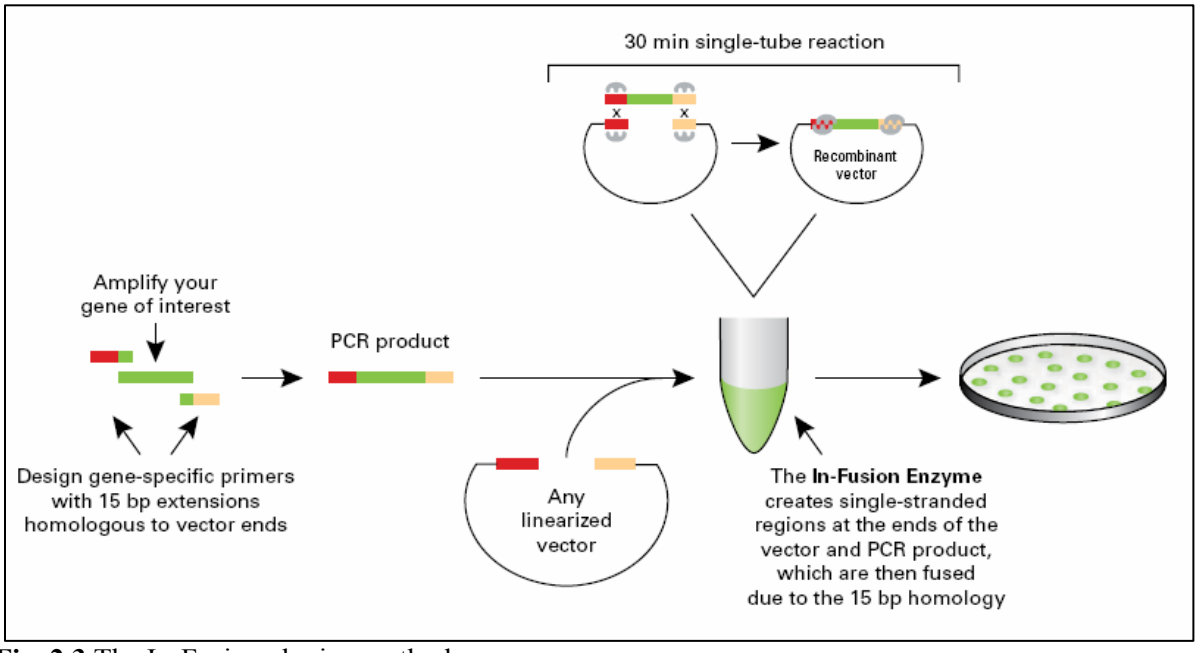


Fig. 2.3 The In-Fusion cloning method.

For each gene, we designed primers that shared 15 bases of homology with the sequences at the ends of pOPIN F vector linearized with *Hind*III and *Kpn*I enzymes. These primers were used for the amplification reaction that were performed in a volume of 25µl with 10ng of plasmid DNA (p*Tdpmei2.1*, p*Tdpmei2.2* and p*Tdpmei7.3*), 2,5 units of FastStart High Fidelity PCR system (Roche Diagnostics, Monza, Italy), 1x Taq PCR buffer, 0,5µM of each of the two primers, and 100µM each deoxyribonucleotide. Amplification conditions were 1 cycle at 95°C for 2 min; 30 cycles at 95°C for 15 sec, 60°C for 30 sec and 68°C for 1 min; and a final step at 68°C for 5 min. The resulting PCR products were recovered and purified and then used in the In-Fusion cloning reaction. The PCR products were incubated with the linearized vector at 30°Cfor 15 min and then at 50°C for other 15 min. This mix was used for the transformation into *E. coli* DH5α strain. The correct coding sequence of *Tdpmei2., Tdpmei2.2* and *Tdpmei7.3* and the insertion sites were confirmed by nucleic acid sequencing.

Plasmids harbouring *Tdpmei2.1*, *Tdpmei2.2* and*Tdpmei7.3* were used for the transformation of *E. coli* Rosetta-gami (DE3)™ strain. We used this strain for heterologous expression because its deficiencies in thioredoxin reductase and glutathione reductase activities provide an oxidizing environment to facilitate disulfide bridges formation.

2.3.2 Expression and purification of recombinant TdPMEI proteins

The expression of recombinant TdPMEI proteins was obtained as follows.

A single recombinant colony of Rosetta-gami transformed with pOPIN F::*Tdpmei2.1*, pOPIN F ::*Tdpmei2.2* and pOPIN F::*Tdpmei7.3* was used to inoculate 3 ml of LB medium plus ampicilline, kanamicine, chloramphenicol and tetracyclin and was grown overnight at 37°C. These pre-cultures were used for the inoculation of 50ml of LB and grown to a density of about 0.4-0.6.

The cultures were then induced by adding 1mM of isopropylthiogalactoside (IPTG) and further grown for 4h at 37°C or overnight at 17°C.

Thereafter, cells were pelleted for 15 min at 10 000 rpm and extracted with lysis buffer (1/10 volume of initial culture volume of 20mM Na_2HPO_4/NaH_2PO_4 pH 7.4, 500mM NaCl, 40mM imidazole, 250U benzonase,1 mg/ml lysozyme). After 30 minutes of incubation, cells were pelletted at 11000 rpm for 20 minutes and supernatant, representing the soluble fraction, was recovered. The insoluble fraction was extracted with the same lysis buffer plus 6M. After 1h incubation in ice, the insoluble fraction was centrifuged at at 11000 rpm for 30 minutes and the supernatant recoverd.

Both soluble and insoluble fractions obtained were loaded into His-Trap HP column (Amersharm Bioscinces) that allows the purification of His-tagged recombinant proteins by immobilized metal affinity chromatography. The column was first equilibrated with 10 volumes of lysis buffer and bound fusion proteins were eluted with 10 volume of elution buffer (20mM Na_2HPO_4/NaH_2PO_4 pH 7.4, 500mM NaCl, 500mM imidazole) with a linear gradient from 0 to 0,5M imidazole using FPLC system Akta® Purifier 10 (GE Healthcare).

2.4 Production of transgenic wheat plants

2.4.1 Construction of the pUBI::Tdpmei7.3 vector

To assemble the expression vector pUBI::*Tdpmei7.3*, the coding region of *Tdpmei7.3* was inserted into the *Bam*HI site of pAHC17 under control of the maize *Ubiquitin1* promoter and nopaline synthase terminator (Christensen and Quail, 1996). The *Bam*HI sites flanking the complete coding region of *pmei* gene was generated by PCR amplification using the forward and reverse primers (Tdpmei3_BamHI/BamHi_Flag_pmei3) (Table 2.2). With these primers, we cloned the coding region of *Tdpmei7.3* gene in fusion with a Flag-tag at the C-terminus. PCR reactions were performed in a reaction volume of 25µl with 10ng of plasmid DNA (p*Tdpmei7.3*), 2,5 units of FastStart High Fidelity PCR system (Roche Diagnostics, Monza, Italy), 1x Taq PCR buffer, 0,5µM of each of the two primers, and 100µM each deoxyribonucleotide. Amplification conditions were 1 cycle at 94°C for 2 min; 30 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min; and a final step at 72°C for 5 min. After *Bam*HI digestion, the amplicon was inserted into the *Bam*HI site of pAHC17 generating a 5422 bp plasmid, pUBI::*Tdpmei7.3*. For the ligation reaction, T4-DNA Ligase (Promega) was used, according to the manufacturer's instructions and then, 10µl of the ligation mix were used to transform DH5α bacterial strains. The correct coding sequence of *Tdpmei7.3* and the insertion sites were confirmed by nucleic acid sequencing.

2.4.2 Media

Modified MS media for wheat cellular cultures were used (Sears and Deckard, 1982) with MS Salt, Maltose, Thiamine-HCl, L-asparagine at pH 5.85 and Phytagel as gelling agent. After autoclaving at 121°C for 20 min, 2,4-D was added and the medium poured in 100mm x 15mm

Petri dishes (except for the bombardment medium for which the Petri dishes were 60mm x 15mm) and let it solidify.

Media compositions are the same as indicated in Weeks *et al.* 1993 (Table 2.1).

Table 2.1 Culture media compositions.

Dissecting medium	Murashige & Skoog Salt mixture 4,3g/l; Maltose 40g/l; Thiamine-HCl (25mg/500ml) 10ml/lt; L-asparagine 0,15g/l 0,25% v/v di phytagel 2,4 D (0,5 mg/ml) 2ml/500 ml
Recovery medium	Murashige & Skoog Salt mixture 4,3g/ Maltose 40g/l; Thiamine-HCl (25mg/500ml) 10ml/lt; L-asparagine 0,15g/l 0,25% v/v di phytagel 2,4 D (0,5 mg/ml) 2ml/500 ml
Bombardment medium (Bomb sucrose)	Murashige & Skoog Salt mixture 4,3g/l; Maltose 40g/l; Thiamine-HCl (25mg/500ml) 10ml/lt L-asparagine 0,15g/l Sucrose 171,5 g/l 2,4 D (0,5 mg/ml) 2ml/500 ml
Regeneration medium	Murashige & Skoog Salt mixture 4,3g/l; Maltose 40g/l; Thiamine-HCl (25mg/500ml) 10ml/l L-asparagine 0,15g/l 2,4 D (0,5 mg/ml) 0,2ml/500 ml Bialaphos (1 mg/ml) 1,5 ml/500 ml
Rooting medium	Murashige & Skoog Salt mixture 2,15g/l; Maltose 20g/l; Thiamine-HCl (25mg/500ml) 5ml/l L-asparagine 0,075g/l Bialaphos (1 mg/ml) 1,5 ml/500 ml
Early germination medium	Murashige & Skoog Salt mixture 4,3g/l; Maltose 40g/l; Thiamine-HCl (25mg/500ml) 10ml/l L-asparagine 0,15g/l Bialaphos (1 mg/ml) 1,5 ml/500 ml

2.4.3 Embryos isolation

For the wheat transformation, we followed the protocol described by Weeks (Weeks *et al*,1993)

Seeds of 10 to 18 days post anthesis were collected from *Triticum durum* cv. Svevo wheat plants, (Zadoks stage 72) grown in the field, and surface-sterilized in 70% ethanol containing 50µl/100ml of Tween 20, by sonication 6 times for 20 sec. Seeds were then sterilized with 70% ethanol for 5 min and 20% sodium hypochlorite for 15 min, followed by three washings with sterile distilled water. Immature embryos, 0,5 to 1 mm long, were aseptically removed under a stereo microscope (Fig. 4a), placed with the scutella exposed on the dissecting medium (Fig. 4b) and stored in the dark at 23°C for 5 days for the callus induction (Fig. 2.4c).

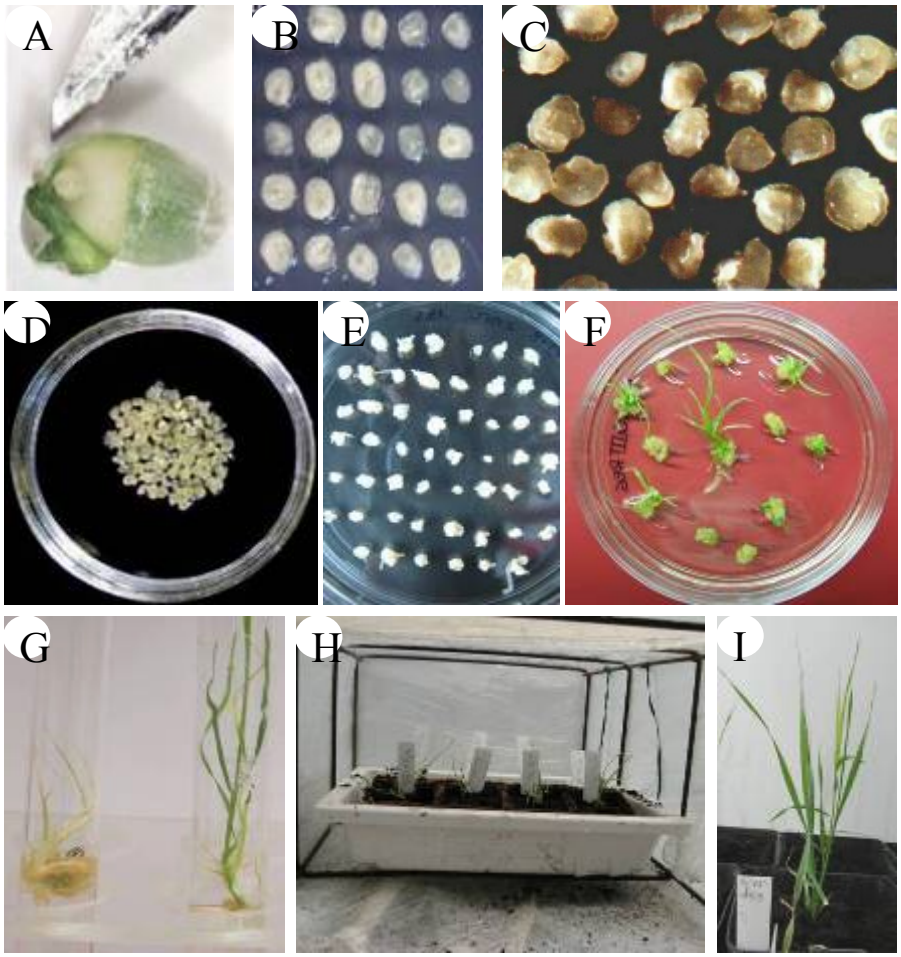


Fig. 2.4 Wheat transformation steps **A)** Isolation of immature embryo from wheat caryopsis. **B)** Immature scutella plated for bombardment. **C)** Formation of embryogenic callus. **D)** calli in the bombardment medium, **E)** Bombarded calli in the recovery medium. **F)** Regeneration of plantlet on selective medium, **G)** plant resistant to the herbicide (right) and susceptible (left), **H)** plants in the acclimatization stage, **I)** plants in pots. A and B images are from "Transgenic Wheat, Barley and Oats" by Jones and Shewry, 2009; C image is from Weeks *et al*. 1993.

2.4.4 Coating gold particles with DNA, bombardment and plant regeneration

Thirty mg of gold particles (0.6µm) were resuspended in 500µl of 100% ethanol. Thirty-five microliters of the suspension were aliquoted into 1,5-mL tubes, spin and the ethanol removed. The microprojectiles were then washed with cold sterile water (200µl), spin and the supernatant discarded.

For the transformation with *Tdpmei7.3* gene, the plasmid pUBI::BAR (5.505 bp), carrying the *bar* gene that confers resistance to the bialaphos herbicide, was co-bombarded with pUBI::*Tdpmei7.3* in a 1:3 molar ratio. Constructs were introduced into immature embryos-derived calli as described above. Fifteen µg of pUBI::BAR containing the marker gene was used.

The following formulas were used to calculate the microliters of plasmid DNA needed for coating the gold particles:

Gene plasmid (µl): bp gene/bp marker x 15µg x (1/plasmid concentration) x 3

Marker plasmid (µl): 15µg x (1/plasmid concentration)

The microprojectiles pellet was resuspended in a solution containing 2.5M $CaCl_2$ (250µl), spermidine (50µl), plasmid DNA and water (250µl - µl of plasmid). The tubes were shaken with a vortex mixer at 4°C for 15-20 min and briefly centrifuged. The supernatant was removed and the pellet was washed with 600µl of 100% ethanol. The DNA-coated gold pellets were resuspended in 36µl of 100% ethanol and stored on ice until they were used. About 30-40 embryos-derived calli were transferred onto the bombardment medium (characterized by a high osmolarity) 4 hours before the bombardment.

For bombardment, 10µl of the DNA-gold suspension was placed in the centre of a macroprojectile and bombarded using a Model PDS-1000/He Biolistic particle delivery system (Bio-Rad, Hercules, CA, U.S.A.) as described in Weeks *et al*. (1993). The distance from the stopping plate to the target was 13 cm, and the rupture disc strength was 1100 p.s.i (Fig. 2.5).

Immediately after the bombardment, calli were kept in the bombardment medium (Fig. 2.4d) and stored in the dark at 23°C for 24 hours; maintained in the recovery medium for 4 weeks and transfer into fresh medium at 2-week intervals (Fig. 2.4e). For the regeneration, calli were transferred to the regeneration medium containing the PPT (DL-Phosphinothricin) as selective agent for 6 weeks at 24°C with 16-h light period (43µE/m^2) and transfer into fresh medium at 2-week intervals. At this stage, new shoots growth from the callus resistant to PPT, usually from the third week of regeneration (Fig. 2.4f).

Shoots were removed from callus and place in Pyrex tubes containing the rooting medium, which not containe the hormone 2,4-D, where they were under herbicide selection for additional 2-3 weeks. At this stage, plants capable to form long, highly branched roots in the PPT-containing medium were defined as resistant (Fig. 2.4g, right).

Once the plants had sufficient leaves and roots, they were transferred into pots and kept in a growth chamber completely covered with plastic bags to increase the humidity, at 23°C, 16-h day light for 5-10 days to allow them to acclimate to greenhouse conditions (Fig. 2.4h). After that, plants were transferred to bigger pots; these primary regenerants are called T_0 plants (Fig. 2.4i).

The total process starting from the excision of the embryos up to the anthesis of regenerated T_0 plants, lasts about 168 days. The presence of the transgene in the regenerated plants were checked by PCR.

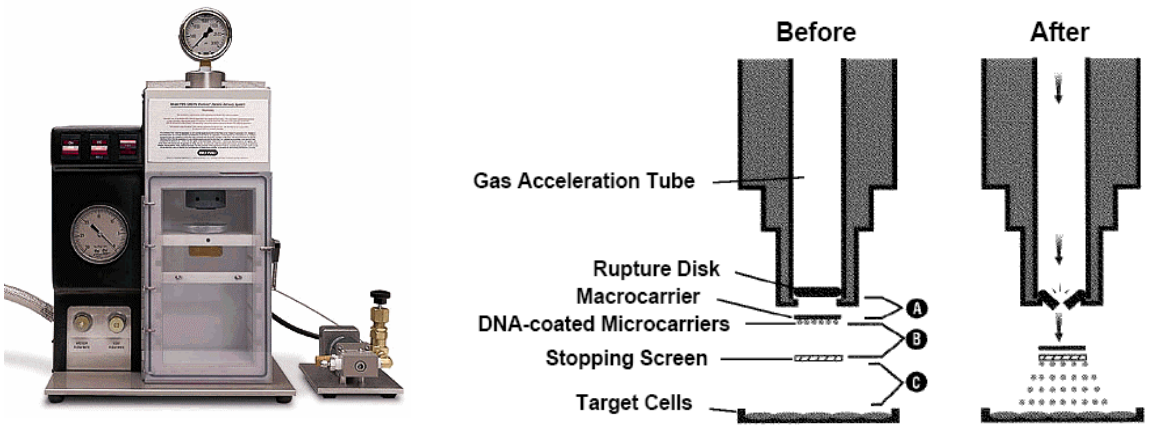


Fig. 2.4 (*Left*) The Biolistic PDS-1000/He instrument. (*Right*) The Biolistic bombardment process. The Biolistic PDS-1000/He system uses high pressure helium, released by a rupture disk, and partial vacuum to propel a macrocarrier sheet loaded with microscopic gold microcarriers toward target cells at high velocity. The microcarriers are coated with DNA for transformation. The macrocarrier is stopped after a short distance by a stopping screen. The DNA-coated microcarriers continue traveling toward the target to penetrate and transform the cells. The launch velocity of microcarriers for each bombardment depends on the helium pressure (rupture disk selection), the amount of vacuum in the bombardment chamber, the distance from the rupture disk to the macrocarrier (A), the macrocarrier travel distance to the stopping screen (B), and the distance between the stopping screen and target cells (C).

2.4.5 Control of transgenic plants

Before putting on germination, seeds were analyzed for the presence of the transgene *Tdpmei7.3* by PCR using total DNA extracted from half-seed (D'Ovidio and Porceddu, 1996). In this rapid procedure, half-seed was homogenized with a pestle, extracted with 80µl of extraction buffer (100mM Tris-HCl pH 8, 50mM EDTA pH 8, 500mM NaCl) containing 32µl of SDS 10% and incubated at 65°C for 10 min. 26,7µl of 5M potassium acetate were added and the samples mixed and kept on ice for 20 min. After a centrifuge at 14000 rpm for 20 min, the supernatant, containing the DNA, was recovered and the DNA precipitated adding isopropanol (in the same volume of the supernatant recovered) and centrifuging for 5 min at 14000 rpm. The pellet of DNA was washed in 70% ethanol, vacuum dried and resuspended in 20µl of sterile water. The same rapid procedure was used to extract DNA from leaf (about 1cm^2).

One microliter of DNA solution was used for PCR analysis. DNA amplification was carried out according to the procedures specified for Ready Mix RedTaq polymerase (Sigma) at an annealing temperature of 60°C.

To avoid interference with the endogenous copy of *Tdpmei7.3*, specific oligonucleotides, designed on the promoter region of pUBI plasmid (UBI -49F) and the transgene coding sequence (Tdpmei3_369R) were used (Table 2.2). Control DNAs included untransformed *T. durum* cv. Svevo (negative) and the vectors pUBI::*Tdpmei7.3* (positive).

2.5 Molecular biology techniques

2.5.1 Total DNA extraction

For total DNA extraction, Tai and Tanksley (1990) method was used. Green leaf tissue (up to 0,15g) was ground with a mortal and pestle in the presence of liquid nitrogen. 700µl of extraction buffer (100mM Tris-HCl pH 8, 50mM EDTA pH 8, 500mM NaCl, 1,25% SDS, 8,3mM NaOH, 0,38% Na bisulphite), pre-heated to 65°C, were added to each sample before the incubation at 65°C in a water bath for 10 min. After the incubation, 220µl of 5M potassium acetate were added and the samples were kept for 40 min on ice, centrifuged for 3 min at 4°C and the supernatant filtrated. For the DNA precipitation, 600µl of isopropanol were added; samples were centrifuged again at 4°C for 3 min, the supernatant was poured out

and the pellet rinsed twice with 800µl of 70% ethanol. The pellet was resuspended with 300µl of T5E (50mM Tris-HCl pH 8, 10mM EDTA pH 8), incubated at 65°C for 5 min and then, 150µl of 7,4M ammonium acetate were added to each sample before centrifuging again for 3 min. The supernatant was collected and 330µl of isopropanol were added; samples were centrifuged for 3 min and the pellet rinsed twice with 70% ethanol, resuspended in 100µl of T5E and incubated at 65°C for 5 min. 10µl of 3M sodium acetate were added and the DNA precipitated with isopropanol (75µl); the pellet was then, rinsed twice with 70% ethanol, dried under vacuum and resuspended overnight at 4°C with 25µl of TE. The day after, the DNA was heated at 65°C for 5 min and stored at 4°C (-20°C for longer periods).
When the initial leaf material was more than 0,15g, the same procedure was used but with increased volumes of extraction buffers.

2.5.2 Total RNA extraction

RNA extraction was carried out using some precautions to inadvertently avoid introducing RNases into the RNA sample and to maintain an RNase-free environment. For this reason, RNA manipulation was done always wearing gloves and using material dedicated only to RNA isolation; in addition, water was treated with diethyl pyrocarbonate (DEPC), a RNase inhibitor, and autoclaved to neutralize the toxic action of this compounds.
Total RNA extraction was performed using "RNeasy Plant mini" Kit (Qiagen), starting from no more than 100mg of frozen leaf tissue ground thoroughly with a mortal and pestle in liquid nitrogen. 450µl of RLT buffer (containing dithiothreitol, DTT). The lysate was then transferred into the column and centrifuged at maximum speed for 2 min in order to remove cell debris and simultaneously homogenize the lysate. 0,5 volume 100% ethanol was added to the cleared lysate and mixed well by pipetting. The sample was applied to the RNeasy column containing a silica-membrane and centrifuged for 15 sec at 10000 rpm. The flow-through was discarded and the column washed adding 700µl of RW1 buffer and centrifuging for 15 sec at 10000 rpm. The column was transferred into a new tube, 500ml of RPE buffer were added and the flow-through discarded after centrifuging for 15s at 10000 rpm. This step was repeated to ensure buffer removal. To elute RNA, 50µl of RNase-free water were used. RNA was stored at -80°C.
Contaminating DNA was removed with DNase treatment using DNase I provided in the "DNA-free™" kit (Ambion) and following the manufacturer's instructions.

2.5.3 Isolation of plasmid DNA

DH5α bacterial strain were transformed with a plasmid or a ligation mix and the DNA was extracted from each colony. Single bacterial colonies were picked to inoculate 1,5ml of LB (Luria Bertani) containing selective antibiotics as described above and and shaken at 250 rpm, 37°C. overnight. The day after, 1,5ml culture were centrifuged in eppendorf tubes at 14000 rpm for 1 min and the pellet resuspended in 1ml TE (1M Tris-HCl pH 8; 1mM EDTA) and centrifuged as above. The pellet obtained was resuspended in 150µl of SET (50mM Tris-HCl pH 8; 50mM EDTA pH 8; 20% Sucrose) using a vortex. 350µl of lysis buffer (0,2M NaOH; 1% SDS) were added and, after mixing gently, tubes were left on ice for 10 min. 250µl of 3M sodium acetate pH 5.2 were added and the tubes left again on ice for 20 min to allow the SDS and chromosome DNA to precipitate. After a centrifuge at 14000 rpm for 10 min the supernatant was collected and the plasmid DNA precipitated adding an equal volume of cold isopropanol and centrifuged for 10 min at 14000 rpm. Pellet was then rinsed with 70% ethanol and dried under vacuum for 10-15 min.
Plasmid DNA was resuspended in 20µl of sterile water and treated with RNase (2µl of 1mg/ml) at 37°C for 20 min. The average yield using this method was about 2µg per 1,5ml of bacterial culture.

2.5.4 Midi-and Maxi preparation of plasmid DNA

Plasmid DNA was extracted using the "PureLink^{TM} HiPure Plasmid Filter Purification" kit from Invitrogen, following the manufacturer's instructions.

2.5.5 Electrophoresis on agarose gel

To identify and separate DNA fragments, 1,2-1,5% agarose gels were prepared in TBE 1x (Tris, Boric acid and EDTA), with 0,5µg/ml Ethidium bromide. DNA samples were prepared by adding a suitable volume of 6x loading Dye ("Fermentas"). As molecular weight markers, either GeneRuler 100 bp DNA Ladder or GeneRuler 1kb DNA Ladder ("Fermentas") were used.

2.5.6 Electrophoresis on agarose gel with formaldehyde

In order to check the integrity and the quantity of the extracted RNA, RNA samples were loaded onto 1,2% formaldehyde gels with MOPS pH7 as running buffer. Samples were prepared adding 3µl of DEPC water and 1µl of 6x loading buffer to 1µl of RNA. After heating for 5 min at 65°C and cooling on ice for 1 min, 1µl of ethidium bromide was added and the samples loaded on the gel.

2.5.7 Gel extraction and purification of DNA fragments

For the purification of DNA fragments from agarose gels a "QIAquick PCR purification "kit (Qiagen) or Wizard® SV Gel and PCR Clean-Up System (Promega) were used according to the procedures specified from the manufacturer.

2.5.8 Digestion with restriction enzymes

The restriction enzymes were purchased from Roche Diagnostics and New England Biolabs® and used with the supplied buffers. Different quantities (from 500ng to 5-20µg) of plasmid or genomic DNA were used whether they were for analytical or preparative digestion. Incubation was performed for 1-2 hours at 37°C with specific restriction enzymes in appropriate reaction conditions. Usually 1U of enzyme were added per µg of DNA.

2.5.9 Southern blotting

Genomic DNA (8 to 10µg) was cleaved with *Pst*I or *Hind*III restriction enzymes, separated on 1,2% agarose gel and transferred to a positively charged nylon membrane (Roche Diagnostics) as described by Sambrook *et al*. (1989). In particular, the gel was incubated for 1 hour in denaturation buffer (0,5M NaOH and 1,5M NaCl) and then for another hour in neutralization buffer (1,5M NaCl; 1M Tris-HCl pH 8.0). DNA fragments in the gel were transferred onto the membrane by capillarity using 10x SSC (150mM NaCl; 15mM sodium citrate) as transferring buffer. After the transfer (about 16 hours), the membrane was washed for 10 min with 2x SSC and air-dried for 30 min. DNA was, then, fixed with UV light at 150 Joule before the hybridization with a digoxigenin-labelled probe.

2.5.10 Molecular hybridizations

These experiments are made of 4 phases: pre-hybridization, hybridization, washing and immunological detection.

Pre-hybridization

The membranes, containing the transferred DNA fragments, were saturated in order to avoid a background on the filter generated by the linkage of the probe. The buffer used for the pre-hybridization was: 5x SSC, 0,1% N-Laurilsarcosine, 0,2% SDS, 0,5% Blocking Reagent (Boehringer Mannheim). The membrane was incubated for at least 3 hour at 65°C.

Hybridization

The probe was first denaturated by keeping it at 95°C for 5 min and on ice for 1 min, and then mixed to a solution similar to the pre-hybridization buffer. The membrane was incubated in this buffer for al least 6 hours at 65°C for high-stringency hybridization.

After the incubation, the probe was recovered and stored at 4°C in order to use it again.

Washing

The membrane was washed twice with a buffer containing 2x SSC and 0,1% SDS for 5 min at room temperature and then twice for 15 min at the hybridization temperature using a buffer containing 0,1x SSC and 0,1% SDS.

The immunological detection was conducted using a chemiluminescence assay.

Detection with chemiluminescence assay

In this assay, a specific anti-digoxigenin-antibody, conjugated to alkaline phosphatase reacts with the CSPD substrate that is dephosphorylated leading to a distinct luminescent signal recorded on a film. The buffers and the washing conditions used were: washing for 5 min with buffer 1 (0,1M Maleic acid, 0,15M NaCl, pH 7.5 containing 0,3% Tween 20 V/V) and washing for 30 min with buffer 2 (10x blocking solution 1:10 in Maleic acid). The antiDIG–AP fragment (Roche Diagnostics) antibody was then added in 1:10000 ratio. The antibody was then removed washing twice with buffer 1 for 15 min and once with buffer 3 (100mM Tris-HCl pH 9.5; 100mM NaCl; 50mM $MgCl_2$).

The filter was put into a plastic bag and 1ml of CSPD (Disodium 3-(4-methoxyspiro {l,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.13,7]decan}-4-yl) phenyl phosphate; Roche Diagnostics) was added. In order to allow the alkaline phosphatase reaction, the bag was kept at 37°C for 15 min. The filter was then exposed on an autoradiography film and developed after 3 hours or overnight.

2.5.11 Labelling of the *Tdpmei* probes by PCR

The coding regions of *Tpmei2.1*, *Tdpmei2.2* and *Tdpmei7.3* were labelled with digoxigenin (digoxigenin-11-uridine-5′-triphosphate; Roche Diagnostics) by PCR following the procedure reported by D'Ovidio and Anderson (1994). One microliter of plasmid DNA (15ng) was added to the reaction mix containing: 73,5µl of H_2O, 10µl of 10x buffer, 10mM dATP, dCTP, dGTP, 1mM dTTP, 0,1mM DIG-11dUTP-alkali labile (Roche Diagnostics) 100mM primers, 2,5 U of RedTaq Polymerase (Sigma).
The DNA amplification conditions were: 94°C 1 min, 60°C 1 min, 72°C 1 min, for 35 cycles.
The amplified DNA was then purified using the "QIAquick PCR purification "kit (Qiagen) or Wizard® SV Gel and PCR Clean-Up System (Promega). The efficiency of the labelling process was verified by DIG Quantification Teststrip (Roche Diagnostics) following the manufacturer's procedure. The probe was stored at 4°C before use.

2.5.12 qPCR and Real time two-step RT-PCR

RNA of *Triticum durum* cv. Svevo obtained from different wheat tissues and from wheat plants infected with fungal pathogen *Bipolaris sorokiniana* was used to generate first strand cDNA following the manufacturer's instructions reported in "QuantiTect Reverse Transcription" kit (Qiagen).
cDNA was used in RT-PCR and also in real-time PCR. This last experiment was performed by using the IQ SYBR GREEN SUPERMIX**,** (Biorad) in a MyCycler™ thermal cycler (Bio-Rad Life Science, Segrate, Italy). Both RT-PCR and qRT-PCR were performed in a total volume of 20µl using 1µl of cDNA) and oligonucleotide primers specific for *Tdpmei2.1*, *Tdpmei2.2* and *Tdpmei7.3* (0,5µM each).
Amplification conditions for *Tdpmei2.1* and*Tdpmei2.2* in RT-PCR were:

Cycle 1 (1X)	95°C for 2 min
Cycle 2 (1X)	95°C for 3 min
Cycle 3 (35X)	95°C for 30 sec 59°C for 30 sec 72°C for 1 min

Amplification conditions for *Tdpmei7.3* in qRT-PCR were:

Cycle 1 (1X)	95°C for 2 min
Cycle 2 (1X)	95°C for 3 min
Cycle 3 (35X)	95°C for 30 sec 59°C for 30 sec 72°C for 1 min

Actin gene was used as housekeeping for the normalization.

Relative expression analysis was determined by using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001; Applied Biosystems User Bulletin No. 2-P/N 4303859). Calculation and statistical analyses were performed by Gene Expression Macro™ Version 1.1 (Bio-Rad Laboratories, Hercules, CA, USA).

2.5.13 Specific oligonucleotide design

Specific primers were designed for *Tdpme2.1, Tdpmei2.2 and Tdpmei7.3* using the function primer design of the DNAMAN software (Lynnon Biosoft, Quebec, Canada). A list of the oligonucleotides used in PCR, RT-PCR and qRT-PCR reactions specific for *Tdpme2.1, Tdpmei2.2 and Tdpmei7.3* and *actin* genes is reported in Table 2.2.

Table 2.2 Oligonucleotides used for the isolation, cloning, chromosomal localization and expression analysis of *Tdpmei2.1*, *Tdpmei2.2* and *Tdpmei7.3* and for the production and screening of transgenic wheat plants.

Primer name	Sequence 5'-3'	Utilization
TapmeiFor	ATGGCATCCTTCTACGCAGC	Isolation of *Tdpmei2.1* and *Tdpmei2.2* genes
TapmeiRev	TCAACGGGGGATAAGCTTGG	Isolation of *Tdpmei2.1* and *Tdpmei2.2* genes
Tc34pmeiF	ATGGCAACAATGGCGCCCTC	Isolation of *Tdpmei7.3* gene
Tc34pmeiR	CTAGGCGAGGAGCGTGATG	Isolation of *Tdpmei7.3* gene r
SOE1_ EcoRI_F	AATAGAATTCTCCCCTGCCGCGACGCCGTCGACC	SOE-PCR and cloning of *Tdpmei2.1* in pPICzαA
SOE2_R	GCTCCACGGCCGCCTCGTACCGCTCCCCACAGTCCTCGAG	SOE-PCR specific for *Tdpmei2.1*
SOE3_F	GAGGACTGTGGGGAGCGGTACGAGGCGGCCGTGGAGC	SOE-PCR specific for *Tdpmei2.1*
SOE4_ EcoRI_R	TATTGAATTCTCAACGGGGGATAAGCTTGGCG	SOE-PCR and cloning of *Tdpmei2.1* and *Tdpmei2.2* in pPICzαA
SOE5_ EcoRI F	AATAGAATTCTCCCCTCCTGCGGCGCCGTC	SOE-PCR and cloning of *Tdpmei2.2* in pPICzαA
SOE6_R	CCACGGCCGCCTCATACCGCTCCCCACAGCCCTCGAG	SOE-PCR specific for *Tdpmei2.2*
SOE7_F	GAGGGCTGTGGGGAGCGGTATGAGGCGGCCGTGGAGC	SOE-PCR specific for *Tdpmei2.2*
TC34_ XhoI F	AAACTCGAGAAAAGAGAGGCTGAAGCTGCACCAGCAGCAACAGCAAC	Cloning of *Tdpmei7.3* in pPICzαA
TC34_XbaI R	ATAATCTAGACTAGGCGAGGAGCGTGATGA	Cloning of *Tdpmei7.3* in pPICzαA
TapmeiFor_EcoRI	ATAGAATTCATGGCATCCTTCTACGCAGCC	Cloning of *Tdpmei2.1* in pPICZ B
SOE4 XbaI_R	TATTTCTAGATCAACGGGGGATAAGCTTGGCG	Cloning of *Tdpmei2.1* in pPICZ B
pOPINF pmei1_F	AAGTTCTGTTTCAGGGCCCGATGTCCCCTGCCGCGACGCCGTC	Cloning of *Tdpmei2.1* in pOPIN F
pOPINF pmei1_R	ATGGTCTAGAAAGCTTTAACGGGGGATAAGCTTGGCGATAGC	Cloning of *Tdpmei2.1* and

		Tdpmei2.2 in pOPIN F
pOPINF pmei2_F	AAGTTCTGTTTCAGGGCCCGATGTCCCCTCCTGCGGCGCCGTC	Cloning of *Tdpmei2.2* in pOPIN F
pOPINF tc34_F	AAGTTCTGTTTCAGGGCCCGATGGCGCCGGCAGCAACAGCAAC	Cloning of *Tdpmei7.3* in pOPIN F
pOPINF tc34_R	ATGGTCTAGAAAGCTTTAGGCGAGGAGCGTGATGATGTCG	Cloning of *Tdpmei7.3* in pOPIN F
Tdpmei1_ 254F	AGACAGCCACATACCTCTCA	Specific of *Tdpmei2.1*
Tdpmei1_ 566R	ATGCTCCGGTGTTCTGGTA	Specific for *Tdpmei2.1*
Tdpmei2_ 64F	AATCTACTGGTTCCCCTCCT	Specific for *Tdpmei2.2*: used in RT-PCR
Turart_391R	AAGTTGCCTTTGGTGATCA	Specific for *Tdpmei2.2*
Tdpmei2.3_69F	TACTGGTTCCCCTGCCGT	Specific for *Tdpmei2.3*
Tdpmei2.3 _393R	TAAAGTTGCCTTTGGTGATTG	Specific for *Tdpmei2.3*
Tdpmei2_ 254F	AGACAGCCACATACCTCGCG	Specific for *Tdpmei2.1;*
TdpmeiN5A_593R	ATGCTCCGGT GTTCTGGTG	Specific for *Tdpmei2.2;* used in RT-PCR
Tdpmei3_ 336F	TCCGCCACCGCGTCCGCGCTT	Specific for *Tdpmei7.3*
Tdpmei3_ 460R	CGTACTTGCCGGCGCAGGTCCTCA	Specific for *Tdpmei7.3*
Tdpmei3_ BamHI F	AATAGGATCCATGGCAACAATGGCGCCCTC	Cloning of *Tdpmei7.3* in pAHC17
BamHI_Flag_PMEI3R	AATAGGATCCTTACTTGTCATCGTCATCCTTGTAGTCGGCGAGGAGCGTGATGA	Cloning of *Tdpmei7.3* in pAHC17
UBI_-49F	TCGATGCTCACCCTGTTGTTT	Screening of transgenic plants
Tdpmei3_ 369R	AGGTGGTGTTGGCAAGCGCG	Screening of transgenic plants
77F (Actin)	TCCTGTGTTGCTGACTGAGG	Reference gene in RT-PCR, qPCR
312R (Actin)	GGTCCAAACGAAGGATAGCA	Reference gene in RT-PCR, qPCR

2.6 Biochemical techniques

2.6.1 Total protein extraction and Bradford assay

Crude protein extracts from wheat plants were obtained from fresh or frozen leaves (at 80°C) using a mortal and pestle under liquid nitrogen. The leaf tissues were homogenized in 20mM sodium acetate and 1M NaCl, pH 4.6 (2mL/g), incubated with gentle shaking for 1h at 4°C, and centrifuged 20 min at 10.000*g*. Supernatants were recovered and, if necessary, centrifuged again 5 minutes to obtain extracts free of debris. Protein concentrations of the

crude extracts were determined with the "Bio-Rad Protein assay" kit (Bio-Rad) which is based on the method of Bradford (Bradford, 1976). In this colorimetric assay, when Coomassie® Brilliant Blue G-250 dye binds protein in an acidic medium, an immediate shift in absorption maximum occurs from 465 nm to 595 nm with a concomitant colour change from brown to blue. After a short mixing and incubation step the absorbance is read on the spectrophotometer at 595 nm and the relative measurement of protein concentration is provided using BSA (Bovine serum albumin) as a standard.

2.6.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed as described by Laemmli (1970) using separating gels containing 15% polyacrylamide and stacking gels with 3,75%. The composition of the running buffer was: 0,2M Glycine, 0,02M Tris pH 8.8, 0,1% SDS.
Samples for SDS-PAGE were denatured by addition of a 4x concentrated SDS-sample buffer containing a reducing agent (DTT) and boiling for 5 min at 95°C. For the conservation of disulfide bridges, the non-reducing buffer was used.

2.6.3 Coomassie staining

Gels were stained in 0.25% Coomassie Brilliant Blue G250, 45% methanol, 10% acetic acid, destained with 45% ethanol, 10% acetic acid.

2.6.4 Silver staining

For silver staining, protein gels were fixed in 30% methanol and 10% glacial acetic acid for at least 30 minutes, washed with 30% methanol for 10 minutes and then rinsed with ultrapure water for 3 minutes. The gels were stained for 30 minutes in the dark with a staining solution (0,1% $AgNO_3$, 0,15% formaldehyde) and developed until bands were visible with a developing solution (2,5% sodium carbonate, 0,05% formaldehyde). Development was stopped by incubating the gel in 30% methanol and 10% glacial acetic acid.

2.6.5 Western blotting

For immunoblotting experiments, the resolving gel was incubated in transfer buffer (25mM Tris-HCl, pH8, 192Mm glycine and 0,04% SDS) for 15 minutes after SDS-PAGE. The blotting was performed in a Mini Trans-blot apparatus (Bio-Rad) using a PVDF membrane (Bio-Rad) according to the manufacturer's protocol. After transferring, the membrane was

saturated in 100ml of Blocking solution (10mM Tris-HCl pH 8, 150mM NaCl, 0,1% Tween20 and 5% not fat dry milk) at room temperature, on an orbital shaker, for 2h. The membrane was then washed twice in Washing buffer (10mM Tris-HCl pH 8, 150mM NaCl and 0,2% Tween20) and incubated overnight with specific primary antibodies from rabbit. In particular, a polyclonal antibody anti-His (His-probe,G-18, sc-804, Santa Cruz Technologies) for the recombinant proteins and a monoclonal anti-Flag antibody (Sigma Aldrich) for the transgenic plants were used. Removed from the incubation buffer, the membrane was washed extensively and incubated with the secondary antibody (goat anti rabbit horseradish peroxidase conjugated) at room temperature, for 1 hour. The antigen-antibody complex was detected using the "Western blotting Luminol reagent" kit (Santa Cruz Technologies) and following the manufacturer's procedure.

2.6.7 Mass spectrometry

The purified TdPMEI proteins, analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue. were excised from the gel and send to the UC Davis Proteomics Core (University of Davis, California) or to Pasteur-Genopole® Ile-de-France / Plate-forme de Proteomique (Institut Pasteur, Paris), where they were digested with trypsin and analysed by LCMS/MS.
Protein identification was performed by using the Mascot search engine based scoring system.

2.6.8 Enzymatic assays

PME activity was quantified by the radial gel diffusion assay as described by Downie *et al*. (1998) with some modifications. A gel was prepared with 0,1% (w/v) of pectin from apple (70-75% esterified) (Sigma-Aldrich), 1% agarose (w/v), 25mM citric acid and 115mM Na_2HPO_4, pH 6.3. The gel was cast into agar plates (15ml per plate) and allowed to polymerize at room temperature. Wells with a diameter of 6mm were made and the protein samples (60µl) were loaded in each well. Plates were incubated at 30°C for 16 h. The gel were stained with 0,02% (w/v) ruthenium red (Sigma) for 45 min, de-stained with water and the diameter of the red-stained zones, resulting from the hydrolysis of esterified pectin in the gel, was measured and compared.
PME activity was determined in protein extracts from wheat tissues carefully chosen at precise stage of development.
For the inhibition assay, TdPMEI purified proteins (0,5µg and 1µg) were combined with crude protein extract obtained from wheat tissues or with 50ng of PME from orange peel (OpPME) or with microbial PME.

The microbial PME used were kindly provided by Prof. Daniela Bellincampi, University of "La Sapienza, Roma, and by Prof. Francesco Favaron, Institute of Plant Pathology, University of Padova.

Inhibition assay of invertase activity was performed following the dinitrosalicylicacid (DNS) assay described by Brutus *et al*, (2004). First the invertase activity was determined as follows: the pure enzyme (20 µl) was mixed with 50µl of sucrose (0.5M) in 50mM phosphate buffer at pH 4.6 (total reaction volume: 200µl) at 46°C for 15 min. The reaction was ended by adding 300µl DNS reagent and boiled for 5 min. The reaction products were cooled and centrifuged for 5 min at 12 000g, and 200µl were transferred onto a microtitre plate. One unit of invertase activity was defined as the amount of protein releasing 1µmol of reducing sugar.min-1. Inhibition was assayed in the presence of 100 mM sucrose after pre-incubating fixed amounts of invertase for 15 min at pH 4.9 at a temperature of 37°C with 0,5µg and 1µg of purified TdPMEI inhibitors

2.7 Infection of plants

2.7.1 Fungal cultures and plant inoculation

Bipolaris sorokiniana (strain 62608) was cultured on potato dextrose agar (PDA; Fluka) (Fig. 2.5). Macroconidia were harvested by gently scraped the culture surface with 3ml of sterile water, and conidia concentration was estimated by a Thoma chamber, adjusting the concentration to 3 x 10^5 conidia/ml for *B. sorokiniana*.

For plant inoculation with *B. sorokiniana* a final concentration of 6000 conidia in 20µl was respectively used. Tween 20 was added to a final concentration of 0,5%.

2.7.2 Leaf infection with *Bipolaris sorokiniana*

The upper surface of the first leaf of wheat plants in the second leaf-emerged stage (Zadoks stage 12) was inoculated with 20µl of conidia suspension of the 62608 strain of *B. sorokiniana*. Plots were covered with plastic film and sprayed with water for the duration of the experiment in order to maintain a high relative humidity. Leaf samples were collected 6h, 12h, 24h, 48h and 72h after the infection to be further analyzed.

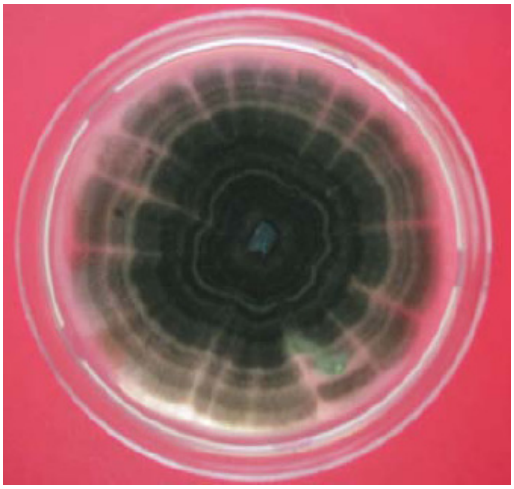


Fig. 2.5 Plate with *Bipolaris sorokiniana*

2.8 Plants growth

Wheat seeds were surface sterilized by immersion in sodium hypochlorite (0,5% v/v) for 15 min and then rinsed thoroughly in sterile water and kept in the dark on filter paper at 4°C for 4 days to ensure synchronous germination. Plants were, then, grown in a climatic chamber with a 16-h light period at 18 to 23°C and fertilized monthly with a 21.7.14 fertilizer until maturation.

Once the Zadoks stage 92 was reached, seeds were trashed by hand and stored at 4°C.

Results

3.1 Identification and characterization of *pmei* genes in durum wheat

3.1.1 *In silico* identification of putative wheat *pmei* sequences

In order to identify wheat *pmei* sequence, the International Nucleotide Sequence Database (http://www.ncbi.nlm.nih.gov/) and The Gene Index (TGI) database (http://compbio.dfci.harvard.edu/tgi/) have been investigated (October 2008) by performing a keyword search. The search on the NCBI databank identified a sequence encoding for a putative *Triticum aestivum* pectin methylesterase inhibitor (accession number FJ006730) and that on the TGI database identified a tentative contig TC344011 (composed by the three overlapping ESTs BE429894, BE426506 and BG905980) annotated as Invertase/pectin methylesterase inhibitor-like.

Sequence similarity searches using the NCBI BLAST (http://www.ncbi.nlm.nih.gov/) with FJ006730 and TC344011 as query did not identify any additional putative wheat *pmei* sequence.

Both FJ006730 and TC344011 were then used to develop specific primers to isolate by PCR the corresponding sequences from genomic DNA of durum wheat (*Triticum durum* Desf.) cv Svevo.

The specific primers developed from the 5' and 3' terminal ends of the FJ006730 sequence, TapmeiFor/TapmeiRev, allowed the isolation of two different sequences named *Tdpmei2.1* and *Tdpmei2.2* (Fig. 3.1).

The complete coding region of *Tdpmei2.1* is 632 bp and contains a 62 bp intron. The *Tdpmei2.2* sequence is 668 bp and is interrupted by a 98 bp intron. Both nucleotide sequences showed a GT-AT intron splice site located after nucleotide 344. Removal of the intron from each sequence, generated two sequence with similar size (570 bp) that shared 97% identity at nucleotide level and 94% identity at amino acid level.

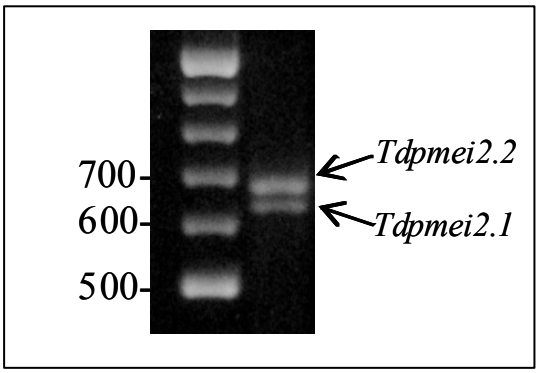


Fig. 3.1 1.5% Agarose gel of the amplification products from genomic DNA of durum wheat cv Svevo. PCR products was obtained using specific primers for FJ006730 sequence which produce two amplification products corresponding to *Tdpmei2.1* and *Tdpmei2.2* sequences.

Sequence comparison between the coding region of *Tdpmei2.1* and *Tdpmei2.2* with FJ006730 showed 92% and 94% identity at the nucleotide level, respectively, and 89% sequence identity at protein level.

SignaIP program (http://www.cbs.dtu.dk/services/SignalP/) predicted that both deduced protein of *Tdpmei2.1* and *Tdpmei2.2* contain three putative cleavage sites for subcellular targeting with the most likely one between position 25 and 26. By using the PSort program (http://psort.hgc.jp) both proteins were predicted to have an apoplastic targeting (Fig.3.2). Removal of the predicted N-terminal signal peptides generates mature TdPMEI2.1 and TdPMEI2.2 proteins of 164 amino acids with a molecular mass of 17492 Da and 17488 Da and predicted isoelectric point of 4.19 and 4.33, respectively (Fig. 3.2). TdPMEI2.1 and TdPMEI2.2 have three potential N-glycosilation sites (predicted with NetNGlyc Server (http://www.cbs.dtu.dk/services/NetNGlyc/) located at position 57, 75 and 153 (Fig. 3.2).

```
TdPMEI2.1    MASFYAAIGFILPFLLTIALPQSTGSPAATPSTTGSLSIEDACKQTAKLYDLCTATLSPD  -60
TdPMEI2.2    MASFYAAIGFILPFLLTIALPQSTGSPPAAPSTTGSLSIEDACKQTTKLYDLCMATLSPD

TdPMEI2.1    RSSLTADAVGLTRAAVLAVQKNASETATYLSNIDEDDNFNKTAQLQQCLEDCGERYEAAV -120
TdPMEI2.2    RSSLTADAVGLTRAAILAVQKNASETATYLANIDEDDNFNKTAQLQQCLEGCGERYEAAV

TdPMEI2.1    EQLADAAIALDMGAYDESQVLVSAGQAEVRLCQKGCQDLPEHRSILMARNTEVDQLCNIT -180
TdPMEI2.2    EQLADATIALDMGAYDESQVLVSAGQAEVKLCQKGCQDSPEHRSIHMARNTEVDQLCNIT

TdPMEI2.1    LAIAKLIPR
TdPMEI2.2    LAIAKLIPR
```

Fig. 3.2 Deduced amino acidic sequences of TdPMEI2.1 and TdPMEI2.2.
Predicted signal peptides are underlined. Amino acid differences are in bold. N-glycosilation sites are boxed.

The TC344011 sequence was also used to develop the specific primers TC34pmeiF/TC34pmeiR and, by using these in PCR reactions on genomic DNA of durum wheat cv Svevo, an additional putative *pmei* sequences was isolated and named *Tdpmei7.3* (Fig. 3.3).

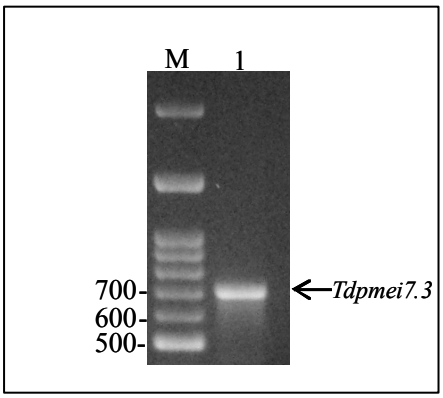


Fig. 3.3 1.5% Agarose gel of the amplification product from genomic DNA of durum wheat cv Svevo. The PCR product was obtained using specific primers TC34pmeiF/TC34pmeiR. M) Marker GeneRuler 100 bp DNA Ladder, 1) Specific PCR amplicon corresponding to *Tdpmei7.3*.

The nucleotide sequence of *Tdpmei7.3* is 681 bp from ATG to TAG and showed only five non-synonymous substitutions with TC344011. The deduced protein of *Tdpmei7.3* contains a signal peptide of 30 residues (predicted by SignaIP program) for targeting to the chloroplast (Fig.3.4). The mature protein is composed of 196 amino acids with a molecular mass of 20233 Da and a predicted p*I*= 6.92. TdPMEI7.3 has two potential N-glycosilation sites at position 44 and 81 (Fig.3.4).

```
MATMAPSSALLPLLLLLPLLLLSVACTAGAAPAATATVPTIPSQPGKPNSNNKQKQGGAP  -60
LGPAVRALVQSTCNATTYYDLCVAALVADPASSTADLRGLCAIAVSAAAANASATASALA -120
NTTWAASGAPETGSDGRAQQVPALLMRTCAGKYGEAREALLEARESVGEEAYDYAFVHVG -180
AAAEYPAVCRTLFRRKRVPYPVELARREQALEHLCTVVIDIITLLA
```

Fig. 3.4 Deduced protein sequence of TdPMEI7.3. Predicted signal peptide is underlined and N-glycosilation sites are box ed.

By searching the the Pfam database (http://pfam.sanger.ac.uk/), we identified a PMEI domain (PF04043) of 147 residues for both TdPMEI2.1 and TdPMEI2.2, and of 154 for TdPMEI7.3 (Fig. 3.5).

A)

B)

C)

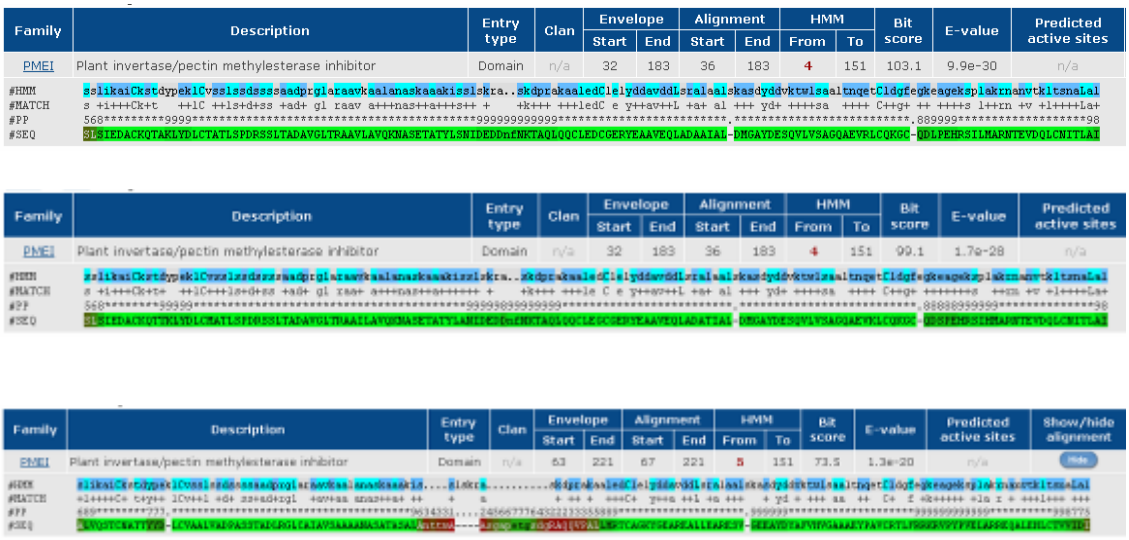


Fig. 3.5 Sequence search results on Pfam database. The three novel PMEI contained a PMEI domain. A) TdPMEI2.1; B) TdPMEI2.2; C)TdPMEI7.3

Sequence comparison between the predicted protein sequences of TdPMEI2.1, TdPMEI2.2 and TdPMEI7.3 with characterized pectin methylesterase inhibitors showed about 20% of sequence similarity (Fig. 3.6). This low level of sequence conservation is a features of most characterized PMEI inhibitors. Nonetheless, all PMEIs, including TdPMEI2.1, TdPMEI2.2 and TdPMEI7.3, share a conserved hydrophobic C-terminal domain and five cysteine residues at conserved position (Fig. 3.7), the first four of which have been showed to be engaged in the disulfide bridges formation.

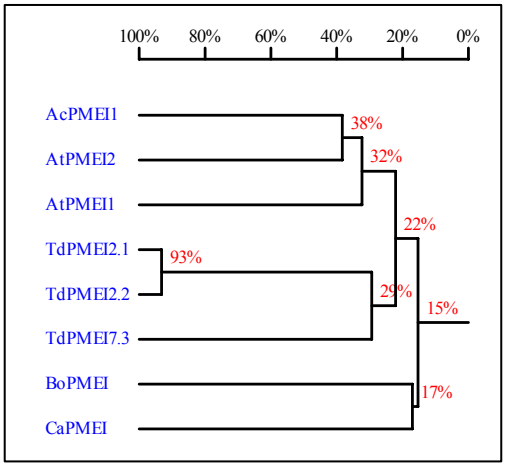


Fig. 3.6 Homology Tree of TdPMEI deduced proteins of characterized plant PMEIs. AcPMEI (*Actinidia chinensis*, P83326); AtPMEI-1 and AtPMEI-2 (*Arabidopsis thaliana*, NP175236 and AtPMEI2), (NP188348), CaPMEI (*Capsicum annum*, AF477956) and BoPMEI (*Brassica oleracea*, AAZ20131).

```
AcPMEI1         ----------------------------------ENHLISEICPKT-RNPSLCLQALESD
AtPMEI1         ----------------------------------AITSSEMSTICDKT-LNPSFCLKFLNTK
AtPMEI2         ----------------------------------QVADIKAICGKA-KNQSFCTSYMKSN
BoPMEI          ----------------------------------SRMMSLC-SHTAYPSLCQPLIKRI
CaPMEI          -----------------NYLKSVSAARPAVGETNTEFIRTSCKSTT-YPNLCFSSLSSR
TdPMEI2.1       ------------------------SPAATPSTTGSLSIEDACKQTAKLYDLCTATLSPD
TdPMEI2.2       ------------------------SPPAAPSTTGSLSIEDACKQTTKLYDLCMATLSPD
TdPMEI7.3       APAATATVPTIPSQPGKPNSNNKQKQGGAPLGPAVRALVQSTCNATT-YYDLCVAALVAD
                                                          .   *        *   .

AcPMEI1         PRSASKDLKGLGQFSIDIAQASAKQTSKIIASLTNQATDPKLKG-----------RYETC
AtPMEI1         FASPNLQALAKTTLDST--QARATQTLKKLQSIIDGGVDPRSKLAY-----------RSC
AtPMEI2         PKTSGADLQTLANITFGSAQTSASEGFRKIQSLVKTATNPTMKKA-----------YTSC
BoPMEI          TNPRRATHKTIQALEAKTKLALADAARYKNGN----------------------QAIATC
CaPMEI          ASAIGASPQLLAHESLTVSLETAQSTSSMMLKLAHGQGMTPRE----------IGAMHDC
TdPMEI2.1       RSSLTADAVGLTRAAVLAVQKNASETATYLSNIDEDDNFNKTAQL--------QQCLEDC
TdPMEI2.2       RSSLTADAVGLTRAAILAVQKNASETATYLANIDEDDNFNKTAQL--------QQCLEGC
TdPMEI7.3       PASSTADLRGLCAIAVSAAAANASATASALANTTWAASGAPETGSDGRAQQVPALLMRTC
                                        *                                 *

AcPMEI1         SENYADAIDSLGQAKQFLTSGDYNSLNIYASAAFD-------GAGTCEDSFEGPPNIPTQ
AtPMEI1         VDEYESAIGNLEEAFEHLASGDGMGMNMKVSAALD-------GADTCLDDVKRLRSVDSS
AtPMEI2         VQHYKSAISSLNDAKQSLASGDGKGLNIKVSAAME-------GPSTCEQDMADFKVDPSA
BoPMEI          YAVFSDAVYNLANARKSIRKRDVMAMNTFLTAAV-------SDYGVCVEGFIDANQVNTV
CaPMEI          VEELSDTVVGLRKSLGEMKQLRGKDFDLKMNDIQTWVSAALTDEDTCTEGFDGKVMNGKV
TdPMEI2.1       GERYEAAVEQLADAAIALDMGAYDESQVLVSAGQ-------AEVRLCQKGCQDLPEHRSI
TdPMEI2.2       GERYEAAVEQLADATIALDMGAYDESQVLVSAGQ-------AEVKLCQKGCQDSPEHRSI
TdPMEI7.3       AGKYGEAREALLEARESVGEEAYDYAFVHVGAAA-------EYPAVCRTLFRRKRVPYPV
                      .   *  .   .                                 *

AcPMEI1         LHQADLKLED--LCDIVLVISNLLPGSK--
AtPMEI1         VVNN-SKTIKN-LCGIALVISNMLPRN---
AtPMEI2         VKNS--GDFQN-ICGIVLVISNMM------
BoPMEI          QNVAVDLRKISSNCLTLSTLVR--------
CaPMEI          KTVVRGKILEVAHLTSNALALINRLAALHG
TdPMEI2.1       LMARNTEVDQ--LCNITLAIAKLIPR----
TdPMEI2.2       HMARNTEVDQ--LCNITLAIAKLIPR----
TdPMEI7.3       ELARREQALEH-LCTVVIDIITLLA-----
```

Fig. 3.7 Multiple amino acid sequence alignment between the mature protein of the three novel TdPMEIs and all characterized plant PMEIs. AcPMEI (*Actinidia chinensis*, P83326); AtPMEI-1 and AtPMEI-2 (*Arabidopsis thaliana*, NP175236 and NP188348), CaPMEI (*Capsicum annum*, AF477956) and BoPMEI (*Brassica oleracea*, AAZ20131). Conserved Cys residues are in yellow; Other conserved residues are in green and grey. Hydrophobic C-terminal domain is highlighted in light-blue.

3.1.2 Heterologous expression, purification and activity of recombinant TdPMEI in *Pichia pastoris* and *Escherichia coli*

In order to verify if the three different wheat *pmei* sequences, *Tdpmei2.1*, *Tdpmei2.2* and *Tdpmei7.3*, code for functional pectin methylesterase inhibitors, the regions encoding the predicted mature proteins were cloned into vectors suitable for the heterologous expression in *Pichia pastoris* and *Escherichia coli*. The cloning of *Tdpmei2.1* and *Tdpmei2.2* into the vectors for heterologous expression required the use of SOE-PCR to remove the intron contained into the coding region of each gene.

For the heterologous expression in *P. pastoris*, the region of *Tdpmei2.1* and *Tdpmei2.2* coding for predicted mature proteins, was inserted into the *Eco*RI site of the pPICZαA® vector under the control of methanol-inducible AOX1 promoter (Fig. 3.8), whereas the insertion of the coding region of *Tdpmei7.3* into pPICZαA® has been realized using *Xho*I and *Xba*I sites (Fig. 3.9).

pPICZαA® vector harbours the sequence for signal peptide of *Saccharomyces cerevisiae* α-actor that allows the secretion of recombinant protein.

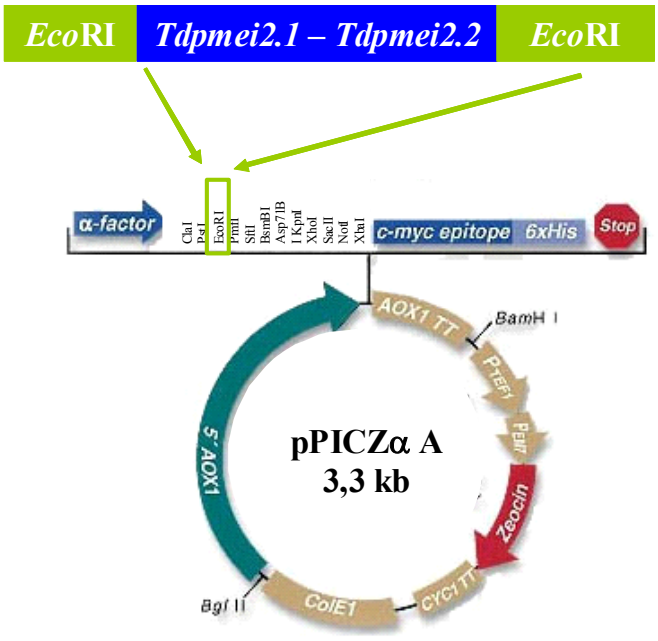


Fig. 3.8 Constructs pPIZαA::*Tdpmei2.1* and pPIZαA::*Tdpmei2.2* used for heterologous expression in *P. pastoris*.

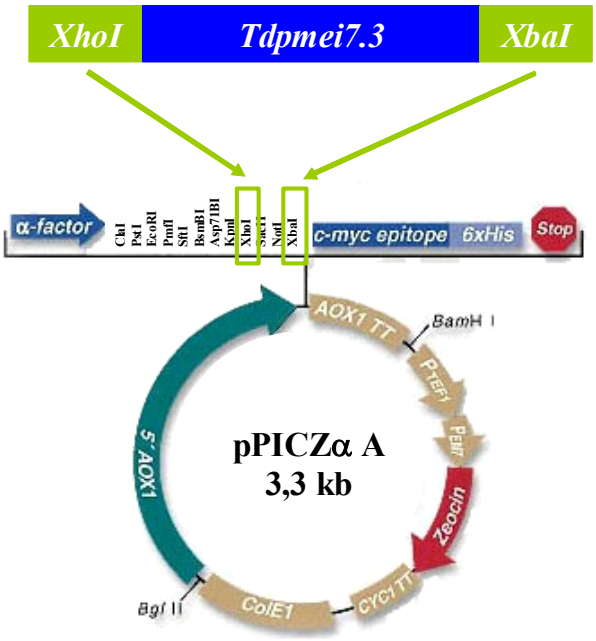


Fig. 3.9 Construct pPIZαA::*Tdpmei7.3*used for heterologous expression in *P. pastoris*.

After yeast transformation and two-step selection, ten multi-copy recombinant colonies were selected and subjected to methanol induction. The secretion and accumulation of the recombinant proteins was verified by SDS-PAGE analysis. Only the supernatant of recombinant colonies harbouring the *Tdpmei2.1* accumulated polypeptides that were absent in the supernatant of *P. pastoris* colonies transformed with the empty vector. The *Tdpmei2.1* colonies showed two major protein bands with a molecular mass of about 25 kDa and 30 kDa and two protein bands that accumulate at very lower level of about 20 kDa and 35 kDa (Fig. 3.10). Time-course induction showed that these polypeptides accumulated at higher level after four days of induction (data not shown). The recombinant colony that accumulated at higher level these polypeptides was used in subsequent analyses.

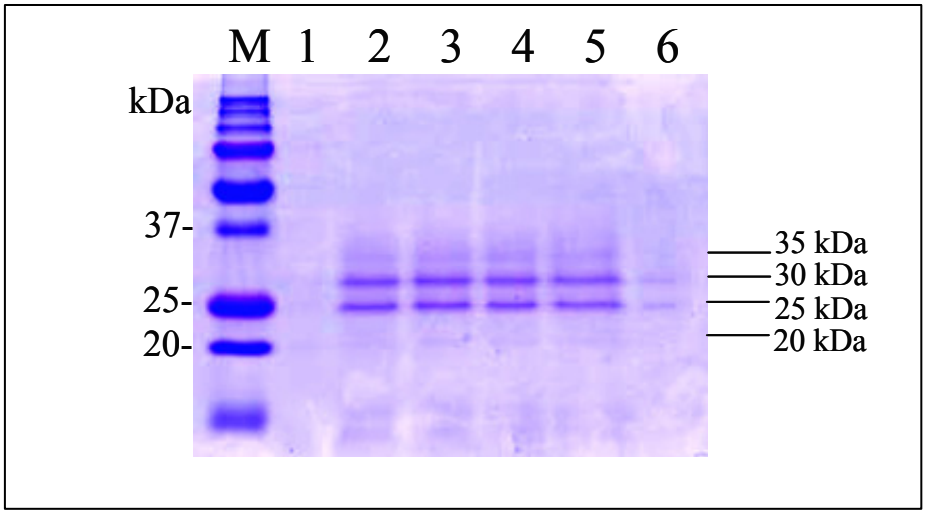


Fig. 3.10 SDS-PAGE analysis of recombinant colonies producing TdPMEI2.1 Two main protein bands were detected and have a molecular mass of about 25kDa and 30kDa. Two additional fainter protein bands of 35kDa and 20kDa were also identified. M) Molecular mass marker; 1) Supernatant of *P. pastoris* transformed with empty vector, 2-6) Supernatant of some recombinant colonies harbouring pPIZαA::*Tdpmei2.1*.

In order to confirm the protein identity, all the four polypeptides visualized on SDS-PAGE were recovered and subjected to trypsin digestion and analysed by mass spectrometry (MS). This analysis confirmed that all the four polypeptides corresponded to the predicted TdPMEI2.1. This result indicates also that the four polypeptides observed on SDS-PAGE are probably due to a different level of glycosilation of TdPMEI2.1.

In order to determine whether TdPMEI2.1 is a functional pectin methylesterase inhibitor, we tested its ability to inhibit the PME activity of crude protein extract from wheat leaf by radial diffusion assays. Different aliquots of TdPMEI2.1 culture medium were combined with 5µg and 10µg of crude protein extract from wheat leaf or wheat seedlings but none reduction of endogenous PME activity was observed (Fig. 3.11). Different aliquots of TdPMEI2.1 culture medium were also tested against a commercial PME from orange peel (OpPME) but similarly to what observed with the wheat protein extract, no reduction of OpPME activity was observed (data not shown).

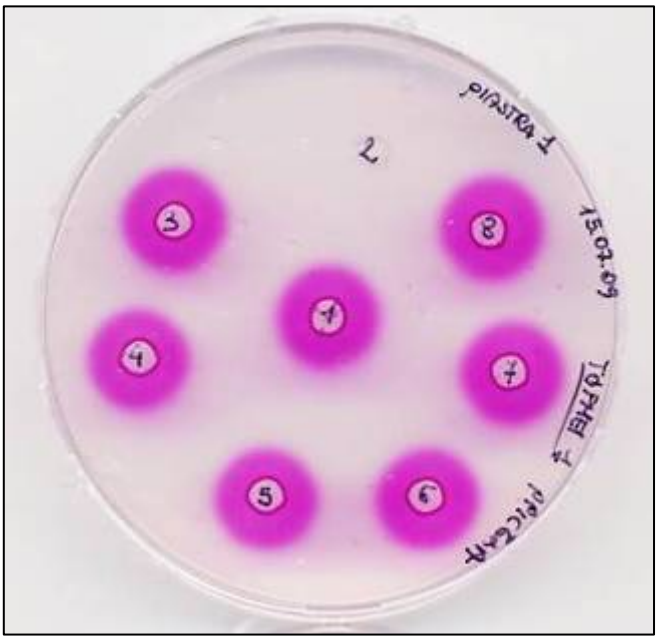


Fig. 3.11 The supernatant of recombinant colonies was combined with 5ug of crude protein extract obtained from wheat leaf. PME activity of *P. pastoris* was also tested, but it was not found.
Samples: 1) Crude protein extract from wheat leaves (10µg), 2) Supernatant of *P. pastoris* transformed with empty vector, 3) *P. pastoris* with empty vector plus 10µg of leaves protein extract, 4-8) Supernatant of recombinant colonies plus 10µg of leaves protein extract.

In order to test higher amount of the inhibitor, the TdPMEI2.1 produced in *P. pastoris* was purified by cation exchange chromatography using a DEAE sepharose column. The eluted fractions were analysed by SDS-PAGE and those containing the recombinant protein (2µg) were used in cup plate assays against the PME activity of crude wheat protein extract or OpPME. As previously observed none inhibition activity was detected (Fig.3.12).

To exclude that the absence of inhibitory activity was due to the failure of the recombinant TdPMEI2.1 to form disulfide bridges, SDS-PAGE performed in reducing and not reducing conditions were performed. As shown in Figure 3.13, a shift of molecular mass of the non-reduced proteins with respect to reduced form was observed, indicating that the cysteine residues of the TdPMEI2.1 recombinant protein have the capacity to form disulfide bridges.

Since PMEI are very similar to invertase inhibitors, we tested TdPMEI2.1 against the tomato invertase using dinitrosalycilicacid (DNS) assay. The result showed that TdPMEI2.1 does not have invertase inhibitor activity (data not shown).

Based on the above results, we hypothesized that the excess of glycosilation of the etherologous proteins could prevent its ability to inhibit the PME activity.

Fig. 3.12 Gel diffusion assay to verify the inhibition capability of purified TdPMEI2.1 against wheat endogenous PME activity. 1) Crude wheat leaf protein extract (10µg), 2-8) Eluted fractions containing TdPMEI2.1 plus 10µg of crude leaf protein extract.

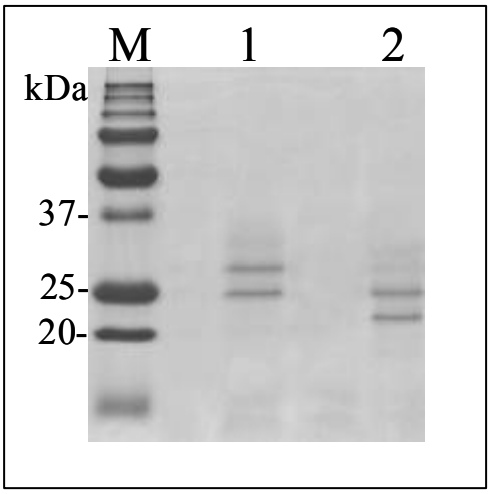


Fig. 3.13 SDS-PAGE analysis of TdPMEI2.1 in reducing and not reducing condition. M) Molecular mass marker; 1) TdPMEI2.1 in reducing conditions; 2) TdPMEI2.1 in non-reducing conditions.

Parallel experiments were performed to verify if the signal peptide predicted by bioinformatics analysis corresponded to the correct one. To this aim, the complete coding region of *Tdpmei2.1* including its signal peptide was inserted into *Eco*RI and *Xba*I sites of pPICZ B® vector. Since bioinformatics analysis revealed that the signal peptide of TdPMEI2.1 targets the mature protein into the extracellular environment, we hypothesized that *P. pastoris* could secrete the recombinant protein under its own signal peptide into the culture medium. After transformation of *P. pastoris* X-33 strain with pPICZB::*Tdpmei2.1*, some recombinant colonies were selected and induced by methanol. SDS-PAGE analysis showed the presence of two protein bands (35 kDa and 30 kDa) in the supernatant of recombinant colonies and not in supernatant of *P. pastoris* transformed with the empty vector (Fig. 3.14). Both protein bands were recovered, digested with trypsin and analyzed by mass spectrometry. The results confirmed the correspondence to TdPMEI2.1 of both protein bands. As suggested above, probably the different molecular mass are due to different level of glycosilation. This mass spectrometry data result did not validate the signal peptide prediction because the analysis showed peptides that included nine additional residues in the N-terminal portion of the protein that based on the signal peptide prediction should be removed during secretion (Fig. 3.15).

We used also this TdPMEI2.1 heterologous proteins in cup plate assays against the PME activity of crude wheat protein extract and OpPME but similarly to what observed previously no inhibition activity was observed (data not shown).

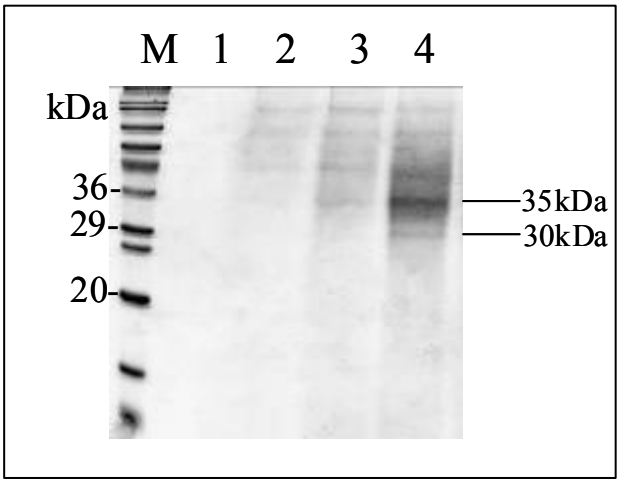


Fig. 3.14 SDS-PAGE analysis of the supernatant of colonies expressing TdPMEI2.1 with its signal peptide. M) Molecular mass marker; 1) Supernatant of *P. pastoris* transformed with empty vector; 2-4) Supernatant of some recombinant colonies harbouring *Tdpmei2.1*.

```
TdPMEI2.1       MASFYAAIGFILPFLLTIALPQSTGSPAATPSTTGSLSIEDACKQTAKLYDLCTATLSPD
Peptide 1       ----------------TIALPQSTGSPAATPSTTGSLSIEDACK----------------
Peptide 2       --------------------QSTGSPAATPSTTGSLSIEDACK----------------
Peptide 3       ----------------------TGSPAATPSTTGSLSIEDACK----------------
Peptide 4       ------------------------------TPSTTGSLSIEDACK----------------
Peptide 5       --------------------------------STTGSLSIEDACK----------------
Peptide 6       -------------------------------------------------LYDLCTATLSPD
Peptide 7       ------------------------------------------------------------
Peptide 8       ------------------------------------------------------------
Peptide 9       ------------------------------------------------------------
Peptide 10      ------------------------------------------------------------
Peptide 11      ------------------------------------------------------------
Peptide 12      ------------------------------------------------------------
Peptide 13      ------------------------------------------------------------
Peptide 14      ------------------------------------------------------------
Peptide 15      ------------------------------------------------------------

TdPMEI2.1       RSSLTADAVGLTRAAVLAVQKNASETATYLSNIDEDDNFNKTAQLQQCLEDCGERYEAAV
Peptide 1       ------------------------------------------------------------
Peptide 2       ------------------------------------------------------------
Peptide 3       ------------------------------------------------------------
Peptide 4       ------------------------------------------------------------
Peptide 5       ------------------------------------------------------------
Peptide 6       R-----------------------------------------------------------
Peptide 7       -SSLTADAVGLTR------------------------------------------------
Peptide 8       -------------AAVLAVQK----------------------------------------
Peptide 9       ----------------------NASETATYLSNIDEDDNFNK--------------------
Peptide 10      ----------------------NASETATYLSNIDEDDNFNKTAQLQQCLEDCGER-----
Peptide 11      -------------------------------------------TAQLQQCLEDCGER-----
Peptide 12      ------------------------------------------------------YEAAV
Peptide 13      ------------------------------------------------------------
Peptide 14      ------------------------------------------------------------
Peptide 15      ------------------------------------------------------------

TdPMEI2.1       EQLADAAIALDMGAYDESQVLVSAGQAEVRLCQKGCQDLPEHRSILMARNTEVDQLCNIT
Peptide 1       ------------------------------------------------------------
Peptide 2       ------------------------------------------------------------
Peptide 3       ------------------------------------------------------------
Peptide 4       ------------------------------------------------------------
Peptide 5       ------------------------------------------------------------
Peptide 6       ------------------------------------------------------------
Peptide 7       ------------------------------------------------------------
Peptide 8       ------------------------------------------------------------
Peptide 9       ------------------------------------------------------------
Peptide 10      ------------------------------------------------------------
Peptide 11      ------------------------------------------------------------
Peptide 12      EQLADAAIALDMGAYDESQVLVSAGQAEVR------------------------------
Peptide 13      -----AAIALDMGAYDESQVLVSAGQAEVR------------------------------
Peptide 14      ------------------------------------GCQDLPEHR---------------
Peptide 15      ------------------------------------------------NTEVDQLCNIT


TdPMEI2.1       LAIAKLIPR
Peptide 1       ---------
Peptide 2       ---------
Peptide 3       ---------
Peptide 4       ---------
Peptide 5       ---------
Peptide 6       ---------
Peptide 7       ---------
Peptide 8       ---------
Peptide 9       ---------
Peptide 10      ---------
Peptide 11      ---------
Peptide 12      ---------
Peptide 13      ---------
Peptide 14      ---------
Peptide 15      LAIAK----
```

Fig. 3.15 Alignment of TdPMEI2.1 protein sequence and peptide obtained from digestion with trypsin and MS analysis. Predicted signal peptide of TdPMEI2.1 is underlined.

Since the heterologous expression of *Tdpmei2.1*, *Tdpmei2.2* and *Tdpmei7.3* in *P. pastoris* did not produced active proteins, we decided to express these gene in *E. coli*. The regions encoding the predicted mature proteins were inserted into the pOPIN F vector (Fig. 3.16) using the In-Fusion™ Advantage Cloning method. This vector allows the production of recombinant protein as fusion to a N-terminal polyhistidine tag (6x His). Vectors harbouring each of the three wheat *pmei* genes were used to transform *E. coli* Rosetta-gami (DE3)™ strain. We used this strain because its deficiencies in thioredoxin reductase and glutathione reductase activities provide an oxidizing environment to facilitate disulfide bridges formation. After selection of the recombinant colonies, the expression of each heterologous protein was obtained following induction with 1mM of isopropylthiogalactoside (IPTG). Each heterologous proteins was extracted from the soluble and insoluble fractions and purified using a His-Trap HP column. SDS-PAGE analysis of the eluted fractions of each heterologous proteins showed a single polypeptide with molecular masses of about 28kDa for TdPMEI2.1 and TdPMEI2.2 and about 24kDa for TdPMEI7.3 (Fig. 3.17). These fraction were also subjected to western blot analysis using an anti-His antibody. The result showed a specific immunodecoration signal corresponding to the heterologous expressed polypeptide, which is not present in the non-transformed *E. coli* strain (Fig. 3.18).

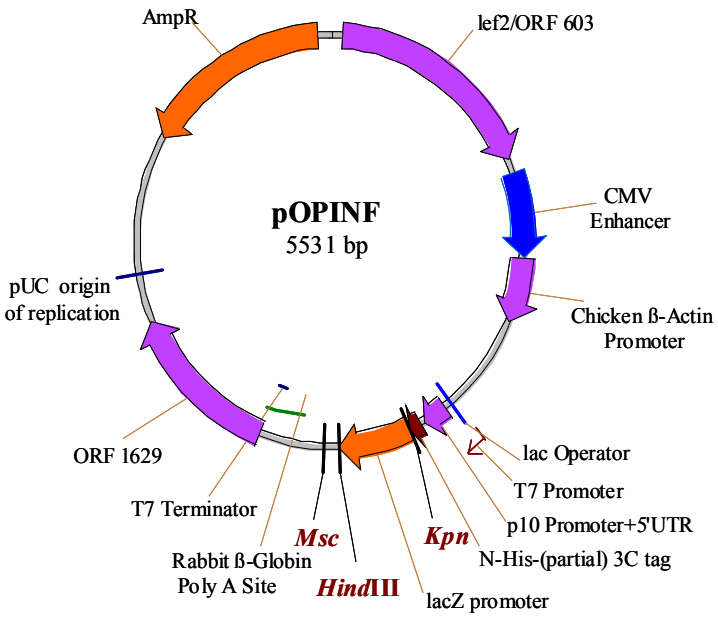


Fig. 3.16 Map of pPOPIN F vector used for heterologous expression in *E. coli*

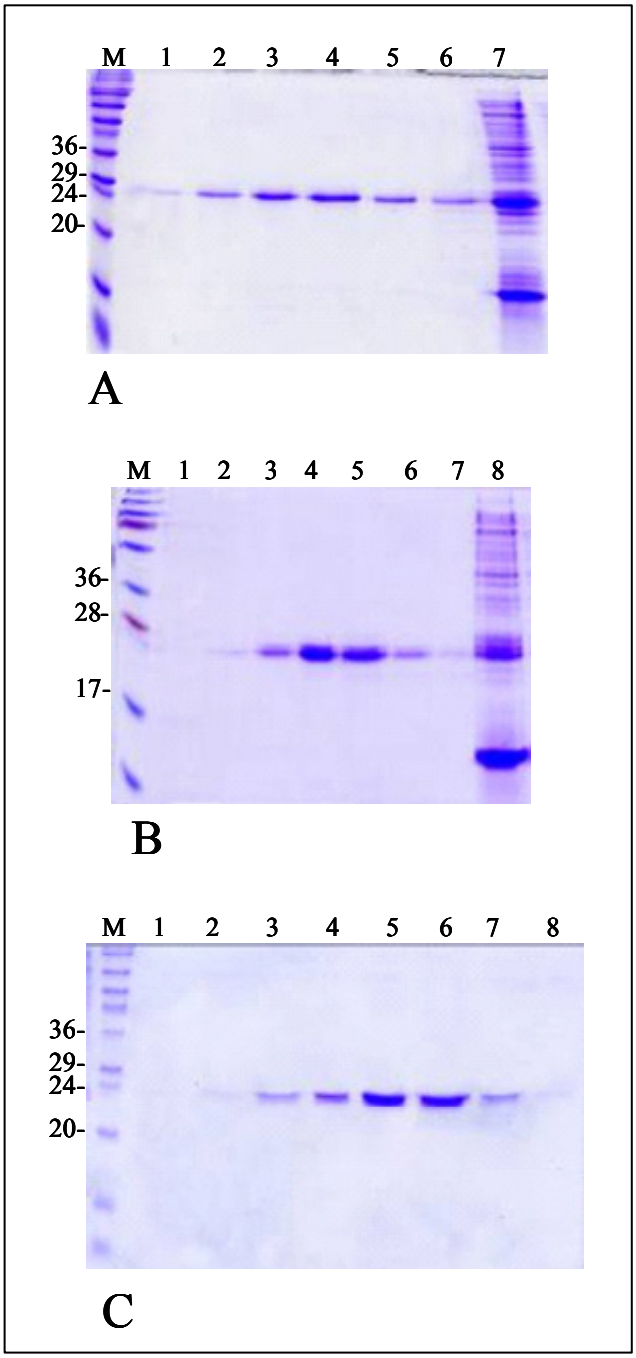


Fig. 3.17 SDS-PAGE analysis of purified TdPMEI proteins produced in *E. coli*.
A) Purified protein from *E. coli* harbouring pOPINF-Tdpmei2.1. M) Molecular mass marker; 1-6) Eluted fraction from His-Trap HP column, 7) Pellet of *E. coli* harbouring pOPIN F-Tdpmei2.1. **B**) Purified protein from *E. coli* harbouring pOPINF-Tdpmei2.2. M) Molecular mass marker; 1-7) Eluted fraction from His-Trap HP column, 7) Pellet of *E. coli* harbouring pOPIN F-Tdpmei2.1. **C**) Purified protein from *E. coli* harbouring pOPINF-Tdpmei7.3. M) Molecular mass marker; 1-8) Eluted fraction from His-Trap HP column.

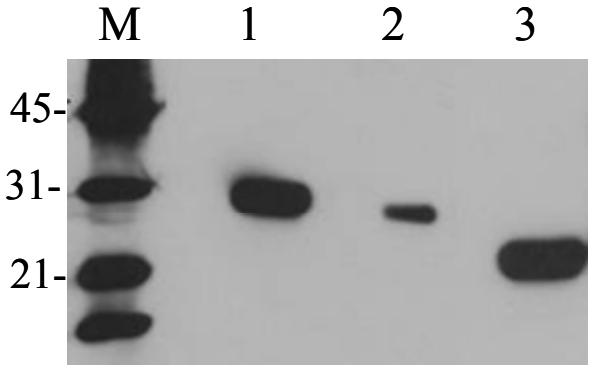


Fig. 3.18 Western blot of purified recombinant proteins performed by using an Anti-His antibody. M) Biotinilated Molecular mass marker. 1) TdPMEI2.2, 2)TdPMEI2.1, 3) TdPMEI7.3

The purified recombinant proteins were analyzed by mass spectrometry (MS). Each purified protein was excided from the gel and subjected to the digestion with trypsin. MS of the tryptic peptides confirmed that all three recombinant proteins corresponded to the predicted TdPMEI2.1, TdPMEI2.2 and TdPMEI7.3.

Before performing PME inhibition assays, purified PMEI proteins were analyzed for the capacity to form intra-molecular disulfide bridges. SDS-PAGE of the purified proteins performed in reducing and not reducing conditions showed different mobility, with the non-reduced samples showing an increased mobility (Fig. 3.19). This result indicate that recombinant proteins are able to form intramolecular disulfide bridges.

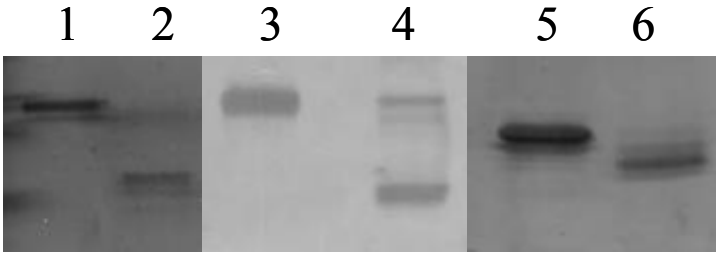


Fig. 3.19 SDS-PAGE analysis of purified TdPMEI recombinant proteins in reducing and not reducing conditions. 1) TdPMEI2.1 reduced, 2) TdPMEI2.1 non reduced, 3) TdPMEI2.2 reduced, 4)TdPMEI2.2 not reduced, 5) TdPMEI7.3 reduced, 6) TdPMEI7.3 not reduced

Since PMEI are very similar to invertase inhibitors, we tested TdPMEI2.1, TdPMEI2.2 and TdPMEI7.3 against the tomato invertase using dinitrosalycilicacid (DNS) assay. The result showed that TdPMEI inhibitors do not have invertase inhibitor activity (data not shown).
In order to determine whether TdPMEI2.1, TdPMEI2.2 and TdPMEI7.3 are functional pectin methylesterase inhibitors, we tested their ability to inhibit OpPME. We performed radial diffusion assays by using 50ng of OpPME combined with 0.5 μg or 1 μg of purified TdPMEIs. We used a larger amount of inhibitor respect to OpPME because we consider the possibility that only a portion of the recombinant protein could reside in the correct folding and consequently could be active. The results of the agarose assays demonstrated that all three TdPMEIs inhibit OsPME (Fig. 3.20 and Fig. 3.21).

These results demonstrated that all three *pmei* genes isolated from durum wheat code for functional pectin methylesterase inhibitors.

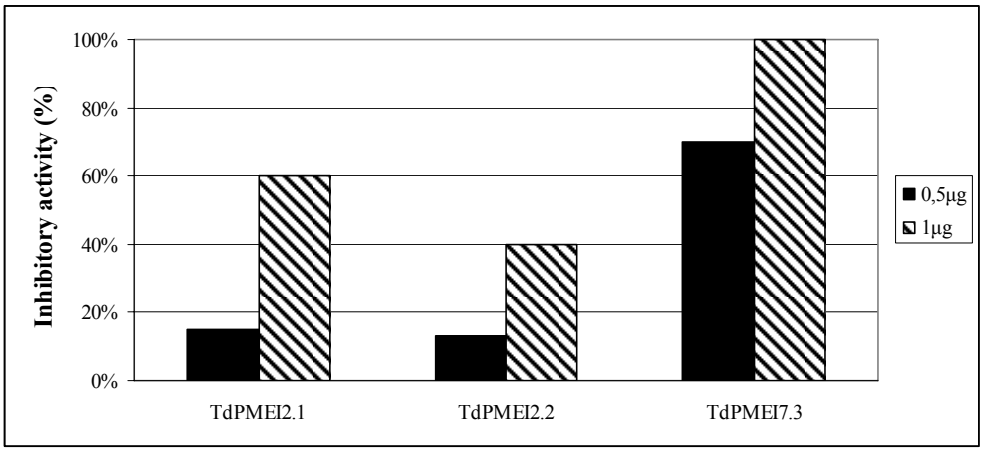


Fig.3.20 Inhibition activity of TdPMEI protein against 50ng of OpPME. Data are based on the halo produced on agarose gel diffusion assay.

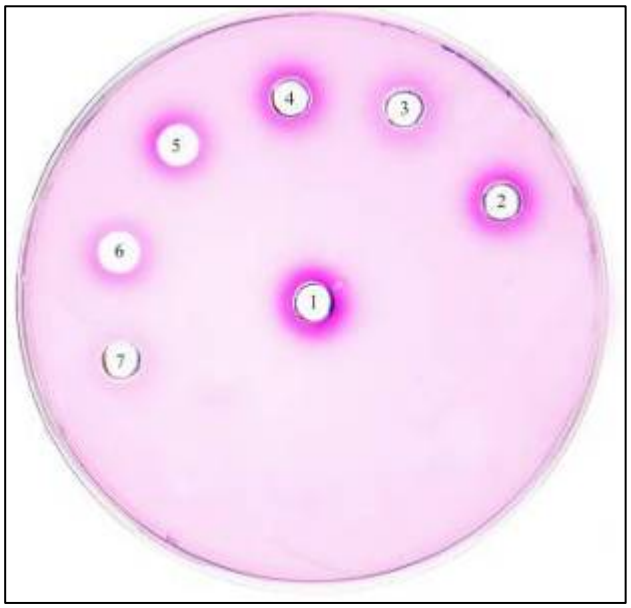
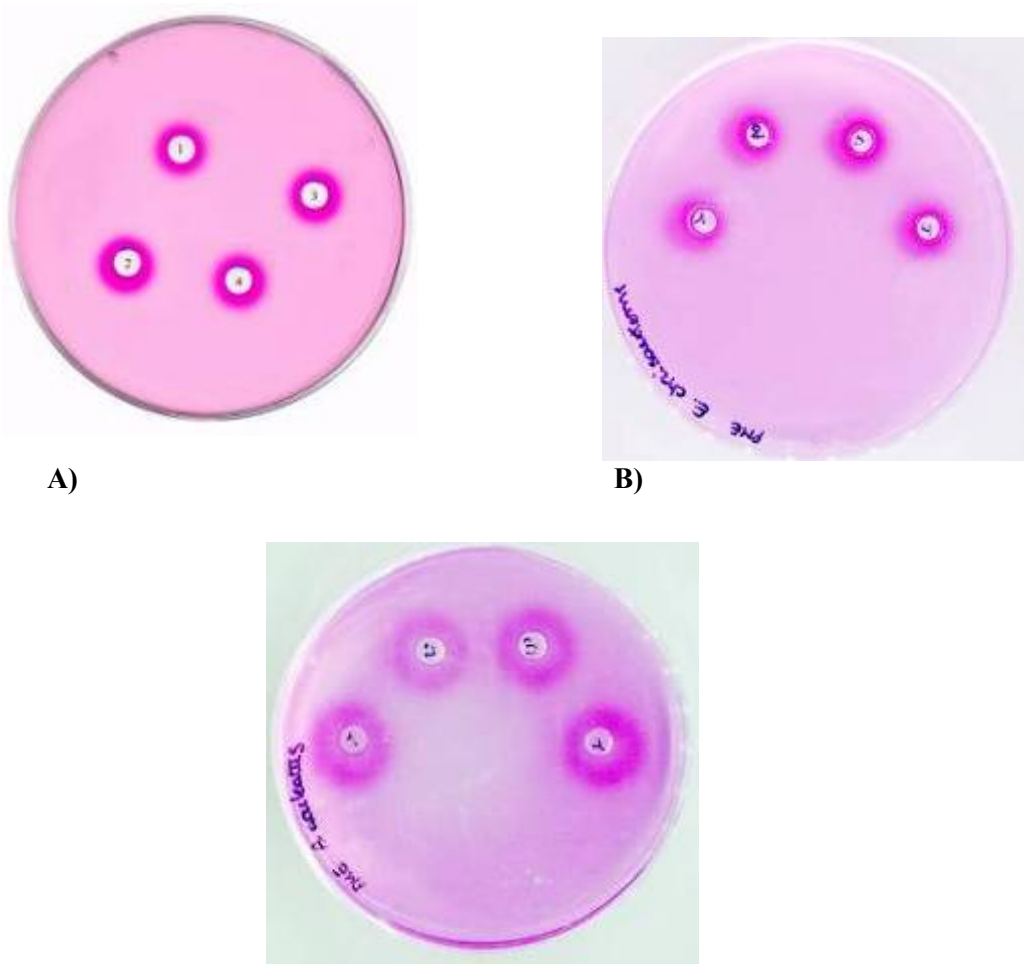


Fig. 3.21 Gel diffusion assay to verify the inhibition capability of recombinant TdPMEI against OpPME.
1) 50ng of OpPME, 2) 50ng of OpPME plus 0.5µg of TdPMEI2.1, 3) 50ng of OpPME plus 1µg of TdPMEI2.1, 4) 50ng of OpPME plus 0.5µg of TdPMEI2.2, 5) 50ng of OpPME plus 1µg of TdPMEI2.2, 6) 50ng of OpPME plus 0.5µg of TdPMEI7.3, 7) 50ng of OpPME plus 1µg of TdPMEI7.3.

3.1.3 TdPMEIs are not active against bacterial and fungal PME

We verified also the ability of TdPMEI proteins to inhibit bacterial and fungal PME activity. We tested their activities against purified PME of *Erwinia chrisantemi*, *Fusarium graminearum* and *Aspergillus aculeatus*. Radial diffusion assays were performed by using the same amount of inhibitors that were effective to inhibit OpPME but none of the microbial PME was inhibited by the TdPMEI (Fig. 3.22).

The results of these analysis and those reported above indicate that TdPMEI2.1, TdPMEI2.2 and TdPMEI7.3 are effective inhibitors of plant PMEs and do not inhibit microbial PMEs.



A) **B)**

C)

Fig. 3.22 Inhibition assay of microbial PME combining with wheat TdPMEI proteins.
A) Samples: 1) *F. graminearum* PME (FgPME), 2) FgPME plus TdPMEI2.1, 3) FgPME plus TdPMEI2.2, 4) FgPME plus TdPMEI7.3
B) Samples: 1) *A. aculeatus* PME (AcPME), 2) AcPME plus TdPMEI2.1, 3) AcPME plus TdPMEI2.2, 4) AcPME plus TdPMEI7.3.
C) Samples: 1) *E. chrisantemi* PME (EcPME), 2) EcPME plus TdPMEI2.1, 3) EcPME plus TdPMEI2.2, 4) EcPME plus TdPMEI7.3.

3.1.4 Genomic organization and chromosomal localization of *Tdpmei2.1, Tdpmei2.2* genes

The genomic organization of *Tdpme2.1* and *Tdpmei2.2* genes has been examined by Southern blot analysis by using the complete coding region of both genes as probe. Two main hybridizing fragments of about 1250 bp and 1200 and a fainter band of about 2100 bp were detected on genomic DNA of *Triticum durum* cv. Svevo and *Triticum aestivum* cv. Chinese Spring digested with *Pst*I (Fig. 3.23).

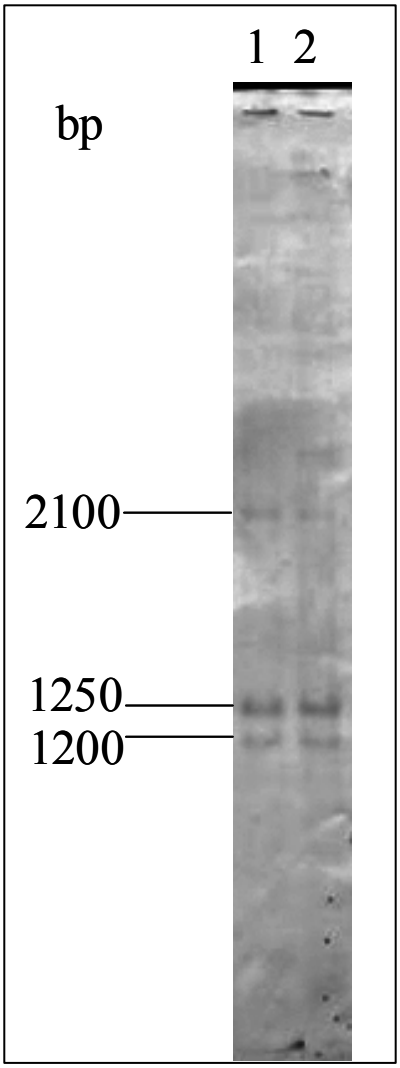


Fig. 3.23 Southern blot of genomic DNA of durum and bread wheat hybridized with *Tdpme2.1* and *Tdpmei2.2*. Genomic DNA (8 µg) was digested with *Pst*I and probed with the complete coding region of *Tdpme2.1* and *Tdpmei2.2* labelled with digoxigenin. 1) *T. durum* cv. Svevo 2) *T. aestivum* cv. Chinese Spring.

In order to identify the chromosomal localization of *Tdpme2.1* and *Tdpmei2.2* genes, we performed PCR assays on genomic DNA of nulli-tetrasomic lines (NT) of *T. aestivum* cv. Chinese Spring (CS). We used the specific primers, TapmeiFor/TapmeiRev, spanning the entire coding region of *Tdpmei2.1* and *Tdpmei2.2*. These primers produced two amplicons of 632bp and 668bp on genomic DNA of CS that correspond in size to those obtained from durum wheat cv. Svevo (Fig. 3.24).

The lack of the amplicon of 632bp on N2BT2A and N2BT2D indicates that this amplicon, corresponding to the *Tdpmei2.1* gene, is localized on chromosome 2B (Fig.3.25). The chromosomal localization of the *Tdpmei2.2* was obtained by developing additional specific primers. Based on sequence comparison between *Tdpmei2.1* and *Tdpmei2*.2, we developed the primer pairs Tdpmei1_254F/Tdpmei1_566R, specific for *Tdpmei2.1*, and Tdpmei2_64F/Turart_391R, specific for *Tdpmei2.2*. By using these primers we confirmed the chromosomal localization of *Tdpmei2.1* on chromosome 2B and we localized *Tdpmei2.2* on chromosome 2A (Fig. 3.26).

The same PCR assays were performed on genomic DNA of ditelosomic lines of *T. aestivum* cv. CS. These analyses assigned *Tdpmei2.1* and *Tdpmei2.2* to the long arm of chromosome 2B and 2A, respectively (Fig. 3.26).

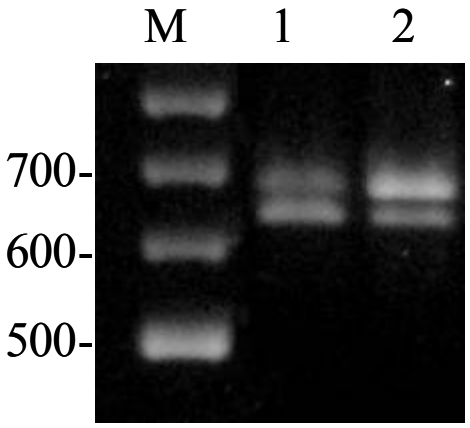


Fig. 3.24 1.5% agarose gel of PCR products obtained with primer pair TapmeiFor/TapmeiRev.
M) Ladder Gene Ruler DNA 100bp 1) *T. durum* cv. Svevo, 2) *T. aestivum* cv Chinese Spring.

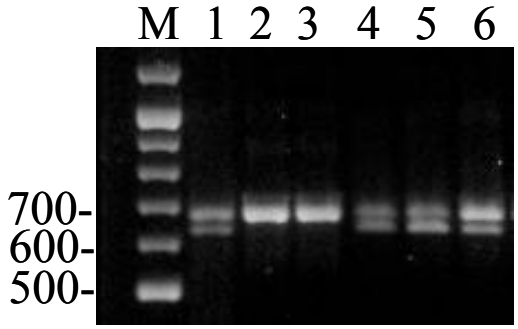


Fig. 3.25 Chromosomal assignment of *Tdpmei2.1*.1.5% agarose gel of PCR products obtained with primer pair TapmeiFor/TapmeiRev. M) Ladder Gene Ruler DNA 100bp 1) N2AT2D 2) N2BT2A 3) N2BT2D 4) N2DT2A 5) N2DT2B 6) Chinese Spring

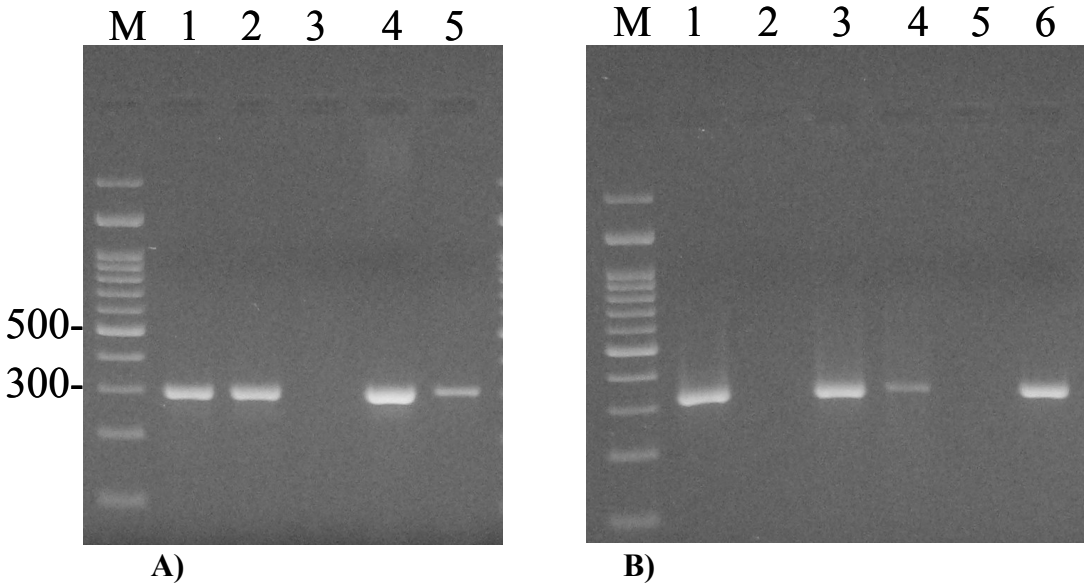


Fig. 3.26 Chromosomal assignment of *Tdpmei2.1.* and *Tdpmei2.1*
A) 1.5% Agarose gel of PCR products obtained with primer pairs Tdpmei1_254F/Tdpmei1_566R, specific for *Tdpmei2.1*. M) Ladder Gene Ruler DNA 100bp, 1) *T. aestivum* cv. Chinese Spring (CS); Nulli-tetrasomic (NT) and ditelosomic (DT) lines of *T. aestivum* cv. Chinese Spring: 2) N2AT2D, 3) N2BT2A, 4) N2DT2A, 5) CS DT2BL; **B)** 1.5% Agarose gel of PCR products obtained with primer pairs Tdpmei2_64F/Turart_391R specific for *Tdpmei2.2*. M) Ladder Gene Ruler DNA 100bp, 1) *T. aestivum* cv. Chinese Spring (CS); Nulli-tetrasomic (NT) and ditelosomic (DT) lines of *T. aestivum* cv. Chinese Spring: 2) N2AT2D, 3) N2BT2A, 4) N2DT2A 5) CS DT2AS, 6) CS DT2AL.

3.1.5 Isolation of *Tdpmei2.1* and *Tdpmei2.2* homeologs in hexaploid wheat and in wild wheat progenitors

In order to isolate wheat *pmei* gene homeologs, we used a strategy based on PCR amplification of genomic DNA of D-genome substitution lines of *T. durum* cv Langdon (LDN). On the basis of the chromosomal assignment described previously, we amplified separately the genomic DNA of LND, LDN 2D(2A) and LDN 2D(2B) by using TapmeiFor/TapmeiRev primers, spanning the complete coding region of *Tdpmei* genes. As expected, the lack of the smaller amplicon of 632 bp confirmed the chromosomal localization of *Tdpmei2.1* on chromosome 2B (Fig. 3.27). Moreover, the presence of the amplicon of 668bp on the PCR sample containing the genomic DNA of LDN 2D(2A) as template, indicated that this amplicon, localized previously on chromosome 2A, originate also from chromosome 2D. To verify the lack of the *Tdpmei2.2* on LDN 2D(2A), we amplified the genomic DNA of this line with the primers Tdpmei2_64F/Turart_391R specific for *Tdpmei2.2* and, as expected, none amplification product was obtained (Fig. 3.28). Consequently, these results indicated that the amplicon of 668 bp obtained from the genomic DNA of CS with the TapmeiFor/TapmeiRev primers is represented by two sequences, one from the genome A and the other from genome D. The amplicon of 668 bp obtained from the genomic DNA of LDN 2D(2A) was purified and subjected to direct nucleotide sequencing (Fig. 3.27). The nucleotide sequence of this amplicon is 668 bp, like that of *Tdpmei2.2*. This novel wheat *pmei* sequence, named *Tapmei2.3*, shared the 85% and 91% nucleotide sequence identity and 93% and 95% amino acid identity with *Tdpmei2.1* and *Tdpmei2.2*, respectively (Fig. 3.29 e Fig. 3.30).

Sequence comparison between *Tapmei2.3* and the other two *pmei* genes (*Tdpmei2.1* and *Tdpmei2.2*) allowed the development of a specific primer pair, Tdpmei2.3_69F/Tdpmei2.3 _393R, that was used to localize the *Tapmei2.3* on the long arm of chromosome 2D (Fig. 3.31).

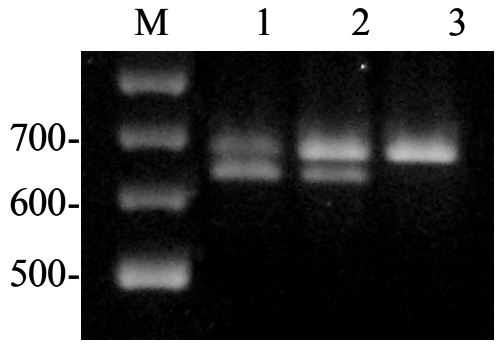


Fig. 3.27 1,5% Agarose gel of PCR products obtained with primer pair TapmeiFor/TapmeRev from D-genome substitution lines of *T. durum* cv. Langdon (LDN): M) Ladder Gene Ruler DNA 100bp, 1) cv.Langdon, 2) LDN 2D(2A), 3) LDN 2D(2B).

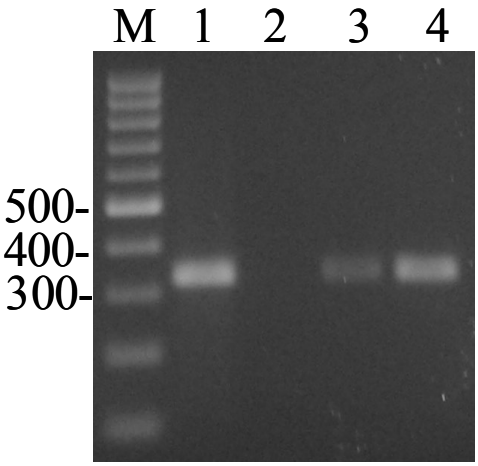


Fig. 3.28 1,5% Agarose gel of PCR products obtained with primer pair Tdpmei2_64F/Turart_391R. from D-genome substitution lines of *T. durum* cv. Langdon (LDN) and nulli tetrasomic lines (NT) of T. *aestivum* cv Chinese Spring (CS): M) Ladder Gene Ruler DNA 100bp, 1)Landgon (LDN), 2) LDN 2D(2A), 3) N2DT2A 4) CS.

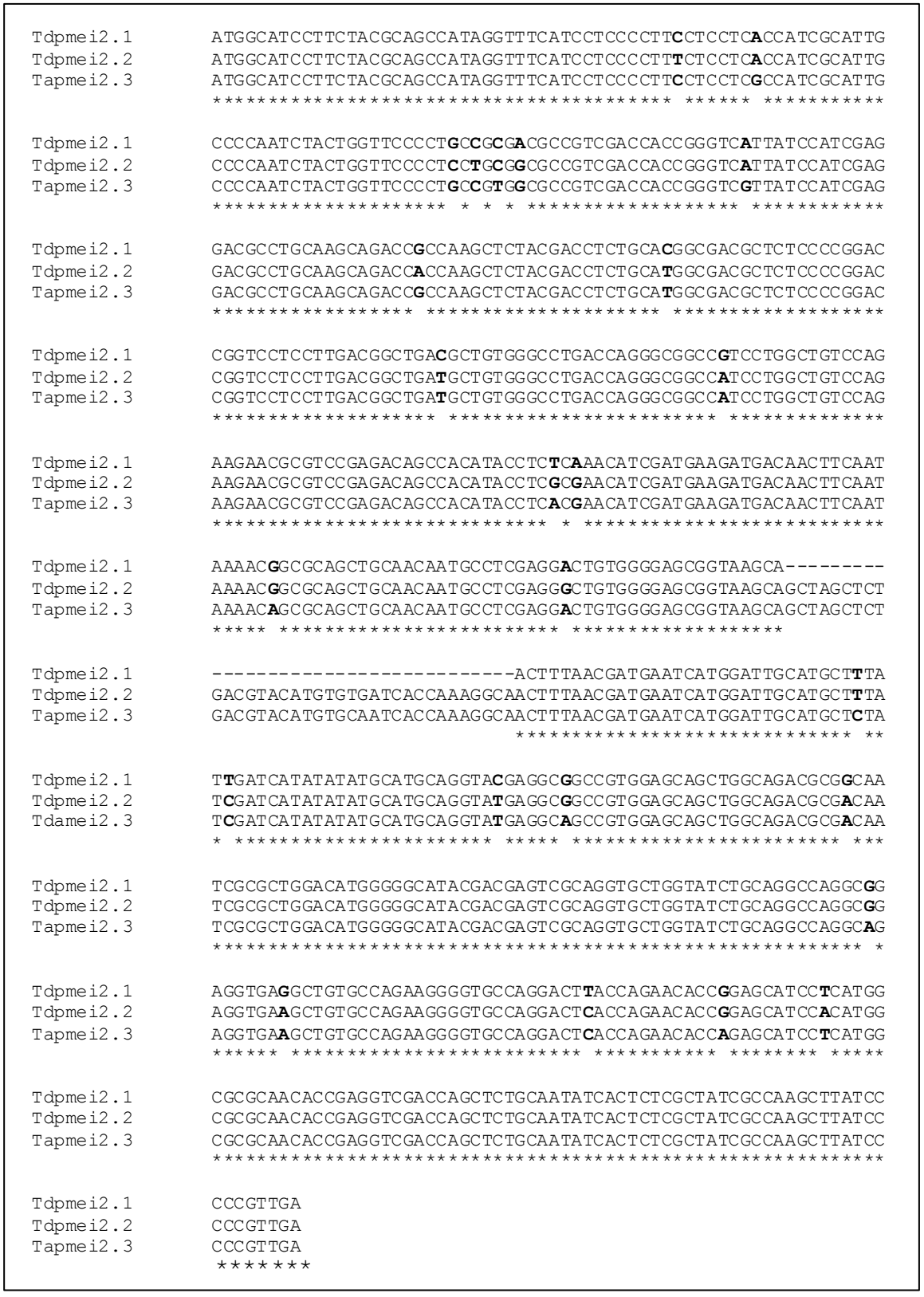


Fig. 3.29 Multiple sequence alignment between nucleotide sequences of *Tdpmei2.1* , *Tdpmei2.2* and *Tapmei2.3*. Single Nucleotide substitutions (SNPs) are in bold.

```
TdPMEI2.1       MASFYAAIGFILPFLLTIALPQSTGSPAATPSTTGSLSIEDACKQTAKLYDLCTATLSPD
TdPMEI2.2       MASFYAAIGFILPFLLTIALPQSTGSPPAAPSTTGSLSIEDACKQTTKLYDLCMATLSPD
TaPMEI2.3       MASFYAAIGFILPFLLAIALPQSTGSPAVAPSTTGSLSIEDACKQTAKLYDLCMATLSPD
                ****************.**********  .****************.****** ******

TdPMEI2.1       RSSLTADAVGLTRAAVLAVQKNASETATYLSNIDEDDNFNKTAQLQQCLEDCGERYEAAV
TdPMEI2.2       RSSLTADAVGLTRAAILAVQKNASETATYLANIDEDDNFNKTAQLQQCLEGCGERYEAAV
TaPMEI2.3       RSSLTADAVGLTRAAILAVQKNASETATYLTNIDEDDNFNKTAQLQQCLEDCGERYEAAV
                **************.*************.****************** *********

TdPMEI2.1       EQLADAAIALDMGAYDESQVLVSAGQAEVRLCQKGCQDLPEHRSILMARNTEVDQLCNIT
TdPMEI2.2       EQLADATIALDMGAYDESQVLVSAGQAEVKLCQKGCQDSPEHRSIHMARNTEVDQLCNIT
TaPMEI2.3       EQLADATIALDMGAYDESQVLVSAGQAEVKLCQKGCQDSPEHQSILMARNTEVDQLCNIT
                ******.**********************.******** ***.** **************

TdPMEI2.1       LAIAKLIPR
TdPMEI2.2       LAIAKLIPR
TaPMEI2.3       LAIAKLIPR
                *********
```

Fig. 3.30 Multiple amino acid sequence alignment between the mature protein of the TdPMEI2.1, TdPMEI2.2 and TaPMEI2.3. Amino acid differences are in bold.

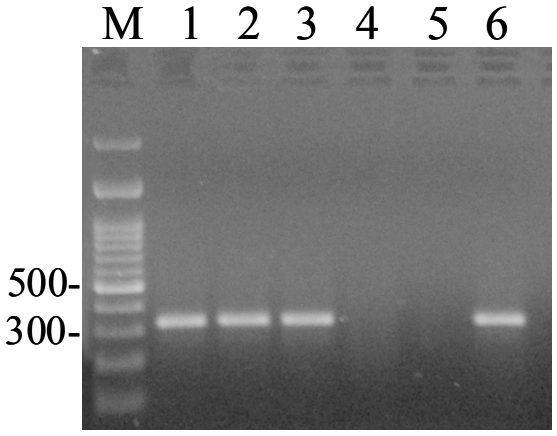


Fig. 3.31 Chromosomal assignment of *Tapmei2.3*. 1.5% Agarose gel of PCR products obtained with primer pair Tdpmei2.3_69F/Tdpmei2.3_393R on *T. aestivum* cv. Chinese Spring (CS), Nulli-tetrasomic (NT) and ditelosomic (DT) lines of *T. aestivum* cv. CS: M) Ladder Gene Ruler DNA 100bp, 1)CS, 2) N2AT2D, 3) N2BT2A, 4) N2DT2A, 5) DT2DS, 6) DT2DL.

In order to verify the conservation of the *Tdpmei2.1*, *Tdpmei2.2 and Tapmei2.3* genes in wild wheat progenitors, we analysed these genes in *T. urartu*, *Ae. speltoides* and *Ae. squarrosa*. PCR assays on the genomic DNA of each of species by using the primer pair TapmeiFor/TapmeiRev, spanning the entire coding region, produced a single amplicon with similar size of those obtained from bread wheat (Fig.3.32). Nucleotide sequence of the amplicon obtained from *T. urartu* showed 99% identity with *Tdpmei2.2* (Fig. 3.33), thus confirming the origin of this gene from the A genome. Nucleotide sequence of the amplicon obtained from *Ae. speltoides*, shared the 98% identity with *Tdpmei2.1* (Fig. 3.34), thus confirming the origin of this gene from the B genome, being *Ae. speltoides* one of the possible donor of the B genome. Finally, nucleotide sequence of the amplicon obtained from *Ae. squarrosa* showed 99% identity with to *Tapmei2.3***,** thus confirming the origin of this gene from the D genome (Fig. 3.35).

All these data demonstrated that the entire coding region of *Tdpmei2.1*, *Tdpmei2.2*, *Tapmei2.3* are strongly conserved in cultivated and wild wheat progenitors.

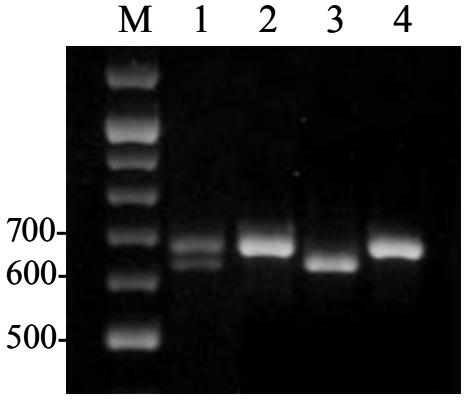


Fig. 3.32 1,5% Agarose gel of PCR products obtained with primer pair TapmeiFor/TapmeiRev from genomic DNA of wild wheat progenitors. M) Ladder Gene Ruler DNA 100bp, 1) *T. aestivum* cv. Chinese Spring, 2) *T. urartu*, 3) *Ae. Speltoides*, 4) *Ae. squarrosa*.

```
Tdpmei2.2       ATGGCATCCTTCTACGCAGCCATAGGTTTCATCCTCCCCTTTCTCCTCACCATCGCATTG
Tupmei          ATGGCATCCTTCTACGCAGCCATAGGTTTCATCCTCCCCTTTCTCCTCACCATCGCATTG
                ************************************************************

Tdpmei2.2       CCCCAATCTACTGGTTCCCCTCCTGCGGCGCCGTCGACCACCGGGTCATTATCCATCGAG
Tupmei          CCCCAATCTACTGGTTCCCCTCCTGCGGCGCCGTCGACCACCGGGTCATTATCCATCGAG
                ************************************************************

Tdpmei2.2       GACGCCTGCAAGCAGACCACCAAGCTCTACGACCTCTGCATGGCGACGCTCTCCCCGGAC
Tupmei          GACGCCTGCAAGCAGACCACCAAGCTCTACGACCTCTGCATGGCGACGCTCTCCCCGGAC
                ************************************************************

Tdpmei2.2       CGGTCCTCCTTGACGGCTGATGCTGTGGGCCTGACCAGGGCGGCCATCCTGGCTGTCCAG
Tupmei          CGGTCCTCCTTGACGGCTGATGCTGTGGGCCTGACCAGGGCGGCCATCCTGGCTGTCCAG
                ************************************************************

Tdpmei2.2       AAGAACGCGTCCGAGACAGCCACATACCTCGCGAACATCGATGAAGATGACAACTTCAAT
Tupmei          AAGAACGCGTCCGAGACAGCCACATACCTCACGAACATCGATGAAGATGACAACTTCAAT
                ***************************** ****************************

Tdpmei2.2       AAAACGGCGCAGCTGCAACAATGCCTCGAGGGCTGTGGGGAGCGGTAAGCAGCTAGCTCT
Tupmei          AAAACGGCGCAGCTGCAACAATGCCTCGAGGGCTGTGGGGAGCGGTAAGCAGCTAGCTCT
                ************************************************************

Tdpmei2.2       GACGTACATGTGTGATCACCAAAGGCAACTTTAACGATGAATCATGGATTGCATGCTTTA
Tupmei          GACGTACATGTGTGATCACCAAAGGCAACTTTAACGATGAATCATGGATTGCATGCTTTA
                ************************************************************

Tdpmei2.2       TCGATCATATATATGCATGCAGGTATGAGGCGGCCGTGGAGCAGCTGGCAGACGCGACAA
Tupmei          TCGATCATATATATGCATGCAGGTATGAGGCGGCCGTGGAGCAGCTGGCAGACGCGACAA
                ************************************************************

Tdpmei2.2       TCGCGCTGGACATGGGGGCATACGACGAGTCGCAGGTGCTGGTATCTGCAGGCCAGGCGG
Tupmei          TCGCGCTGGACATGGGGGCATACGACGAGTCGCAGGTGCTGGTATCTGCAGGCCAGGCGG
                ************************************************************

Tdpmei2.2       AGGTGAAGCTGTGCCAGAAGGGGTGCCAGGACTCACCAGAACACCGGAGCATCCACATGG
Tupmei          AGGTGAAGCTGTGCCAGAAGGGGTGCCAGGACTCACCAGAACACCAAGCATCCTCATGG
                ******************************************** ******** *****

Tdpmei2.2       CGCGCAACACCGAGGTCGACCAGCTCTGCAATATCACTCTCGCTATCGCCAAGCTTATCC
Tupmei          CGCGCAACACCGAGGTCGACCAGCTCTGCAATATCACTCTCGCTATCGCCAAGCTTATCC
                ************************************************************

Tdpmei2.2       CCCGTTGA
Tupmei          CCCGTTGA
                ********
```

Fig. 3.33 Nucleotide alignment between *Tdpmei2.2* and *Tupmei*. The two sequences share 99% identity. Nucleotide differences are in bold.

```
Tdpmei2.1       ATGGCATCCTTCTACGCAGCCATAGGTTTCATCCTCCCCTTCCTCCTCACCATCGCATTG
Aesppmei        ATGGCATCCTTCTACGCAGCCATAGGTTTCATCCTCCCCTTCCTCCTCACCATCGCATTG
                ************************************************************

Tdpmei2.1       CCCCAATCTACTGGTTCCCCTGCCGCGACGCCGTCGACCACCGGGTCATTATCCATCGAG
Aesppmei        CCCCAATCTACTGGTTCCCCTGCCGCGACGCCGTCGACCACCGGGTCATTATCCATCGAG
                ************************************************************

Tdpmei2.1       GACGCCTGCAAGCAGACCGCCAAGCTCTACGACCTCTGCACGGCGACGCTCTCCCCGGAC
Aesppmei        GACGCCTGCAAGCAGACCGCCAAGCTCTACGACCTCTGCATGGCGACGCTCTCCCCGGAC
                ************************************** ******************

Tdpmei2.1       CGGTCCTCCTTGACGGCTGACGCTGTGGGCCTGACCAGGGCGGCCGTCCTGGCTGTCCAG
Aesppmei        CGGTCCTCCTTGACGGCTGACGCTGTGGGCCTGACCAGGGGGGCCATCCTGGCTGTCCAG
                ************************************** **** **************

Tdpmei2.1       AAGAACGCGTCCGAGACAGCCACATACCTCTCAAACATCGATGAAGATGACAACTTCAAT
Aesppmei        AAGAACGCGTCCGAGACAGCCACATACCTCTCGAACATCGATGAAGATGACAAATTCAAT
                ******************************** ******************** ******

Tdpmei2.1       AAAACGGCGCAGCTGCAACAATGCCTCGAGGACTGTGGGGAGCGGTAAGCAACTTTAACG
Aesppmei        AAAACGGCGCAGCTGCAACAATGCCTCGAGGACTGTGGGGAGCGGTAAGCAACTTTAACG
                ************************************************************

Tdpmei2.1       ATGAATCATGGATTGCATGCTTTATTGATCATATATATGCATGCAGGTACGAGGCGGCCG
Aesppmei        ATGAATCATGGATTGCATGCTTTATCGATCATATATATGCATGCAGGTATGAGGCGGCCG
                ************************ ********************** **********

Tdpmei2.1       TGGAGCAGCTGGCAGACGCGGCAATCGCGCTGGACATGGGGGCATACGACGAGTCGCAGG
Aesppmei        TGGAGCAGCTGGGAGACGCGGCAATCGCTCTGGACATGGGGGCATACGACGAGTCGCAGG
                ************ *************** ******************************

Tdpmei2.1       TGCTGGTATCTGCAGGCCAGGCGGAGGTGAGGCTGTGCCAGAAGGGGTGCCAGGACTTAC
Aesppmei        CGCTGGTATCTGCAGGCCAGGCGGAGGTGAAGCTGTGCCAGAAGGGGTGCCAGGACTTAC
                 ***************************** ******************************

Tdpmei2.1       CAGAACACCGGAGCATCCTCATGGCGCGCAACACCGAGGTCGACCAGCTCTGCAATATCA
Aesppmei        CAGAACACCGGAGCATCCTCATGGCGCGCAACACCGAGGTCGACCAGCTCTGCAATATCA
                ************************************************************

Tdpmei2.1       CTCTCGCTATCGCCAAGCTTATCCCCCGTTGA
Aesppmei        CTCTTGCTATCGCCAAGCTTATCCCCCGTTGA
                **** *************************
```

Fig. 3.34 Nucleotide alignment between *Tdpmei2.1* and *Aesppmei*. The two sequences share 98% identity. Nucleotide differences in bold.

```
Tapmei2.3       ATGGCATCCTTCTACGCAGCCATAGGTTTCATCCTCCCCTTCCTCCTCGCCATCGCATTG
Aesqpmei        ATGGCATCCTTCTACGCAGCCATAGGTTTCATCCTCCCCTTCCTCCTCGCCATCGCATTG
                ************************************************************

Tapmei2.3       CCCCAATCTACTGGTTCCCCTGCCGTGGCGCCGTCGACCACCGGGTCGTTATCCATCGAG
Aesqpmei        CCCCAATCTACTGGTTCCCCTGCCGTGGCGCCGTCGACCACCGGGTCGTTATCCATCGAG
                ************************************************************

Tapmei2.3       GACGCCTGCAAGCAGACCGCCAAGCTCTACGACCTCTGCATGGCGACGCTCTCCCCGGAC
Aesqpmei        GACGCCTGCAAGCAGACCGCCAAGCTCTACGACGTCTGCATGGCGACGCTCTCCCCGGAC
                ********************************* ************************

Tapmei2.3       CGGTCCTCCTTGACGGCTGATGCTGTGGGCCTGACCAGGGCGGCCATCCTGGCTGTCCAG
Aesqpmei        CGGTCCTCCTTGACGGCTGATGCTGTGGGCCTGACCAGGGCGGCCATCCTGGCTGTCCAG
                ************************************************************

Tapmei2.3       AAGAACGCGTCCGAGACAGCCACATACCTCACGAACATCGATGAAGATGACAACTTCAAT
Aesqpmei        AAGAACGCGTCCGAGACAGCCACATACCTCACGAACATCGATGAAGATGACAACTTCAAT
                ************************************************************

Tapmei2.3       AAAACAGCGCAGCTGCAACAATGCCTCGAGGACTGTGGGGAGCGGTAAGCAGCTAGCTCT
Aesqpmei        AAAACAGCACAGCTGCAACAATGCCTCGAGGACTGTGGGGAGCGGTAAGCAGCTAGCTCT
                ******** ***************************************************

Tapmei2.3       GACGTACATGTGCAATCACCAAAGGCAACTTTAACGATGAATCATGGATTGCATGCTCTA
Aesqpmei        GACGTACATGTGCGATCACCAAAGGCAACTTTAACGATGAATCATGGATTGCATGCTCTA
                ************ ***********************************************

Tapmei2.3       TCGATCATATATATGCATGCAGGTATGAGGCAGCCGTGGAGCAGCTGGCAGACGCGACAA
Aesqpmei        TCGATCATATATATGCATGCAGGTATGAGGCAGCCGTGGAGCAGCTGGCAGACGCGACAA
                ************************************************************

Tapmei2.3       TCGCGCTGGACATGGGGGCATACGACGAGTCGCAGGTGCTGGTATCTGCAGGCCAGGCAG
Aesqpmei        TCGCACTGGACATGGGGGCATACGACGAGTCGCAGGTGCTGGTATCTGCAGGCCAGGCGG
                **** ******************************************************* *

Tapmei2.3       AGGTGAAGCTGTGCCAGAAGGGGTGCCAGGACTCACCAGAACACCAGAGCATCCTCATGG
Aesqpmei        AGGTGAAGCTGTGCCAGAAGGGGTGCCAGGACTCACCAGAACACCAGAGCATCCTCATGG
                ************************************************************

Tapmei2.3       CGCGCAACACCGAGGTCGACCAGCTCTGCAATATCACTCTCGCTATCGCCAAGCTTATCC
Aesqpmei        CGCGCAACACCGAGGTCGACCAGCTCTGCAATATCACTCTCGCTATCGCCAAGCTTATCC
                ************************************************************

Tapmei2.3       CCCGTTGA
Aesqpmei        CCCGTTGA
                ********
```

Fig. 3.35 Nucleotide alignment between *Tapmei2.3* and *Aesqpmei*. The two sequences share 99% identity. Nucleotide differences in bold.

3.1.6 Genomic organization and characterization of *Tdpmei7.3* homeologs

Southern blot analysis performed on genomic DNA of *Triticum aestivum* cv. Chinese Spring digested with *Hind*III by using the complete coding region of *Tdpmei7.3* as probe showed a main hybridization fragment of 3.0 kbp and two additional weaker hybridizing fragments of about 3.2 kbp and 3.3 kbp (Fig. 3.36).

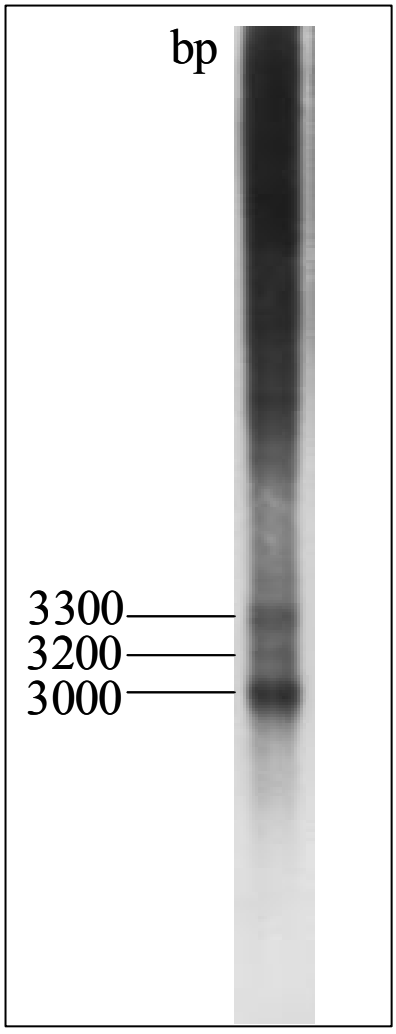


Fig. 3.36 Southern blot of genomic DNA of bread wheat hybridized with *Tdpme7.3*. Genomic DNA of *T. aestivum* cv. Chinese Spring (8 µg) was digested with *Hind*III and probed with the complete coding region of *Tdpme7.3* labelled with digoxigenin.

Parallel experiments were performed to determine the chromosomal localization of *Tdpmei7.3*. Unfortunately, the richness in guanine and cytosine and their uniform distribution along the entire *Tdpmei7.3* sequence prevented the development of primers that gave reproducible PCR amplification results.

To isolated homeologs of the *Tdpmei7.3* gene, the primer pair spanning the entire coding region, Tc34pmeiF/TC34pmeiR, was used to amplify separately the genomic DNA of *T. durum* cv. Svevo and *T. aestivum* cv Chinese Spring (Fig. 3.37).

The single amplicon obtained from each cultivar was recovered and subjected to direct nucleotide sequence. The nucleotide sequence from cv. Chinese Spring showed a single sequence with 98% identity to *Tdpmei7.3*, whereas the nucleotide sequence from the amplicon of cv. Svevo showed the occurrence of multiple sequences, since the electropherogram showed twenty positions composed by two peaks (Table 3.1). To verify this result, the nucleotide sequence was repeated on a different amplicon from cv. Svevo but the same multiple sequence was obtained. To identify the single *Tdpmei* sequence we cloned this amplicon into pGEM T-easy vector and sequenced six recombinant clones. Nucleotide comparison between the nucleotide sequences of the amplicon and those obtained from each clones confirmed all nucleotide substitutions observed in the electropherogram of the amplicon and allowed the identification of six different sequences, one of which corresponded to *Tdpmei7.3* (Table 3.2). These sequences, named *Tdpmei7.1* and *Tdpmei7.2, Tdpmei7.4*, *Tdpmei7.5* and *Tdpmei7.6* showed each a few nucleotide substitutions with *Tdpmei7.3* (Table 3.2).

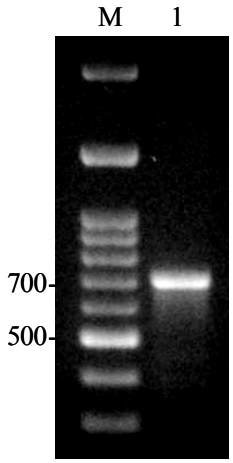


Fig. 3.37 1.5% agarose gel of 1,5% Agarose gel of PCR products obtained with primer pair Tc34pmeiF/Tc34pmeiR from genomic DNA of *T. aestivum* cv. Chinese Spring (CS). M) Ladder Gene Ruler DNA 100 bp, 1)CS.

The primer pair Tc34pmeiF/TC34pmeiR spanning the entire coding region of *Tdpmei7.3*, was also used to amplify separately the genomic DNA of the wild wheat progenitors, *T. urartu*, *Ae. speltoides* and *Ae. squarrosa*. In all three cases only non-specific amplicons were obtained. Direct nucleotide sequencing of the recovered amplicons from all three genotypes did not show any similarity to *Tdpme*i sequences.

Table 3.1 Nucleotide differences found on the PCR amplification product obtained from durum wheat cv Svevo using the primer pair spanning the entire coding region, Tc34pmeiF/TC34pmeiR.

nt	SNP
22	C/T
60	G/T
85	T/G
90	A/G
93	G/A
114	G/A
129	T/C
180	A/G
243	C/G
270	C/G
298	C/A
300	C/G
327	A/C
351	C/G
357	C/T
390	A/G
437	T/C
439	A/C
444	C/T
549	T/C

Table 3.2 Sequences comparison between *Tdpmei7.3* sequence and the other *Tdpmei7* sequences isolated from *T. durum* cv. Sevo. Nucleotide substitution and relative position are shown.

Position	PCR amplicon from Svevo	*Tdpmei7.3*	*Tdpmei7.1*	*Tdpmei7.2*	*Tdpmei7.4*	*Tdpmei7.5*	*Tdpmei7.6*
22	C/T	T	C	C	C	T	T
60	G/T	G	G	G	G	T	G
85	T/G						
90	A/G					G	
93	G/A					A	
114	G/A					A	
129	T/C					C	
180	A/G					G	
243	C/G				G	G	
270	C/G				G	G	
298	C/A		A		A	A	
300	C/G		G		G	G	
327	A/C		C		C	C	
351	C/G		G		G	G	T
357	C/T	T	C	T	C	C	T
390	A/G		G		G	G	
437	T/C	T	C	T	C	C	C
439	A/C		C		C	C	C
444	C/T	C	T	C	T	T	T
549	T/C			T			

3.2Functional analysis of *Tdpmei* genes and their encoded products

Since it has been demonstrated that the modulation of PME activity by the interaction with its specific inhibitor plays a key role during plant growth, we analyze the transcript accumulation of the three *Tdpmei* genes isolated and tested the inhibiting capacity of their encoded products against the PME activity from different wheat tissues at different developmental stages. We demonstrate also the inhibiting properties of TdMEI7.3 *in vivo* by expressing it in durum wheat transgenic plants. Finally, since pmei genes have been involved in plant defence, we determined the expression level of all three *Tdpmei* genes following fungal pathogen infection.

3.2.1 Expression analysis of *Tdpmei* genes in wheat tissues at different developmental stages and following fungal infection

We analysed the expression of *Tdpmei2.1* and *Tdpmei2.2* in roots of plants at Zadoks stage7; stem, second and third leaves of plants at Zadoks stage 13; lemma, palea, ovary and anther of plants at Zadoks stage 65; we also analyzed the transcript accumulation of these genes in grains 1-4 days after pollination (dap), 5-10 dap and 11-17 dap and in roots and coleoptile grown in dark conditions. Transcript accumulation was analyzed by RT-PCR using the primers TapmeiFor/TapmeiRev that amplify the complete coding region of these genes. The use of these primers highlighted that *Tdpmei2.1* and *Tdpmei2.2* undergo intron retention. In particular, our results showed that in root, second and third leaves the transcript of *Tdpmei2.1* and *Tdpmei2.2* is represented only by unprocessed transcripts (Fig. 3.38), whereas in stem, lemma, palea, ovary and in all developmental stages of grains analyzed, the transcript is partially processed. The processed transcript is abundant in lemma and palea, ovary and grains 1-4 dap, whereas is low in the stem and caryopsis 5-10 dap and 11-17 dap. The only tissue analysed where the *Tdpmei2.1* and *Tdpmei2.2* transcripts were completely processed was the anther.

To verify the sequence composition of the processed transcript, the amplicon from anther was recovered and subjected to direct nucleotide sequencing. The result showed that the amplicon is represented by both *Tdpmei2.1* and *Tdpmei2.2*. The composition of the processed transcript in the other tissues was determined by using primers specific for each *Tdpmei2.1* and *Tdpmei2.2*. RT-PCR assays on the total RNA from the different tissues with these new primers confirmed the presence of both processed transcript in the anther and showed that the processed transcript was represented by both *Tdpmei2.1* and *Tdpmei2.2* in all tissues

analyzed, except in the grains 5-10 dap and 15-17 dap, that showed only the processed transcript of *Tdpmei2.2* (Fig.3.39).

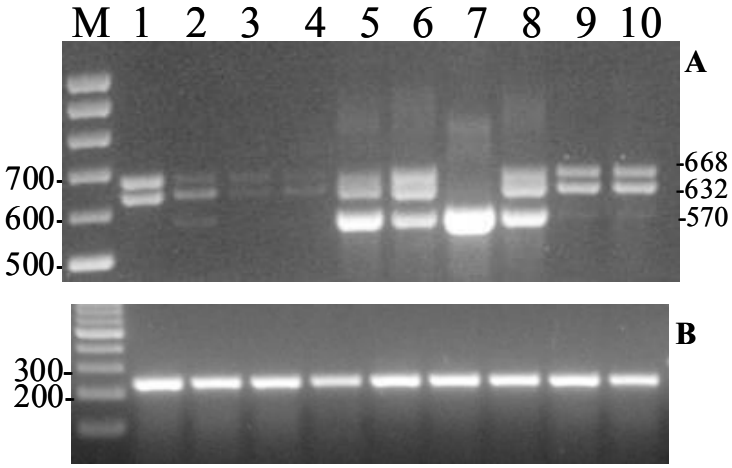


Fig. 3.38 Semi-quantitative RT-PCR analysis for the expression analysis of *Tdpmei2.1* and *Tdpmei2.2* in different wheat tissues. **A)** 1,5% agarose gel of RT-PCR products obtained with primer pair TapmeiFor/TapmeiRev showing intron retention. M) Ladder Gene Ruler DNA 100bp, 1) Roots Z7, 2) Stem Z13, 3) Second leaf Z13, 4) Third leaf Z13, 5) Lemma and Palea Z65, 6) Ovary Z65, 7) Anther Z65, 8) Grains 1-4 dap, 9) Grains 5-10 dap, 10) Grains 11-17dap. **B)** 1,5% Agarose gel of RT-PCR products obtained with primer pair TaAct77F/TaAct312R specific for *Actin*.

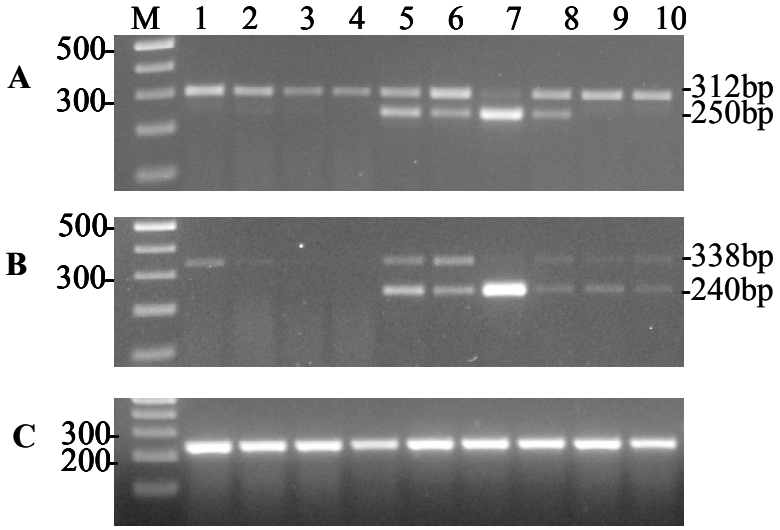


Fig. 3.39 Semi-quantitative RT-PCR analysis for the expression analysis of *Tdpmei2.1* and *Tdpmei2.2* in different wheat tissues. **A)**1.5% agarose gel of RT-PCR products obtained with primer pair Tdpmei1_254F/Tdpmei1_566R, specific for *Tdpmei2.1*. The amplicon representing unprocessed transcript is 312bp, whereas that representing the processed one is 250bp; **B)** 1.5% agarose gel of RT-PCR products obtained with primer pairs Tdpmei2_64F/TdpmeiN5A_593R, specific for *Tdpmei2.2.* The amplicon representing unprocessed transcript is 338bp, whereas that representing the processed one is 240bp.**C)** 1,5% agarose gel of RT-PCR products obtained with primer pair TaAct77F/TaAct312R specific for Actin. M) Ladder Gene Ruler DNA 100bp, 1) Roots Z7, 2) Stem Z13, 3) Second leaf Z13, 4) Third leaf Z13, 5) Lemma and Palea Z65, 6) Ovary Z65, 7) Anther Z65, 8) Grains 1-4 dap, 9) Grains 5-10 dap, 10) Grains 11-17dap.

The expression of *Tdpmei2.1* and *Tdpmei2.2* was also analyzed in dark-grown wheat seedlings. We collected 2 cm long and 5 cm long coleoptiles and relative roots. In all tissues partially processed transcript was, present. Nucleotide sequence analysis of the processed amplicon obtained from 5 cm long coleoptile demonstrated that it corresponded to only *Tdpmei2.1*.

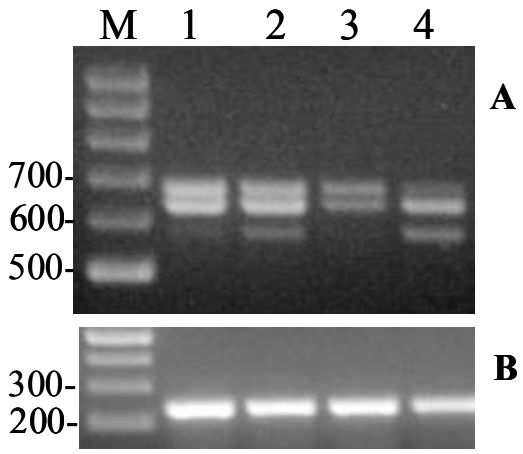


Fig. 3.40 RT-PCR products from tissue of plant grown un dark condition obtained with primer pair TapmeiFor/TapmeiRev (A) and TaAct77F/TaAct312R (B) .M) Ladder Gene Ruler DNA 100bp, 1)Root 2cm, 2) Coleoptile 2cm, 3) Root 5cm, 4) Coleoptile 5cm

Taken all together, these results demonstrated that *Tdpmei2.1* and *Tdpmei2.2* expression is modulated during plant development by intron retention mechanism and that the products of these two genes should play a particular role in anther and or pollen development.

Transcript accumulation was also analyzed for the *Tdpmei7.3*. Since this gene does not contain intron, we used real-time PCR to evaluate its expression level in different tissues and developmental stages. The analysis was performed on the same tissues specified above by using the primer pair Tdpmei3_336F/Tdpmei3_460R and the *Actin* gene as housekeeping gene.

qRT-PCR showed that *Tdpmei7.3* accumulated in all tissues examined. However, transcript accumulation was very low in roots, anther and grains, whereas was particularly high in the stem where the expression was about 200-fold higher than in grain 11-17 dpa, where the expression was the lowest (Fig.3.41).

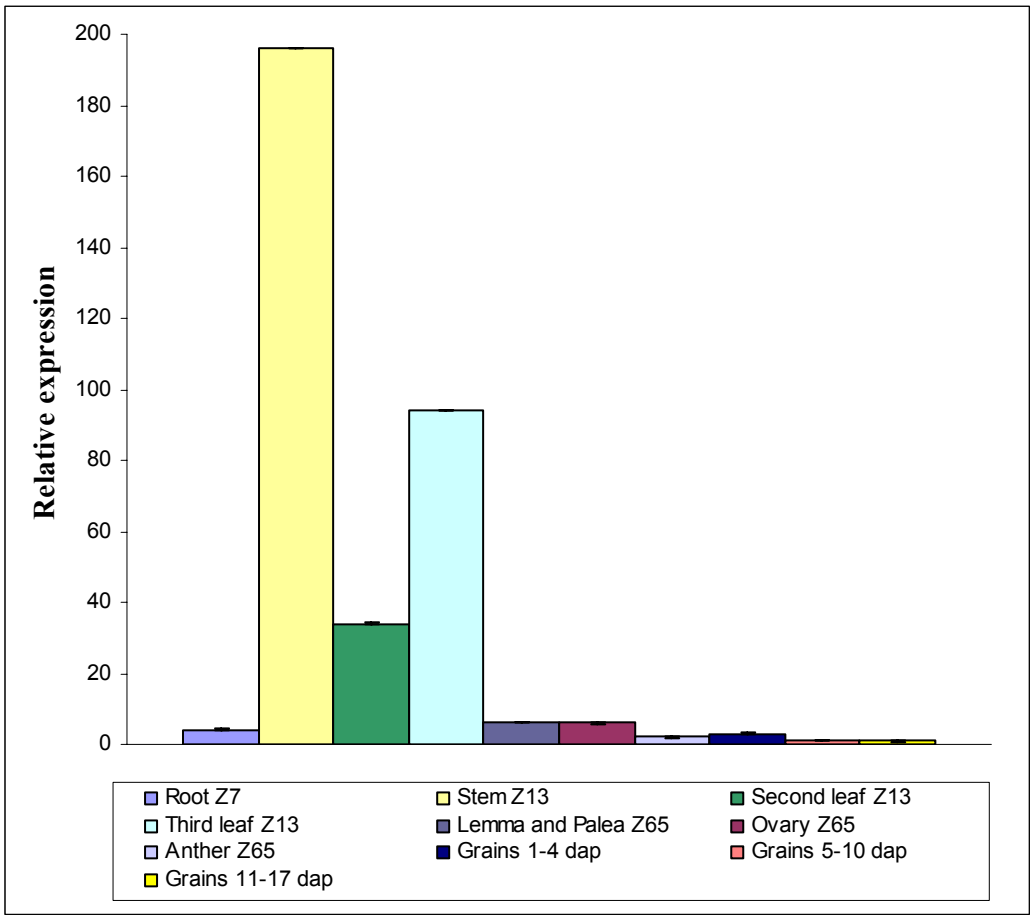


Fig. 3.41 qRT-PCR analysis of *Tdpmei7.3* in different wheat tissues. *Tdpmei7.3* is expressed in all tissues analysed but it strongly accumulates in stem tissue, where the expression is about 200-fold-higher than in 11-17 dap grains..

To verify in more details the region of the stem responsible for the strong accumulation of the *Tdpmei7.3* transcript, we extended the qRT-PCR analysis to the nodal and internodal regions of the stem and found that the *Tdpmei7.3* transcript accumulated about 8-fold more in the internode as compared to the node that showed a similar transcript accumulation as in root (Fig. 3.42).

The expression of *Tdpmei7.3* was also analyzed in dark-grown wheat seedlings and compared with wheat seedlings germinated in light conditions. We collected 2 cm and 5 cm dark-grown coleoptiles and the corresponding roots, and 2 cm coleoptile and corresponding roots grown in light conditions. qRT-PCR analysis showed that *Tdpmei7.3* transcript mainly accumulate in coleoptile. In light-grown tissue, *Tdpmei7.3* accumulated 20-fold more in coleoptile than in roots. In 2cm dark-grown coleoptile transcript accumulation was 14-fold higher than in the corresponding roots, whereas in 5cm dark-grown coleoptile the expression was 42-fold higher than in the corresponding roots (Fig. 3.43). The relative transcript accumulation between light- and dark-grown tissues showed that *Tdpmei7.3* transcripts accumulated less in light-grown roots, where the expression was 18-fold and 7-fold lower than in 2 cm and 5 cm dark-grown roots, respectively (Fig. 3.44). Moreover, *Tdpmei7.3* transcripts in 2cm and 5 cm dark-grow coleoptiles was 14-fold and 20-fold higher than in 2 cm coleoptile grown in light conditions, respectively (Fig. 3.44).

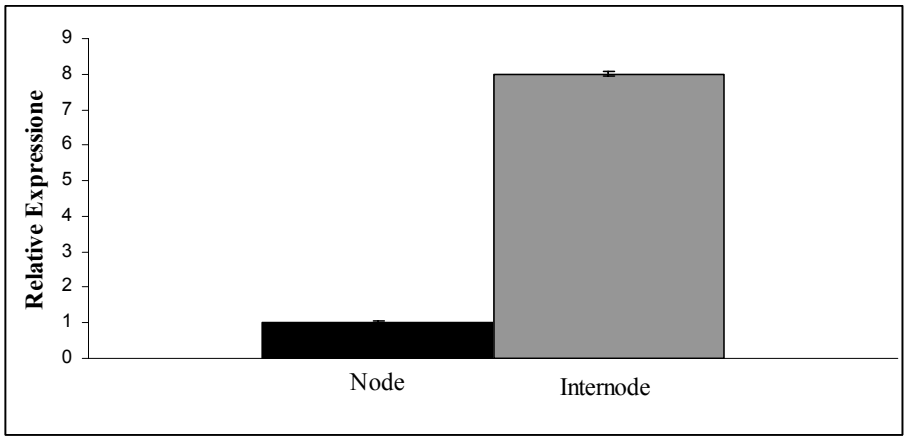
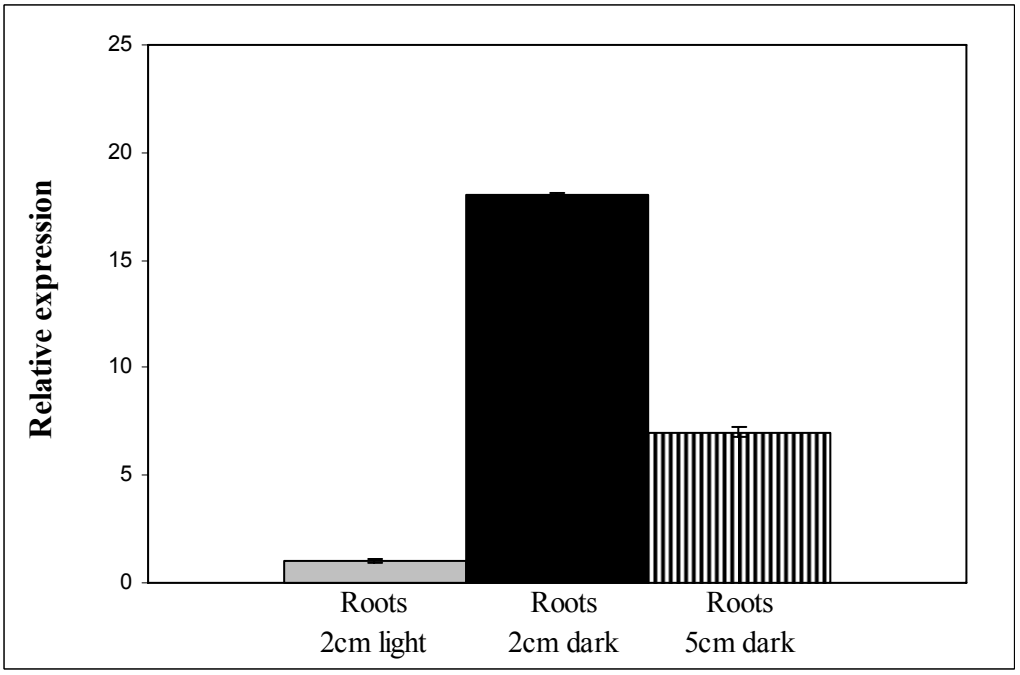
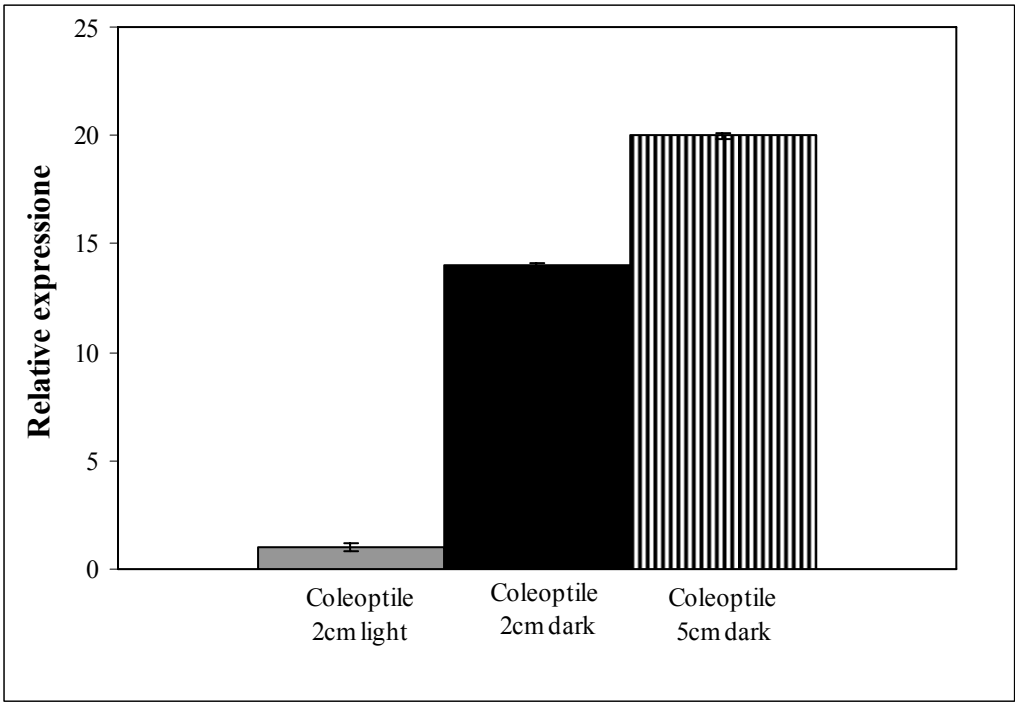


Fig. 3.42 qRT-PCR expression of *Tdpmei7.3* transcript in node and internode.

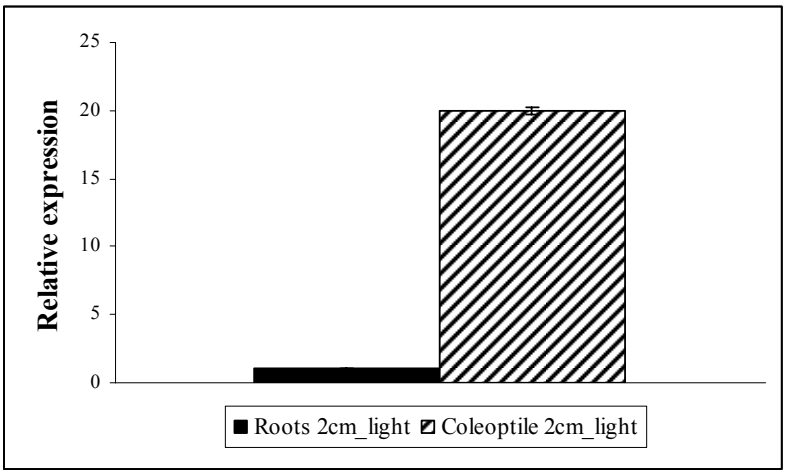


A)

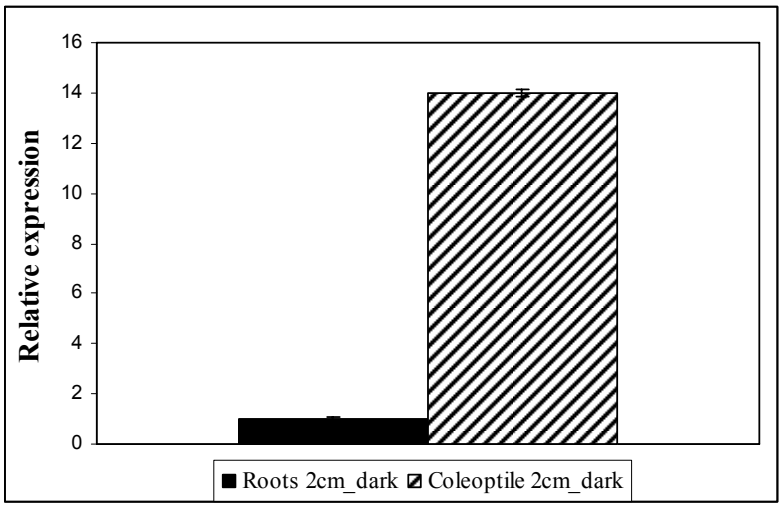


B)

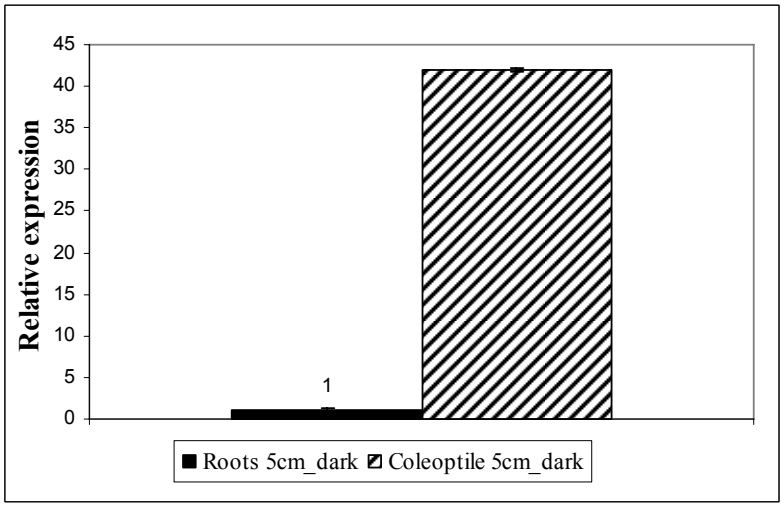
Fig. 3.43 qRT-PCR expression of *Tdpmei7.3* transcript in tissues grown in dark and light conditions. **A)** *Tdpmei7.3* transcript accumulation in roots collected from seed germinated in light and dark condition; **B)** *Tdpmei7.3* transcript accumulation in coleoptiles collected from seed germinated in light and dark condition.



A)



B)



C)

Fig.3.44 Relative transcript accumulation of *Tdpmei7.3* in roots and coleoptiles light and dark grown.

Since the expression of PMEI can be induced following pathogen infections, we analysed also the expression of *Tdpmei2.1*, *Tdpmei2.2* and *Tdpmei7.3* following the infection with the fungal pathogen *Bipolaris sorokiniana*.

RT-PCR analysis with primers TapmeiFor/TapmeiRev specific for the complete coding region of *Tdpmei2.1* and *Tdpmei2.2* genes showed no clear increase of processed transcripts in infected plants compared to non inoculated ones.

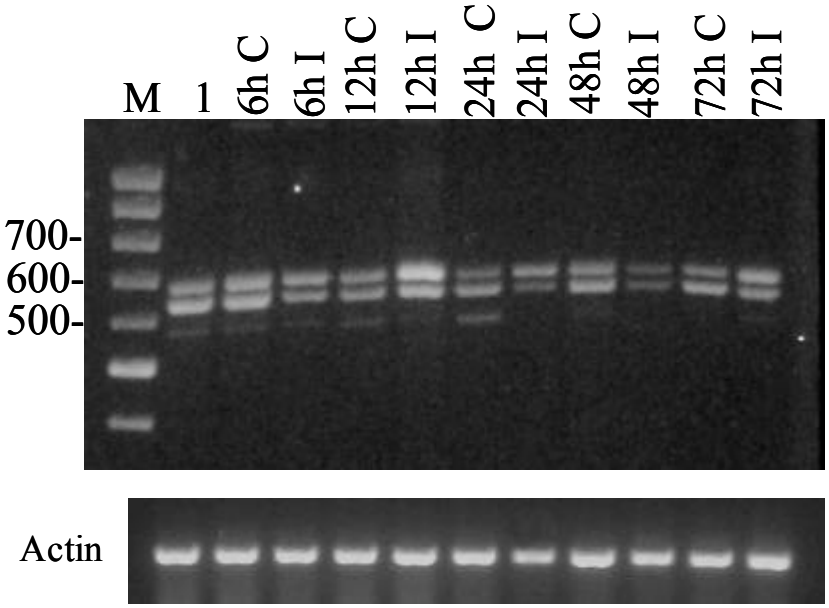


Fig. 3.45 1,5% Agarose gel of RT-PCR products obtained with primer pair TapmeiFor/TapmeiRev on total RNA isolated from plants infected with the fungus *B. sorokiniana*. Processed transcript does not increase in infected plants compared to non-inoculated plants.
C = control plant; I = infected plants.
M) Ladder Gene Ruler DNA 100bp, 1)First leaf_Z12, 2) 6h H_2O, 3) 6h BS, 4) 12h H_2O, 5) 12h BS, 6) 24h H_2O, 7) 24h BS, 8) 48h H_2O, 9) 48h BS, 10) 72h H_2O, 11) 72h BS.

We verify also the expression of *Tdpmei7.3* following infection with *B. sorokiniana*. The analysis was performed by qRT-PCR by using the primer pair Tdpmei3_336F/Tdpmei3_460R and the *Actin* gene as housekeeping gene. Since PR genes can be used as marker of induced gene expression, we analyzed the expression of *PR1.1*, that is known to be induced following fungal pathogen infection. The results showed that *PR1.1* transcript undergoes time-course induction following *B. sorokiniana* infection and its expression was 6 fold higher than in control plants at 6 hours post infection (hpi) and reached the maximum at 48 hpi, when its expression was 22 fold higher than in control plants. Conversely, the expression of *Tdpmei7.3* was down-regulated following fungal infection and at 48 hpi its transcript was 7-fold lower than in control plants (Fig. 3.46).

Taken all together these results indicate that *B. sorokiniana* infection does not induced an accumulation of processed transcript of *Tdpmei2.1* and *Tdpmei2.2*, nor the expression of *Tdpmei7.3* that conversely undergoes a down regulation.

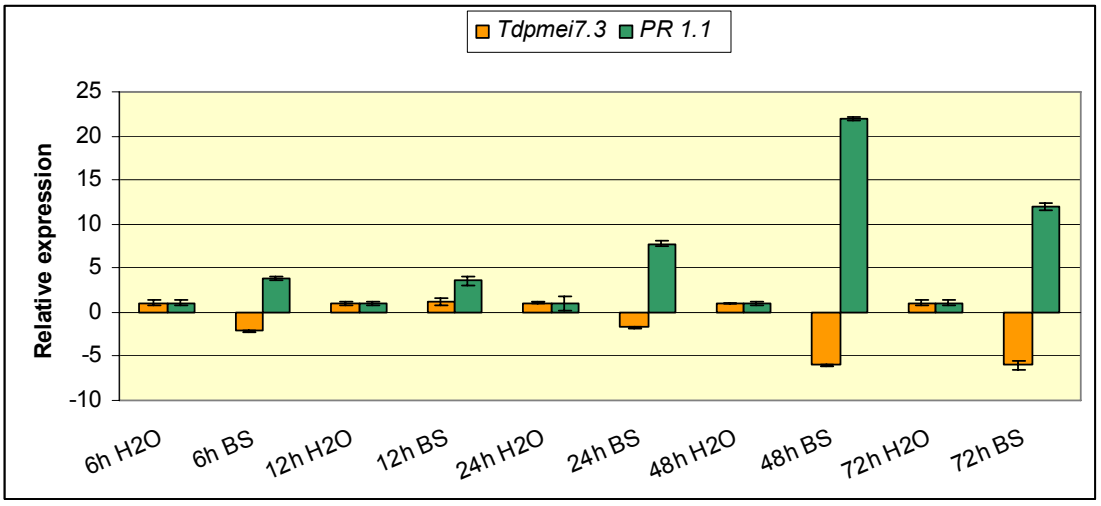


Fig. 3.46 qRT-PCR analysis of *Tdpmei7.3* expression following infection with fungus *B. sorokiniana*.

3.2.2 Characterization of TdPMEI inhibition properties against PME activity from different plant tissues

In order to verify the extent of inhibition of TdPMEI2.1, TdPMEI2.2. and TdPMEI7.3 against endogenous PME activity, we performed radial diffusion assays against the PME activity of different plant tissues.

As preliminary step, we determined the PME activity using different amount of crude protein extracts obtained from roots of wheat seedlings at Zadoks stage 7, second leaf, third leaf and stem of plants at Zadoks stage 13, lemma and palea, ovary and anther of flowering plants at Zadoks stage 65, caryopsis 1-4 dap, 5-11 dap and 11-17 dap and mature dry grain.

PME activity was found in all the tissues analysed, except in dry caryopsis, and showed a broad range of variability between each tissues.(Fig. 3.47). By considering the PME activity obtained from 5µg of crude protein extract of all tissues, higher PME activity was found in ovary, whereas the lower in root (Fig. 3.48).

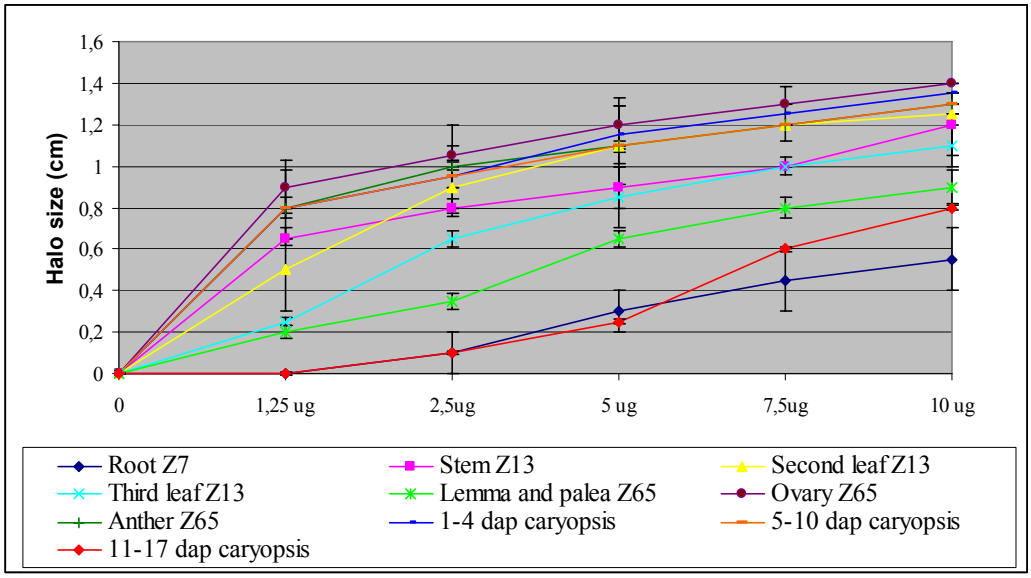


Fig. 3.47 PME activity of wheat tissues. Data are based on the halo produced on agarose gel diffusion assay.

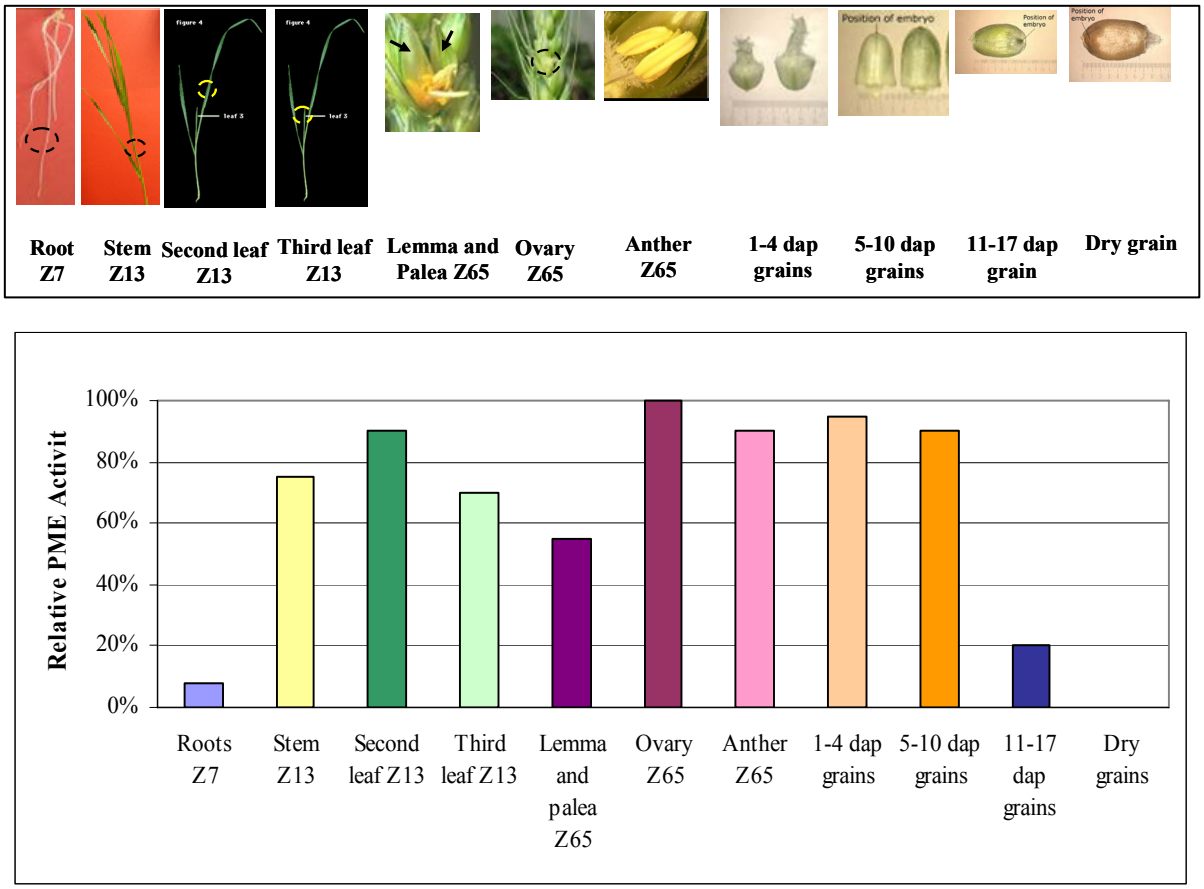


Fig.3.48 Relative PME activity obtained from 5µg of crude protein extract form different wheat tissues. Ovary PME activity was considered as 100% and relative PME activity of other tissues is expressed as a percentage of this activity. Data are based on the halo produced on agarose gel diffusion assay.

The inhibition activity of 1 µg of each TdPMEI2.1, TdPMEI2.2 and TdPMEI7.3 was then verified against the crude protein extracts from the tissues analyzed above. In order to perform the analysis on similar levels of PME activity, different quantities of crude protein extract from the different tissues were used.

TdPMEI2.1 showed the stronger inhibition activity against the PME activity of crude protein extract of the third leaf (25%). This protein inhibits also the PME activity of the roots, second leaf, lemma and palea (15%), root and grains at 1-4 dap (10%). TdPMEI2.1 showed very low inhibition activity against the PME activity of stem, ovary and anther (5%). (Table 3.3).

Table 3.3 Inhibition activity of TdPMEI2.1.The inhibition capability of purified TdPMEI2.1 was assayed against PME activity from wheat tissues. Different quantities of crude protein extract from each tissue were used because they produce different PME activity. Data are based on the halo produced on agarose gel diffusion assay of three replicates ± standard error.

Wheat Tissues	Crude protein extract (µg)	Relative PME activity (%)
Root Z7	10	90±2,8
Stem Z13	2	95±1,7
Second Leaf Z13	2	85±1,1
Third Leaf Z13	1	75±0,29
Lemma and Palea Z65	4	85±0,5
Ovary Z65	1	95±0,58
Anther Z65	1	95±0,23
1-4 dap caryopsis	1	90±0,58

TdPMEI2.2 showed the stronger inhibition activity against the PME activity of crude protein extract of 1-4 dap caryopsis (25%). This protein inhibits also the PME activity of lemma and palea (15%), roots, second leaf, third leaf and ovary (10%). TdPMEI2.1 showed very low inhibition activity against the PME activity of stem and anther (5%). (Table 3.4).

Table 3.4 Inhibition activity of TdPMEI2.2.The inhibition capability of purified TdPMEI2.2 was assayed against PME activity from wheat tissues. Different quantities of crude protein extract from each tissue were used because they produce different PME activity. Data are based on the halo produced on agarose gel diffusion assay of three replicates ± standard error.

Wheat Tissues	Crude protein extract (µg)	Relative PME activity (%)
Root Z7	10	90±1,4
Stem Z13	2	95±1,1
Second Leaf Z13	2	90±1,1
Third Leaf Z13	1	90±1,15
Lemma and Palea Z65	4	85±0,23
Ovary Z65	1	90±1,15
Anther Z65	1	95±0,58
1-4 dap caryopsis	1	75±0,6

TdPMEI7.3 showed the stronger inhibition activity against the PME activity of crude protein extract of stem (60%). This protein inhibits also the PME activity of roots (25%), second leaf, third leaf and lemma and palea (20%) and also of ovary and anther (15%). TdPMEI2.1 did not show any inhibition activity against the PME activity of 1-4 dap caryopsis. (Table 3.5)

Table 3.5 Inhibition activity of TdPMEI7.3. The inhibition capability of purified TdPMEI7.3 was assayed against PME activity from wheat tissues. Different quantities of crude protein extract from each tissue were used because they produce different PME activity. Data are based on the halo produced on agarose gel diffusion assay of three replicates ± standard error.

Wheat Tissues	Crude protein extract (µg)	Realtive PME activity (%)
Root Z7	10	75±1,4
Stem Z13	2	40±2,8
II Leaf Z13	2	80±1,4
III Leaf Z13	1	80±1,44
Lemma and Palea Z65	4	80±1,15
Ovary Z65	1	85±0,87
Anther Z65	1	85±0,29
1-4 days ap grain	1	95±0,58

Since the inhibitors, especially TdPMEI2.1 and TdPMEI2.2, showed a very low inhibition activity against PME activity of mature ovary and anther, we tested their activity against the crude protein extract of these tissues in plants at Zadoks stage 59, when ovary and anther are still immature.

One microgram of each TdPMEI2.1, TdPMEI2.2 and TdPMEI7.3 showed 20%, 30% and 25% inhibition against crude protein extract from immature ovary, respectively (Tab.3.6), whereas their inhibition against PME activity of immature anther was about of 30%, 20%, respectively (Table 3.6).

Table 3.6 Inhibition activity of TdPMEI protein against crude protein extract form immature ovary and anther.

	TdPMEI2.1	TdPMEI2.2	TdPMEI7.3
Immature Ovary (1µg)	20%	30%	25%
Immature Anther (1µg)	30%	20%	20%

These results demonstrated that the three wheat PMEIs are able to inhibit the endogenous wheat PME activity of all tissues analyzed with a slightly different degree of efficacy, except TdPMEI7.3 that resulted particularly efficient against the PME activity of crude protein extract from stem. TdPMEI2.1 inhibitory capability was weakly when assayed against PME activity of almost all tissues, except for that of third leaf that was reduced of 25%. For TdPMEI2.2 we observed the same behaviour, but the stronger inhibition was found against PME activity of 1-4 dap caryopsis

All the three TdPMEI proteins were able to reduce the PME activity from immature ovary and anther with similar degree of efficacy.

3.2.3 Production and characterization of wheat transgenic plants overexpressing *Tdpmei7.3*

In order to verify the capability to modulate *in planta* the wheat endogenous PME activity, we produced wheat transgenic plants overexpressing the *Tdpmei7.3* gene that encoded an inhibitor particularly efficient against the PME activity of wheat stem. For this purpose, the complete coding region of *Tdpmei7.3*, including the *leader* sequence, was inserted into the pAHC17 vector under the control of the constitutive maize *Ubi1* promoter. To facilitate the identification of the transgenic product, a FLAG-tag was fused to the C-terminus of the sequence. The resulting construct, pUBI::*Tdpmei7.3* (Fig.3.49a), and the pUBI::BAR construct (Fig 3.49b), carrying the *bar* gene that confers resistance to the bialaphos herbicide, were co-transformed into immature wheat embryos (*Triticum durum* cv. Svevo) by particle bombardment following the procedure reported by Weeks *et al*, 1993 with some modifications as described by Janni *et al*, 2008.

In total, 1035 immature wheat embryos were co-transformed and forty-one independent regenerated plants were obtained from the bombardment experiment MJ54 (Table 3.7). All regenerated plants were analysed by PCR for the presence of the *Tdpmei7.3* transgene. To avoid interference with the endogenous copy of *Tdpmei7.3*, a primer pair composed by the forward primer specific for the Ubiquitin promoter region of pUBI::*Tdpmei7.3* (UBI_49F) and a reverse primer specific for the transgene (Tdpmei3_369R) were used. The size of the expected amplicon was 420bp. The amplification reaction were conducted on genomic DNA extracted from leaf of transgenic plants (T_0), using genomic DNA of cv. Svevo as negative

control and pUBI::*Tdpmei7.3* as positive control (Fig. 3.50). PCR analysis showed that twenty-two plants were positive for the presence of transgene with a transformation efficiency of 2% (Table 3.7). Of the twenty-two plants, three died in soil during the acclimatation period and the remaining nineteen were further characterized.

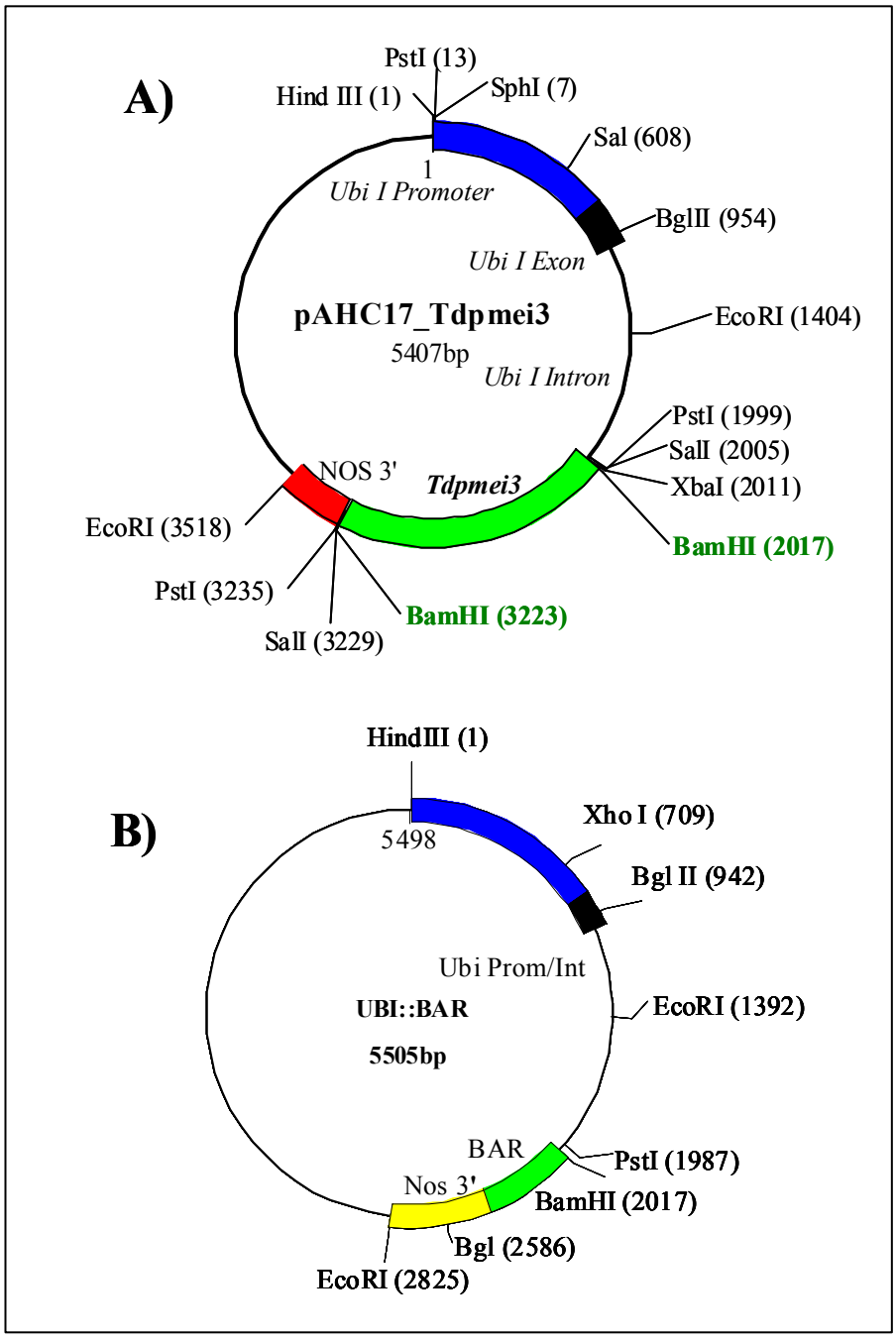


Fig. 3.49 Plasmids used for biolistic transformation of wheat **A)** pUBI::*Tdpmei7.3*, containing the *Ubi*1 promoter and *Tdpmei7.3* gene; **B)** pUBI::BAR containing the *Ubi*1 promoter and the *bar* gene, used for the biolistic bombardment.

Table 3.7 Bombardment experiments with pUBI::*Tdpmei7.3* and transgenic lines obtained and analyzed by PCR.

Cv	# Bombardment	Construct	# bombarded embryos	# Shoots regenerated	#Positive T_0 plants	# T_0 Lines
Svevo	MJ 54	pAHC17_*Tdpmei7.3*	1035	41	22	19

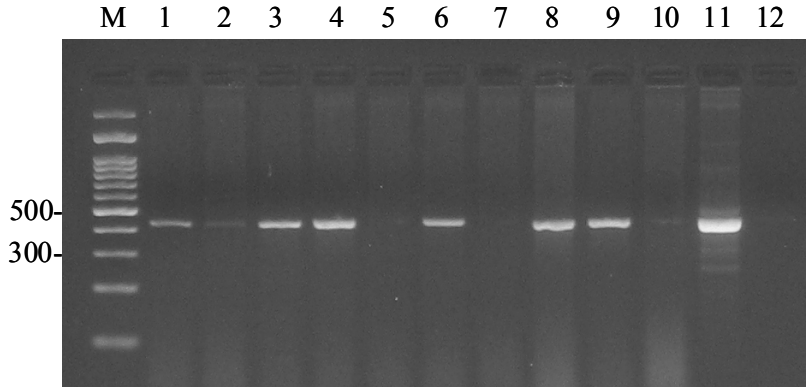


Fig. 3.50 1.5% agarose gel of the amplification products from T_0 leaf DNA obtained using the specific primers for *Tdpmei7.3* and Ubi promoter region, which produce an amplicon of about 420bp. M) Marker GeneRuler 100 bp DNA Ladder, 1-11) some of the regenerated T_0 plants, 11) plasmid DNA pUBI::*Tdpmei7.3*, used as positive control, 12) genomic DNA of durum wheat cv. Svevo used as negative control.

Total leaf protein extracts (10 μg) of all T_0 positive plants were subjected to western blot analysis using a monoclonal antibody anti-FLAG. The result revealed a specific signal with a molecular mass of about 21 kDa corresponding to the transgenic protein, which is not present in the wild-type cv. Svevo (Fig. 3.51).

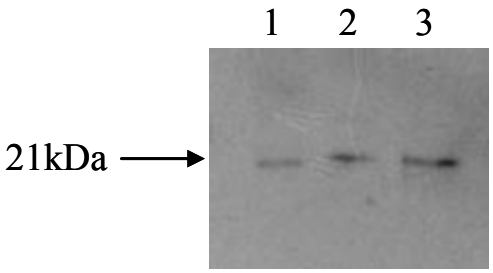


Fig. 3.51 Western Blot analysis of crude protein extract (10 μg) of T_0 positive plants performed with the anti-FLAG antibody. Immunodecoration signal of about 21kDa. 1) MJ54-31(1), 2) MJ54-34(2), 3) MJ54-33,

The T_0 plants were then analysed for their endogenous PME activity. The analysis was performed by radial diffusion assay by using 5 μg of crude protein extract. Relative PME activity of transgenic plants was calculated considering the halo produced by the crude protein extract of the wild-type plants as 100% of PME activity. The nineteen T_0 plants analysed showed different level of PME activity reduction that varied from about 10% to 85% of the control PME activity (Fig.3.52 e Fig. 3.53).

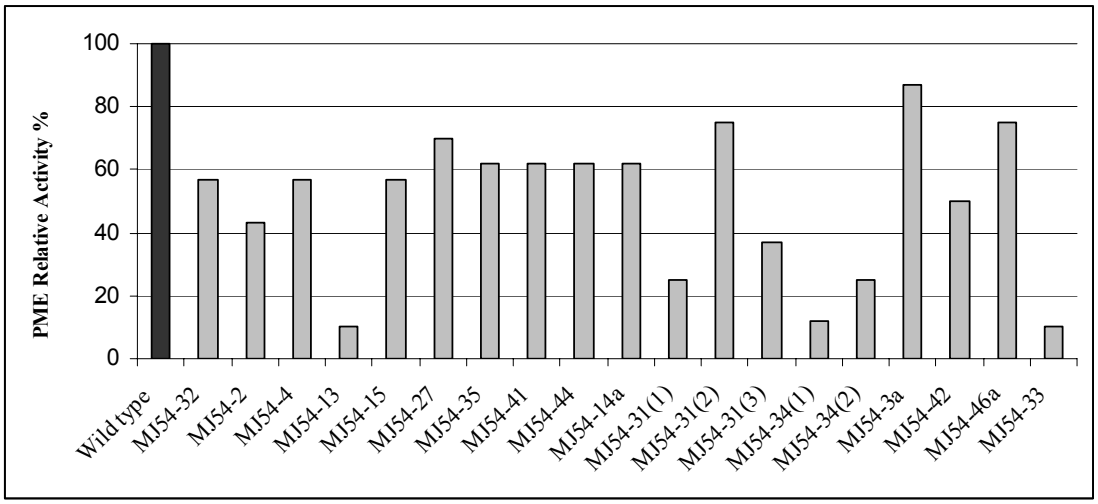


Fig 3.52 Quantification of endogenous PME activity in 19 *Tdpmei7.3* plants and wild type (WT) plants determined by radial gel diffusion assay. PME activity of each transgenic plant is expressed as relative percentage of PME activity of the wild type plants by considering the halo produced by the protein extract of the wild-type plants as 100% of PME activity.

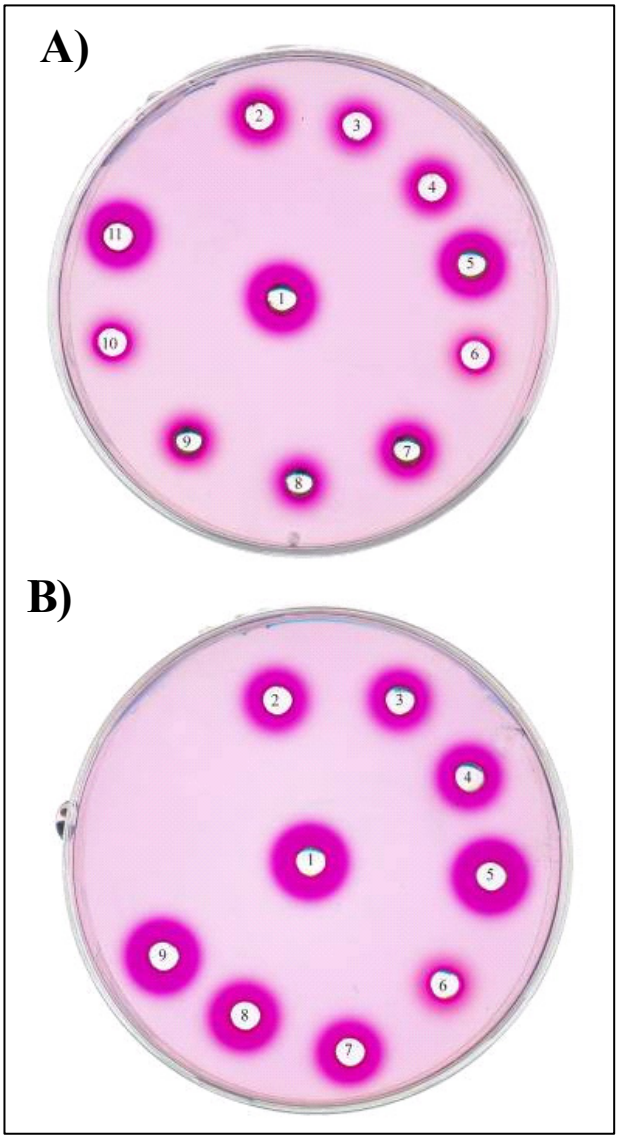


Fig. 3.53 Example of the radial diffusion assay used to verify the endogenous PME activity in transgenic plants overexpressing *Tdpmei7.3*. **A)** 1) 5µg of crude protein extract from Wild type(cv. Svevo), 2-11) 5µg of crude protein extract of T_0 plants; **B)** 1) 5µg of crude protein extract from Wild type(cv. Svevo), 2-9) 5µg of crude protein extract of T_0 plants.

The T_0 positive plants were self-pollinated to obtain the T_1 lines that will be further characterized for the PME activity in the different tissues.

Discussion

In this thesis work, we reported the isolation and functional characterization of three novel wheat *pmei* genes. *In silico* analysis (October 2008) identified a sequence encoding for a putative *T. aestivum* pectin methylesterase inhibitor, now reported as *TaPMEI* (Hong *et al*, 2010), and a tentative contig TC344011 annotated as Invertase/pectin methylesterase inhibitor-like. On the basis of the these two sequences, we isolated three different *pmei* genes, *Tdpmei2.1*, *Tdpmei2.2* and *Tdpmei7.3*. Deduced TdPMEI proteins showed low similarity (about 20%) to other previously described PMEIs. This low level of sequence conservation is a features of most characterized PMEI inhibitors (Zhang *et al*, 2010). Nonetheless, all TdPMEIs possess a conserved hydrophobic C-terminal domain and five cysteine residues at conserved position, the first four of which have been showed to be engaged in the disulfide bridges formation (Camardella *et al*, 2000; Hothorn *et al*, 2004). The deduced mature proteins TdPMEI2.1 and TdPMEI2.2 contain 164 amino acids with a molecular weight of 17492 Da and 17488 Da respectively and predicted p*I* of 4.19 and 4.33. TdPMEI7.3 deduced protein is composed of 196 amino acids with a molecular mass of 20233 Da, a predicted p*I*= 6.92. Since the optimal activity of different PME isoforms can vary substantially with pH, as suggested by their wide p*I* range (Pelloux *et al*, 2007), the different p*I* observed also in TdPMEIs could be correlated to the interaction with the corresponding PME partners. TdPMEI7.3 possesses a signal peptide for targeting to the chloroplast, whereas both TdPMEI2.1 and TdPMEI2.2 have a signal peptide for the apoplastic targeting. The heterologous expression of TdPMEI2.1 in *Pichia pastoris* under its own *leader* signal sequence demonstrated that the protein is secreted into the culture medium, confirming that the signal peptide targets the protein into the extracellular environment. This result was also confirmed by Hong *et al*, (2010) by expressing *TaPMEI*, that share 90% identity with both TdPMEI2.1 and TdPMEI2.2, fused to GFP in onion epidermal cells. Mass spectrometry analysis of TdPMEI2.1 expressed under its own *leader* signal sequence did not confirm the predicted signal peptide since additional residues were found at the N-terminal portion of the putative mature protein.

The recombinant TdPME2.1 accumulated in *P. pastoris* with a molecular mass higher than expected, probably due to higher level of glycosilation. We hypothesized that this feature can be responsible for the lack of inhibition capability of TdPMEI2.1 against PME activity of wheat leaf crude protein extract and orange PME (OpPME).

Functional TdPMEI2.1, TdPMEI2.2 and TdPMEI7.3 were obtained by expressing them in *E. coli*. In i*n vitro* activity assays, the purified heterologous proteins inhibited the PME activity of OpPME, demonstrating that the three *pmei* genes isolated in durum wheat encode for functional inhibitors. Similar activity assays showed that these proteins did not show any

inhibition activity against microbial PME, as already described for others plant PMEIs (Giovane *et al*, 2004; Di Matteo *et al*, 2005). Moreover, all three PMEIs did not showed any inhibition activity against tomato invertase.

Genomic organization of *Tdpmei* has been analyzed by Southern blot and gene-specific PCR. The results showed that *Tdpmei2.1, Tdpmei2.2 and Tapmei2.3* are represented by a single copy or a few copies per genome and are localized on the long arm of chromosome 2B, 2A and 2D, respectively. Sequence analysis of homeologous genes from wild progenitors showed also that all three sequences are strongly conserved, suggesting that their sequence structure is well adapted and important for its physiological role.

Five additional homologs of *Tdpmei7.3* were also isolated from durum wheat cv. Svevo and they showed very little variation, similarly to what observed between *Tdpmei2.1*, *Tdpmei2.2* and *Tapmei2.3*, and also between *pmei* sequences of kiwi (Giovane *et al*, 2004; Irifune *et al*, 2004).

The expression patterns of PMEIs are regulated in a tissue-specific manner (Wolf *et al*, 2003; Raiola *et al*, 2004; An *et al*, Hong *et al*, 2010). We analyzed the transcripts accumulation of *Tdpmei2.1*, *Tdpmei2.2* and *Tdpmei7.3* in different tissues from plants at different stage of development. Our result showed that *Tdpmei2.1* and *Tdpmei2.2* transcript undergo alternative splicing. In particular intron retention was observed in almost all tissues analyzed. The only tissue analysed where the *Tdpmei2.1* and *Tdpmei2.2* transcripts were completely processed was the anther.

It is estimated that alternative splicing in *Arabidopsis* occurs in 7-10% of the gene; about 30%of them are reported as intron retention (Ner-Gaon *et al*, 2004). Stress and other stimuli affect the efficiency or patterns of splicing, however, the mechanisms are largely unknown. Thus, if particular stimuli influence intron retention, is then conceivably that the presence or absence of the intron either stabilizes the transcript or serves to modify its biological function (Ner-Gaon *et al*, 2004). In this context, we hypothesized that *Tdpmei2.1* and *Tdpmei2.2* gene products should play a particular role in anther and or pollen development. It was previously reported that *pmei* genes in *Arabidopsis* are high expressed in flower (Wolf et al, 2003) and in *Brassica oleracea* in mature pollen grains and pollen tube (Zhang *et al*, 2010). These observations suggest that pollen-specific PME enzymes may be under post-translational control of inhibitor proteins and that TdPMEI2.1 and TdPMEI2.2 could be specific for some PME isoforms involved in microsporogenesis and pollen tube growth.

The expression pattern of *Tdpmei7.3* reveals transcript accumulate in all tissues analyzed, but mainly in stem tissue where the expression was very high. We demonstrated also that a

stronger accumulation of *Tdpmei7.3* was found in internodal region of the stem. Similar result has been reported also for *CaPMEI1* (*Capsicum annum* PMEI), where higher level of transcription were found in stem compared to leaf, root and green fruit (An *et al*, 2008). Moreover we investigated *Tdpmei7.3* transcript accumulation in root and coleoptile grown in dark condition and we observed that in tissues grown in dark condition the accumulation was higher when compared with light-grown tissues. In addition, the expression level in coleoptiles dark and light-grown, was about 20-fold higher than in corresponding roots.
Since PMEs are involved in cell wall extension (Moustacas *et al*, 1991) and also in stem elongation (Pilling *et al*, 2000) and internode growth (Saher *et al*, 2005), these finding highlighted that the TdPMEI7.3 inhibitor could interact and modulate the activity of specific PME isoforms involved in these processes.
We investigated also the expression pattern of *Tdpmei2.1*, *Tdpmei2.2* and *Tdpmei7.3* following leaf infection with the fungal pathogen *B. sorokiniana*. The results obtained showed that the accumulation of processed transcripts of *Tdpmei2.1* and *Tdpmei2.2* in inoculated plants was similar to that of the non-inoculated ones, reinforcing the hypothesis that these genes are mainly involved in the modulation of PME activity during floral development. Differently, transcript accumulation of *Tdpmei7.3* showed that this gene is down-regulated following fungal infection. This finding can be explained with a decrease in leaf expansion during infection or with a specific down-regulation of this gene operated by the pathogen to increase the activity of endogenous PME, thus decreasing the methyl esterification of cell wall pectin. This possibility is supported by a recent evidence that necrotrophic pathogens such as *Pectobacterium carotovorum* and *Botrytis cinerea* induced the expression of specific endogenous PME in Arabidopsis during infection (Raiola *et al*, 2011).

The capability of the three TdPMEI proteins to reduce the endogenous wheat PME activity from different tissues was also analyzed. Firstly we characterized the PME activity on different wheat tissues and it was found that all tissues showed PME activity, except dry grain. The higher PME activity was found in ovary and the lower in roots. The subsequent analyses with the TdPMEI inhibitors showed that all three TdPMEIs are able to inhibit the endogenous wheat PME activity of all tissues analyzed with a slightly different degree of efficacy, except for TdPMEI7.3 that resulted particularly efficient against the PME activity of crude protein extract from stem. This results can be correlated with the expression pattern of *Tdpmei7.3*, which was highly expressed in the stem tissue,. These observation support to the hypothesis that this inhibitor may be specific of PME isoforms expressed in the stem tissue. A

similar correspondence was not found between the presence of *Tdpmei2.1* and *Tdpmei2.2* processed transcript in the anther and the inhibition activity of the corresponding proteins against the PME activity of the anther. Probably, the PME activity of this tissue is so high and determined by a number of PMEs that the effective inhibition of TdPMEI2.1 and TdPME2.2 against the corresponding PME partners is not detectable..

The inhibiting properties of TdMEI7.3 were demonstrated also *in vivo* by expressing it in durum wheat transgenic plants. The nineteen T_0 wheat plants obtained showed different level of PME activity reduction that varied from about 10% to 85% of the control PME activity. This finding confirms that the *Tdpmei7.3* gene codes for a functional inhibitor able to reduce *in vivo* the endogenous PME activity. These transgenic plants represent a very important material to shed light on the physiological role of this inhibitor and to verify the possibility to manipulate the pectin methyl esterification for wheat improvement.

Conclusions

This thesis reports the isolation and functional characterization of three novel *pmei* genes from durum wheat. This important class of genes and their encoded products have been poorly characterized in wheat, for which only one gene and corresponding product have been reported so far (Hong *et al*, 2010).

In particular, the results obtained during this work were:

- Three genes, named *Tdpmei2.1*, *Tdpmei2.2* and *Tdpmei7.3*, have been identified in durum wheat and their encoded products have been characterized.
- Genomic organization of *Tdpmei2.1* and *Tdpmei2.2* has been analyzed: these genes are located on the long arms of chromosome 2B and 2A respectively. The homeolog from hexaploid wheat, *Tapmei2.3*, has been isolated and localized on the long arm of chromosome 2D. Isolation of homeologous genes from wild progenitors showed a stronger conservation of their sequence.
- Five additional homologs of *Tdpmei7.3* were isolated from durum wheat cv. Svevo and they showed very little sequence variation.
- The transcription pattern of *Tdpmei* genes was investigated. Regulation of *Tdpmei2.1* and *Tdpmei2.2* transcript undergo intron retention. The transcript of these genes was completely processed only in the anthers. This observation suggests that these genes may be involved in pollen development. *Tdpmei7.3* does not contain intron and transcript analysis revealed a tissue-specific modulation: the stronger accumulation of *Tdpmei7.3* was found in stem tissue and in particular in the internodal region of stem, suggesting that the encoded product of this gene may interact with PME involved in cell expansion and internodal growth.
- The heterologous expression of TdPMEI2.1 in *P. pastoris* produced inactive protein probably due to high level of glycosilation .The expression of TdPMEI2.1 in the same heterologous system with its own *leader* sequence demonstrated that the signal peptide targets the protein into the extracellular environment.
- Functional TdPMEI2.1, TdPMEI2.2 and TdPMEI7.3 were obtained by expressing them in *E. coli*. The purified heterologous protein showed inhibitory capability against orange PME (OpPME) demonstrated that the three wheat *pmei* genes isolated encode for functional inhibitors.
- All the three inhibitors did not have any inhibitory activity against microbial PME and tomato invertase.
- The inhibiting capacity of TdPMEI recombinant protein was assayed against the PME activity from different wheat tissues at different developmental stages. TdPMEI2.1

and TdPMEI2.2 were able to inhibit endogenous PME activity with a slightly degree of efficacy. In particular TdPMEI2.1 inhibits mainly the PME activity of third leaf obtained from plant at Zadoks stage 13 (25%), whereas TdPMEI2.2 shows the stronger inhibition against PME activity from 1-4dap caryopsis (25%). TdPMEI7.3 results particularly efficient against PME activity from stem that was reduced of about 60%.

- The inhibition capability of these TdPMEI7.3 was confirming also in durum wheat transgenic plants by expressing the encoding gene under the maize Ubiquitin promoter. The different regenerated transgenic plants showed a reduced level of endogenous PME activity that varied from 10%to 85%.

Bibliography

Alghisi, P. and Favaron, F. (1995). Pectin-degrading enzymes and plant-parasite interactions. *Eur. J. Plant Pathol*. 101:365-375.

An S.H., Sohn K.H., Choi H.W., Hwang I.S., Lee S.C. and Hwang B.K. (2008) Pepper pectin methylesterase inhibitor protein CaPMEI1 is required for antifungal activity, basal disease resistance and abiotic stress. *Planta*. 268:61-78.

Balestrieri, C., Castaldo, D., Giovane, A., Quagliuolo, L. and Servillo, L. (1990). Aglycoprotein inhibitor of pectin methylesterase in kiwi fruit (Actinidia chinensis). *Eur. J.Biochem*., 193, 183-187.

Bellincampi D., Camardella L., Delcour J.A., Dessaux V., D'Ocidio R., Durand A., Elliot G., Gebruers K., Giovane A., Juge N., Sorensen J.F., Svensson B. and Vairo D. (2004) Potential physiological role of plant glycosidase inhibitors. *Biochimica et Biophysica Act*a 1696: 265– 274.

Beaulieu, C., Boccara, M. and Van Gijsegem, F. (1993). Pathogenic behavior of pectinasedefective Erwinia chrysanthemi mutants on different plants. *Mol. Plant-Microbe Interact*. 6,197-202.

Bonnin, E., Le Goff, A., Korner, R., Vigouroux, J., Roepstorff, P. and Thibault, J.-F. (2002). Hydrolysis of pectins with different degrees and patterns of methylation by the endopolygalacturonase of Fusarium moniliforme. *Biochim. Biophys Acta* 1596:83-94.

Boccara M. and Chatain V. (1989). Regulation and role in pathogenicity of Erwinia chrysanthemi 3937 pectin methylesterase. *J. Bacteriol*. 171: 4085-4087.

Bordenave M. and Goldberg R. (1993). Purification and characterization of pectin methylesterases from Mung bean hypocotyl cell walls. *Phytochemistry* 33: 999-1003.

Bosch M., Cheung A.Y. and Hepler P.K. (2005) Pectin Methylesterase, a Regulator of Pollen Tube Growth. *Plant Physiology*,138:1334–1346.

Bosch M. and Hepler P.K. (2006). Silencing of the tobacco pollen pectin methyl-esterase NtPPME1 results in retarded in vivo pollen tube growth. *Planta* 223: 736-745.

Boudart G., Lafitte C., Barthe J.P., Frasez D. and Esquerre-Tugayé M.T. (1998). Differential elicitation of defence responses by pectin fragments in bean seedlings. *Planta* 206: 86-94.

Bradford M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. *Anal Biochem* 72: 248-254.

Brummell D.A. and Harpster M.H. (2001). Cell wall metabolism in fruit softening and quality and its manipulation in transgenic plants. *Plant Mol. Biol*. 47: 311-340.

Brutus A., Villard C., Durand A., Tahir T., Furniss C., Puigserver A., Juge N. and Giardina T. (2004). The inhibition specificity of recombinant Penicillium funiculosum xylanase B towards wheat proteinaceous inhibitors. B*iochimica et Biophysica Acta-Proteins and Proteomics* 1701: 121-128.

Camardella L., Carratore V., Ciardiello M.A., Servillo L., Balestrieri C. and Giovane A. (2000). Kiwi protein inhibitor of pectin methylesterase amino-acid sequence and structural importance of two disulfide bridges. *Eur. J. Biochem*. 267: 4561-4565.

Carpita N.C. (1996) Structure and biogenesis of the cell walls of grasses. *Annu Rev Plant Physiol Plant Mol Biol* . 47:445-476.

Carpita N.C. and Gibeaut D.M. (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *Plant J*. 3: 1-30.

Carpita N.C. and McCann M.C. (2010) The Maize Mixed-Linkage (1→3),(1→4)-β-D-Glucan Polysaccharide Is Synthesized at the Golgi Membrane. *Plant Physiology* 153:1362-1371.

Catoire L., Pierron M., Morvan C., Du Penhoat C.H. and Goldberg R. (1998). Investigation of the action patterns of pectinmethylesterase isoforms through kinetic analyses and NMR spectroscopy. Implications In cell wall expansion. *J. Biol. Chem.* 273: 33150-33156.

Chen M.H., Sheng J., Hind G., Handa A.K. and Citovsky V. (2000). Interaction between the tobacco mosaic virus movement protein and host cell pectin methylesterases is required for viral cell-to-cell movement. *EMBO J*. 19: 913-920.

Christensen A.H. and Quail P.F. (1996). Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. *Trans. Res.* 5:213-218.

Christensen T.M.I.E., Nielsen J.E., Kreiberg J.D., Rasmussen P. and Mikkelsen J.D. (1998). Pectin methyl esterase from orange fruit: characterization and localization by in-situ hybridization and immunohistochemistry. *Planta* 206: 493-503.

Ciardiello M.A., D'Avino R., Amoresano A., Tuppo L., Carpentieri A., Carratore V., Tamburrini M., Giovane A., Pucci P. and Camardella L. (2008). The peculiar structural features of kiwi fruit pectin methylesterase: Amino acid sequence, oligosaccharides structure, and modelling of the interaction with its natural proteinaceous inhibitor. *Proteins* 71: 195-206.

Collmer, A. and Keen, N. T., (1986). The role of pectic enzymes in plant pathogenesis. *Annu. Rev. of Phytopathol*. 24:383-409.

Cosgrove D.J. (1997) Assembly and enlargement of the primary cell wall in plants. *Annu. Rev. Cell Dev. Biol*. 13, 171–201.

Cosgrove D.J. (2005). Growth of the plant cell wall. *Mol. Cell. Biol.*, 6: 850-861.

D'Avino, R., Camardella, L., Christensen, T.M., Giovane, A. and Servillo, L. (2003). Tomato pectin methylesterase: modeling, fluorescence, and inhibitor interaction studies-comparison with the bacterial (Erwinia chrysanthemi) enzyme. *Proteins* 53: 830-839.

Denes J.M., Baron A., Renard C.M., Pean C. and Drilleau J.F. (2000). Different action patterns for apple pectin methylesterase at pH 7.0 and 4.5. *Carbohydr. Res*. 327: 385-393.

De Paepe A., Vuylsteke M., Van Hummelen P., Zabeau M. and van der Straeten D. (2004). Transcriptional profiling by cDNA-AFLP and microarray analysis reveals novel insights into the early response to ethylene in Arabidopsis. *Plant Journal* 39: 537-559.

Dedeurwaerder S., Menu-Bouaouiche L., Mareck A., Lerouge P. and Guerineau F. (2008). Activity of an atypical Arabidopsis thaliana pectin methyl-esterase. *Planta*. 229: 311-321.

Di Matteo A., Giovane A., Raiola A., Camardella L., Bonivento D., De Lorenzo G., Cervone F., Bellincampi D. and Tsernoglou D. (2005). Structural Basis for the Interaction between Pectin Methylesterase and a Specific Inhibitor Protein. *Plant Cell* 17: 849-858.

Divol F., Vilaine F., Thibivilliers S., Amselem J., Palauqui J.C., Kusiak C. and Dinant S. (2005). Systemic response to aphid infestation by Myzus persicae in the phloem of Apium graveolens. *Plant. Mol. Biol*. 57: 517-540.

Dongowski G and Bock W (1984). On the effects of molecular-parameters of pectin-substrates on the activities of pectin-esterases from Aspergillus niger and from higher plants. *Nahrung-Food* 28: 507-516.

D'Ovidio R. and Anderson O.D. (1994). Purification and molecular characterization of a soybean polygalacturonase-inhibiting protein. *Theor. Appl. Genet.* 88:759-763.

D'Ovidio R., Mattei B., Roberti S. and Bellincampi D. (2004a). Polygalacturonases, polygalacturonaseinhibiting proteins and pectic oligomers in plant–pathogen interactions. *Biochim Biophys Acta* 1696: 237-244.

Dorokhov Y.L., Frolova O.Y., Skurat E.V., Ivanov P.A., Gasanova T.V., Sheveleva A.A., Ravin N.V., Makinen K.M., Klimyuk V.I., Skryabin K.G., Gleba Y.Y. and Atabekov J.G. (2006a). A novel function for a ubiquitous plant enzyme pectin methylesterase: The enhancer of RNA silencing. *FEBS Lett*. 580: 3872-387.

Dorokhov Y.L., Skurat E.V., Frolova O.Y., Gasanova T.V., Ivanov P.A., Ravin N.V., Skryabin K.G., Makinen K.M., Klimyuk V.I., Gleba Y.Y. and Atabekov, J.G. (2006b). Role of the leader sequence in tobacco pectin methylesterase secretion. *FEBS Lett*. 580: 3329-3334.

Duvetter T., Fraeye I., Sila D.N., Verlent I., Smout C., Hendrickx M. and Van Loey A. (2006). Mode of de-esterification of alkaline and acidic pectin methyl esterases at different pH conditions. *J Agric FoodChem* 54: 7825-7831.

Downie B., Dirk L.M.A., Hadfeild K.A., Wilkins T.A., Bennet A.B. and Bradfrod K.J. (1998). A gel diffusion assay for quantification of pectin methylesterase activity. *Analytical Biochemistry* 264:149-157.

Dvorák J. and Zhang H.B. (1990). Variation in repeated nucleotide sequences sheds light on the phylogeny of the wheat B and G genomes. *Proc Natl Acad Sci USA* 87:9640-9644.

Dvorák J., Terlizzi P.D., Zhang H.B. and Resta P. (1993). The evolution of polyploid wheats: identification of the A genome donor species. *Genome* 36: 21-31.

Farrokhi N., Burton R.A., Brownfield L., Hrmova M., Wilson S.M., Bacic A. and Fincher G.B. (2006) Plant cell wall biosynthesis: genetic, biochemical and functional genomics approaches to the identification of key genes. *Plant Biotechnology Journal*.4: 145–167.

Feldman M., Liu B., Segal G., Abbo S., Levy A.A. and Vega J.M. (1997) Rapid elimination of low-copy DNA sequences in polyploid wheat: A possible mechanism for differentiation of homoeologous chromosomes. *Genetics* 147, 1381–1387.

Fleming A., McQueen-Mason S., Mandel T. and Kuhlemeier C. (1997). Induction of leaf primordia by the cell wall protein expansin. *Science* 276: 1415-1418.

Francis K.E., Lam S.Y. and Copenhaver G.P. (2006). Separation of Arabidopsis pollen tetrads is regulated by QRT1, a pectin methylesterase gene. *Plant Physio*l 142: 1004-1013.

Fries M., Ihrig J., Brocklehurst K., Shevchik V.E. and Pickersgill R.W. (2007). Molecular basis of the activity of the phytopathogen pectin methylesterase. *EMBO J* 26: 3879-3887.

Futamura N., Mori H., Kouchi H. and Shinohar K. (2000) Male Flower-Specific Expression of Genes for Polygalacturonase, Pectin Methylesterase and β-1,3-Glucanase in a Dioecious Willow (Salix gilgiana Seemen) *Plant and Cell Physiology* 41(1): 16-26.

Geisler-Lee J., Geisler, M., Coutinho, P.M., Segerman, B., Nishikubo, N., Takahashi, J., Aspeborg, H., Djerbi, S., Master, E., Andersson-Gunneras, S., Sundberg, B., Karpinski, S., Teeri, T.T., Kleczkowski, L.A., Henrissat, B. and Mellerowicz, E.J. (2006). Poplar carbohydrate-active enzymes. Gene identification and expression analyses. *Plant Physiology* 140, 946-962.

Giovane A., Balestrieri C., Quagliulo L, Castaldo D. and Sevillo L. (1995) A glycoprotein inhibitor of pectin methylesterase in kiwi fruit: Purification by affinity chromatography and evidence of a ripening-related precursor . *Eur. J. Biochem*. 233, 926-929.

Giovane A., Servillo L., Balestrieri C., Raiola A., D'Avino R., Tamburrini M., Ciardiello M.A. and Camardella L. (2004). Pectin methylesterase inhibitor. *Biochim. Biophys. Acta* 1696: 245-252.

Glazebrook, J. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. (2005) *Annu. Rev. Phytopathol*. 43, 205–227.

Goldberg R., Morvan C., Jauneau A. and. Jarvis M.C. (1996). Methyl-esterification, deesterification and gelation of pectins in the primary cell wall. In Pectins and Pectinases. *Progress in Biotechnology*, Vol. 14 Visser, J. and Voragen, A.G.J., eds, 151-172, Elsevier Science.

Hall T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp*. Ser. 41: 95-98.

Hasunuma T., Fukusaki E. and Kobayashi A. (2004). Expression of fungal pectin methylesterase in transgenic tobacco leads to alteration in cell wall metabolism and a dwarf phenotype. *J. Biotechnol*. 111: 241-251.

Haun J.R. (1973). Visual Quantification of Wheat Development. *Agronomy Journal* 65:116-119.

Hématy Johnson K.L., Jones B.J., Bacic A. and Schultz C.J. (2003). The Fasciclin-Like Arabinogalactan Proteins of Arabidopsis. A Multigene Family of Putative Cell Adhesion Molecules. *Plant Physiology* 133:1911-1925.

Hong M.J., Kim D.Y., Lee T.G., Jeon W.B. and Seo Y.M. (2010) Functional characterization of pectin methylesterase inhibitor (PMEI) in wheat. *Genes Genet. Syst.* 85:97-106

Hothorn M., Wolf S., Aloy P., Greiner S. and Scheffzek K. (2004). Structural insights into the target specificity of plant invertase and pectin methylesterase inhibitory proteins. *Plant Cell* 16: 3437-3447.

Irifune K., Nishida T., Egawa H. and Nagatani A. (2004). Pectin methylesterase inhibitor cDNA from kiwi fruit. *Plant Cell Rep*. 23: 333-338.

Janni M., Sella L., Favaron F., Blechel A.E., De Lorenzo G. and D'Ovidio R. (2008). The expression of a bean pgip in transgenic wheat confers increased resistance to the fungal pathogen Bipolaris sorokiniana. *Mol. Plant-Microbe Interact*. 21: 171-177.

Jenkins J., Mayans O., Smith D., Worboys K. and Pickersgill R.W. (2001). Three dimensional structure of Erwinia chrysanthemi pectin methylesterase reveals a novel esterase active site. *J. Mol. Biol*. 305: 951-960.

Jenkins J. and Pickersgill R. (2001). The architecture of parallel beta-helices and related folds, *Progress in Biophysics and Molecular Biology* 77: 111-175.

Jiang L., Yang S.L., Xie L.F., Puah C.S., Zhang X.Q., Yang W.C., Sundaresan V. and Ye D. (2005). VANGUARD1 Encodes a pectin methylesterase that enhances pollen tube growth in the Arabidopsis style and transmitting tract. *Plant Cell* 17: 584-596.

Johansson K., El Ahmad M., Friemann R., Jornvall H., Markovic O. and Eklund H. (2002). Crystal structure of plant pectin methylesterase. *FEBS Lett*. 514: 243-249.

Johnson K.L., Jones B.J., Bacic A. and Schultz C.J. (2003). The Fasciclin-Like Arabinogalactan Proteins of Arabidopsis. A Multigene Family of Putative Cell Adhesion Molecules. *Plant Physiology* 133:1911-1925.

Jolie R.P., Duvetter T., Van Loey A.M. and Hendrickx E. (2010) Pectin methylesterase and its proteinaceous inhibitor: a review. *Carbohydrate Research* 345: 258.

Jones H.D. and Shewry P.R. (2009) Transgenic wheat, barley and oats. *Methods in Molecular Biology* 478

Juge N. (2006). Plant protein inhibitors of cell wall degrading enzymes. *Trends Plant Sci*. 11: 359-367.

Kars, I., Krooshof, G. H., Wagemakers, L., Joosten, R., Benen, J. A. and van Kan, J. A. (2005). Necrotizing activity of five Botrytis cinerea endopolygalacturonases produced in Pichia pastoris. *The Plant Journal* 43:213-225.

Kihara T. (1924) Cytologische und genetische studien bei wichtigen getreidearten mit besonderer rucksicht auf das verhalten der chromosomen und die sterilitat in den bastarden. In Men. Coli. Sci. Ser. B. Kyoto.

Kohn R., Dongowski G. and Bock W. (1985). The distribution of free and esterified carboxyl groups within the pectin molecule after the influence of pectin esterase from Aspergillus niger and oranges. *Nahrung-Food* 29: 75-85

Konarev V.G. (1983). The nature and origin of wheat genomes on the data of grain protein immunochemistry of grain proteins. *Cereal Chem.* 56: 272-278.

Korner E., von Dahl C.C., Bonaventure G. and Baldwin I.T. (2009). Pectin methylesterase NaPME1 contributes to the emission of methanol during insect herbivory and to the elicitation of defence responses in Nicotiana attenuata. *Journal of Experimental Botany* 60: 2631-2640.

Laemmli U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.

Lafiandra D., Ciaffi M. and Benedettelli S. (1993). Seed storage proteins of wild wheat progenitors. In: Damaina A.B (ed). *Biodiversity and wheat improvement*. John Wiley and Sons.

Large E.C. (1954). Growth Stages in Cereals, Illustration of the Feekes Scale. *Plant Pathology* 3:128-129.

Lee, J.Y. and Lee, D.H. (2003). Use of serial analysis of gene expression technology to reveal changes in gene expression in Arabidopsis pollen undergoing cold stress. *Plant Physiology*., 132, 517-529.

Limberg, G., Korner, R., Buchholt, H.C., Christensen, T.M., Roepstorff, P. and Mikkelsen, J. D. (2000). Analysis of different de-esterification mechanisms for pectin by enzymatic fingerprinting using endopectin lyase and endopolygalacturonase II from A. Niger. *Carbohydr. Res*. 327:293-307.

Lionetti V., Raiola A., Camardella L., Giovane a., Obel N., Pauly M., Favaron F., Cervone F. and Bellincampi D. (2007) Overexpression of Pectin Methylesterase Inhibitors in Arabidopsis Restricts Fungal Infection by Botrytis cinerea1. *Plant Physiology*143: 1871–1880.

Livak K.J. and Schmittgen T.D. (2001). Analysis of relative gene expression data using Real-Time 17 quantitative PCR and $2^{-\Delta\Delta CT}$ method. *Methods* 25: 402-8.

Lohani S., Trivedi P.K. and Nath P. (2004). Changes in activities of cell wall hydrolases during ethylene-induced ripening in banana : effect of 1-MCP, ABA and IAA. *Postharvest Biology and Technology* 31: 119-126.

Ly-Nguyen B., Van Loey A.M., Smout C., Verlent I., Duvetter T. and Hendrickx M.E. (2004). Effect of intrinsic and extrinsic factors on the interaction of plant pectin methylesterase and its proteinaceous inhibitor from kiwi fruit. *J. Agric. Food Chem*. 52: 8144-8150.

Markovic O. and Janecek S. (2004). Pectin methylesterases: sequence-structural features and phylogenetic relationships. *Carbohydr. Res*. 339: 2281-2295.

McMillan G.P., Hedley D., Fyffe L. and Pérombelon M.C.M. (1993). Potato resistance to soft-rot erwinias is related to cell wall pectin esterification. *Physiol Mol Plant Pathol.* 42: 279-289.

Marty P., Jouan B., Bertheau Y., Vian B. and Goldberg R. (1997). Charge density in stem cell walls of solanum tuberosum genotypes and susceptibility to blackleg. *Phytochemistry* 44: 1435-1441.

Mattei B., Raiola A., Caprari C., Federici L., Bellincampi D., De Lorenzo G., Cervone F., Giovane A. and Camardella L. (2002). Studies on plant inhibitors of pectin modifying enzymes: polygalacturonase-inhibiting protein (PGIP) and pectin methylesterase inhibitor (PMEI). In *Carbohydrate Bioengineering-Interdisciplinary approaches*, T.T.Teeri, B.Svensson, H.J.Gilbert, and T.Feizi, eds pp. 160-167.

Micheli F., Sundberg B., Goldberg R. and Richard L. (2000). Radial distribution pattern of pectin methylesterases across the cambial region of hybrid aspen at activity and dormancy. *Plant Physiology* 124: 191-199.

Micheli F. (2001). Pectin methylesterases: cell wall enzymes with important roles in plant physiology. *Trends Plant Science* 6: 414-419.

Moscatiello R., Mariani P., Sanders D. and Maathuis F.J.M. (2006). Transcriptional analysis of calcium-dependent and calcium-independent signalling pathways induced by oligogalacturonides. *Journal of Experimental Botany* 57: 2847-2865.

Moustacas AM., Nari J., Borel M., Noat G. and Ricard J. (1991) Pectin methylesterase, metal ions and plant cell-wall extension. *Biochem. J.* 279: 351-354

Ner-Goan H., Halachmi1 R., Savaldi-Goldstein S., Rubin E., Ophir R. and Fluhrl R. (2004) Intron retention is a major phenomenon in alternative splicing in Arabidopsis. *The Plant Journal* 39: 877–885.

Nesbitt M. & Samuel D. (1995) From staple crop to extinction? The archeology and history of the hulled wheats. In Hulled Wheats. *Proceedings of the First International Workshop on Hulled Wheats*, 1st ed. 40-99.

Orth R.A. & Shellenberger J.A (1988) Origin, production, and utilization of wheat. *In Wheat Chemistry and Tecnology*, 1-14, vol.3. Pomeranz Y., (ed) American Association of Cereal Chemists; St Paul, MN, USA.

Peaucelle A., Louvet R., Johansen J.N., Hofte H., Laufs P., Pelloux J and Mouille G. (2008)Arabidopsis Phyllotaxis Is Controlled by the Methyl-Esterification Status of Cell-Wall Pectins. *Current Biology* 18:1943–1948.

Pelloux J., Rustérucci C. and Mellerowicz E.J.(2007) New insights into pectin methylesterase structure and function. *Trends in Plant Science* Vol.12 No.6.

Penuelas J., Filella I., Stefanescu C. and Llusia J. (2005). Caterpillars of Euphydryas aurinia (Lepidoptera : Nymphalidae) feeding on Succisa pratensis leaves induce large foliar emissions of methanol. *New Phytol*. 167: 851-857.

Perez S., Rodríguez-Carvajal M.A and Doco T. (2003). A complex plant cell wall polysaccharide: rhamnogalacturonan II. A structure in quest of a function. *Biochimie* 85: 109-121.

Pilling J., Willmitzer L. and Fisahn J. (2000). Expression of a Petunia inflata pectin methyl esterase in Solanum tuberosum L. enhances stem elongation and modifies cation distribution. *Planta* 210: 391-399.

Pina C., Pinto F., Feijo J.A. and Becker J.D. (2005). Gene family analysis of the Arabidopsis pollen transcriptome reveals biological implications for cell growth, division control, and gene expression regulation. *Plant Physiol* 138: 744-756.

Raiola A., Camardella L., Giovane A., Mattei B., De Lorenzo G., Cervone F. and Bellincampi D. (2004). Two Arabidopsis thaliana genes encode functional pectin methylesterase inhibitors. *FEBS Lett*. 557: 199-203.

Raiola A., Lionetti V., Elmaghraby1 I., Immerzeel P., Mellerowicz E.J., Salvi G., Cervone F. and Bellincampi D. (2011) Pectin Methylesterase Is Induced in Arabidopsis upon Infection 1 and Is Necessary for a Successful Colonization by Necrotrophic Pathogens. *Molecular Plant Microbe Interaction*, doi: 10.1094/MPMI-07-10-0157

Rausch, T. and Greiner, S. (2004). Plant protein inhibitors of invertases. *Biochim. Biophys. Acta* 1696, 253-261

Rautengarten C. (2005). Inferring hypotheses on functional relationships of genes: analysis of the Arabidopsis thaliana subtilase gene family. *PLoS Comput. Biol.* 1: 297-312.

Reiter F.D. (2002) Biosynthesis and properties of the plant cell wall. *Current Opinion in Plant Biology* 5:536–542.

Rhee S.Y., Osborne E., Poindexter P.D. and Somerville C.R. (2003). Microspore separation in the quartet 3 mutants of Arabidopsis is impaired by a defect in a developmentally regulated polygalacturonase required for pollen mother cell-wall degradation. *Plant Physiology* 133: 1170-1180.

Ren C. and Kermode A.R. (2000). An increase in pectin methyl esterase activity accompanies dormancy breakage and germination of yellow cedar seeds. *Plant Physiology* 124: 231-242.

Ricard J. and Noat G. (1986). Electrostatic effects and the dynamics of enzyme reactions at the surface of plant cells. 1. A theory of the ionic control of a complex multi-enzyme system. *Eur. J. Biochem.* 155: 183-190.

Ridley B.L., O'Neill M.A., Mohnen D. (2001) Pectins: structure, biosynthesis, and oligogalacturonide-related signalling. *Phytochemistry* 57: 929–967.

Riley R. and Chapman V. (1958). Genetic control of cytologically diploid behaviour of hexaploid wheat. *Nature* 182: 713-715.

Röckel N., Wolf S., Kost B., Rausch T. and Greiner S. (2008). Elaborate spatial patterning of cell-wall PME and PMEI at the pollen tube tip involves PMEI endocytosis, and reflects the distribution of esterified and de-esterified pectins. *Plant Journa*l 53 133-143.

Sakamura T. (1918) Kurze mitteilung uber die chromosomenzahlen und die verwandt schaftsverhaltnisse der Triticum arten. Tokyo.

Saher S., Piqueras A., Hellin E and Olmos E.(2005) Pecin methylesterase and pectins in normal and hyperhydric shoots of carnation cultured in vitro. *Plant Physiol.Biochem*. 43:155-159.

Sambrook J., Fritsch E.F. and Maniatis T. (1989). Molecular Cloning-A Laboratory Manual, 2nd ed. *Cold Spring Harbor Laboratory* Press, Cold Spring Harbor, NY, U.S.A.

Sears E.R. (1956). The B genome of Triticum. *Wheat Info. Serv*. 4: 8-10.

Sears R.G. and Deckard E.L., (1982). Tissue culture variability in wheat: callus induction and plant regeneration. *Crop Sci.* 22: 546-550.

Siedlecka A., Wiklund S., Péronne M.A., Micheli F., Leśniewska J., Sethson I., Edlund U., Richard L., Sundberg B. and Mellerowicz E.J. (2008). Pectin Methyl Esterase Inhibits Intrusive and Symplastic Cell Growth in Developing Wood Cells of Populus. *Plant Physiology* 146: 554-565.

Stephenson, M.B. and Hawes, M.C. (1994). Correlation of pectin methylesterase activity in root caps of pea with root border cell separation. *Plant Physiol*., 106, 739-745.

Tai T.H. and Tanksley S.D., (1990). A rapid and inexpensive method for isolation of total DNA from dehydrated plant tissue. *Plant Mol Biol Rep*; 8:297-303.

Thakur B.R., Singh R.K. and Handa A.K. (1996). Effect of an antisense pectin methylesterase gene on the chemistry of pectin in tomato (Lycopersicon esculentum) juice. *Journal of Agricultural and Food Chemistry* 44: 628-630.

Tian G.W., Chen M.H., Zaltsman A. and Citovsky V. (2006). Pollen-specific pectin methylesterase involved in pollen tube growth. *Dev. Biol*. 294: 83-91.

Thompson G.A. and Goggin F.L. (2006). Transcriptomics and functional genomics of plant defence induction by phloem-feeding insects. *Journal of Experimental Botany* 57: 755-766.

Tian G.W., Chen M.H., Zaltsman A. and Citovsky V. (2006). Pollen-specific pectin methylesterase involved in pollen tube growth. *Dev. Biol.* 294: 83-91.

Valette-Collet O., Cimerman A., Reignault P., Levis C. and Boccara M. (2003). Disruption of Botrytis cinerea pectin methylesterase gene Bcpme1 reduces virulence on several host plants. *Mol. Plant Microbe Interact*. 16: 360-367.

van Alebeek G.J., van Scherpenzeel K., Beldman G., Schols H.A. and Voragen A.G. (2003). Partially esterified oligogalacturonides are the preferred substrates for pectin methylesterase of Aspergillus niger. *Biochem. J.* 372: 211-218.

Venkatesh B. (2002). Characterization of bacterial lipopolysaccharides (Pseudomonas syringae pv. tomato and Pseudomonas syringae pv. apii) and pectins of tomato and celery plants (Lycopersicon esculentum and Apium graveolens) regarding their possible role in host/pathogen interaction. PhD thesis, University of Göttingen, Germany.

Vogel J. (2008) Unique aspects of the grass cell wall. *Current Opinion in Plant Biology* 11:301–307.

von Dahl C.C., Havecker M., Schlogl R. and Baldwin I.T. (2006). Caterpillar-elicited methanol emission: a new signal in plant-herbivore interactions. *Plant Journal* 46: 948-960.

Vorangen A.G.J., Coenen G.J., Verhoef R.P. and Schols H.A. (2009)Pectin, a versatile polysaccharide present in plant cell walls. *Struct Chem*. 20:263–275.

Vorwerk S., Somerville S. and Somerville C.(2004) The role of plant cell wall polysaccharide composition in disease resistance. *Trends in Plant Scienc*e Vol.9 (4).

Yoder, M.D., Keen, N.T. and Jurnak, F. (1993). New domain motif: The structure of pectate lyase C, a secreted plant virulence factor. *Science* 260, 1503-1507.

Wakabayashi K., Chun J.-P., and Huber D.J. (2000). Extensive solubilisation and depolymerization of cell-wall polysaccharides during avocado (Perseaamericana) ripening involves concerted action of polygalacturonase and pectinmethyl-esterase. *Physiologia Plantarum* 108: 345-352.

Wakeley P.R., Rogers H.J., Rozycka M., Greenland A.J. and Hussey P.J. (1998). A maize pectin methylesterase-like gene, ZmC5, specifically expressed in pollen. *Plant Mol. Biol.* 37: 187-192.

Warren A-N., Jones M.D. and Lechler R. (1997)Splicing by overlap extension by PCR using asymmetric amplification: an improved technique for the generation of hybrid proteins of immunological interest *Gene* 186: 29–35.

Weeks J.T., Anderson O.D. and Blechl A.E. (1993). Rapid production of multiple independent lines of fertile transgenic wheat (Triticum aestivum). *Plant Physiology*. 102: 1077-1084.

Wrigley C.W. (2009) Wheat: a unique grain for the world. In Wheat Chemistry and Tecnology, 1-17 vol.4. Khan K. and Shewry P.R. (ed) *American Association of Cereal Chemist*s; St Paul, MN, USA.

Weeks J.T., Anderson O.D. and Blechl A.E. (1993). Rapid production of multiple independent lines of fertile transgenic wheat (Triticum aestivum). *Plan Physiology* 102: 1077-1084.

Wen F.S., Zhu Y.M. and Hawes M.C. (1999). Effect of pectin methylesterase gene expression on pea root development. *Plant Cell* 11: 1129-1140.

Wietholter N., Graebner B., Mierau M., Mort A.J. and Moerschbacher B.M. (2003). Differences in the methyl ester distribution of homogalacturonans from near-isogenic wheat lines resistant and susceptible to the wheat stem rust fungus. *Mol. Plant Microbe Interact*.16: 945-952.

Wolf S., Grsic-Rausch S., Rausch T. and Greiner S. (2003). Identification of pollen-expressed pectin methylesterase inhibitors in Arabidopsis. *FEBS Lett.* 555: 551-555.

Wolf S., Mouille G. and pelloux J. (2009a). Homogalacturonan Methyl-Esterification and Plant *Development. Mol. Plant* 2: 851-869.

Wolf S., Rausch T. and Greiner S. (2009b). The N-terminal pro region mediates retention of unprocessed type I PME in the Golgi apparatus. *Plant J*. 58: 361-375.

Zadoks J.C., Chang T.T. and Konzak C.F. (1974). A decimal code for the growth stages of cereals. *Weed Res*. 14: 415-421.

Zhang G.Y., Feng J., Wu J. and Wang X.W. (2010) BoPMEI1, a pollen-specific pectin methylesterase inhibitor, has an essential role in pollen tube growth. *Planta* 231:1323–1334.

Zohary D. and Feldman M. (1962). Hybridization between amphidiploids and the evolution of polyploids in the wheat (Aegilops-Triticum) group. *Evolution* 16:44-61.

Aknowledgements

First of all I would like to thanks my professor Renato D'Ovidio to give me the possibility to do my PhD in his laboratory of Plant Physiology and Biotechnology. He received me in his laboratory seven years ago and little by little he taught me how to make research but above all he taught me to make research with passion. All my gratitude for believed in me all this time.

I would like to thank my French professor Thierry Giardina to have accepted to be my thesis co-director and gives me the opportunities to work in his laboratory. Thanks for sustaining me in all difficulties and also for giving me the opportunities to make a beautiful experiences in Marseille.

Thanks also to Prof. Stefania Masci, the coordinator of my PhD course, for her professional behaviour as well as for her availability. Thanks to her and Prof. Domenico Lafiandra for giving me hospitality in their laboratories.

I would like to thank the Dipartimento di Agrobiologia e Agrochimica and the director professor Saladino, for accepted me for my thesis work and also to the administration people for their help with bureaucratic matters.

Thank to the Insitut de Science Moléculaire de Marseille (ISM2)and its director Prof. Rodriguez to give me the opportunity to make a part of my thesis work in their laboratories. Thanks to the Équipe BiosCience, its director Dr. Reglier and all members to receive me in their structure and let me feel a part of the equipe from the beginning of my stage there.

I would like to thaks the University Italo-Francese that financially sustain my stage in France.

My immense gratitude is of course to the people of my laboratory, Dr. Janni Michela, Dr. Volpi Chiara, Raviraj Kalunke and Samuela Palombieri. In particular I would like to thank Dr. Janni Michela. I learned to much from her, from the beginning of my stage in the laboratory seven years ago until now. She was always available to answer to all my question, give me useful advice and support me during my research.

In this sense, I would like also to thank the people from the laboratory of Genetics and Biochemistry of the Plant Proteins with which I share every day my lunch-time and also my research problems. Thanks to Francesco, Linda, Federica, Daniela, Lina Maria, Roberta, Gaetan and in particular to Eleonora. We met at university course and we became true friends. Thanks Ele for all you give me during these years, I known that I can rely on you. Thanks to Tiziana for her friendship and for making funny moments in the laboratory.

I would like also to thank the people of the french laboratory. Thanks to my French Family to let me feel everyday at home, to be everyday available to answer to my question, to sustain me in some difficult moments. Thanks to “ma petite poule” Alexandra, to be always available to help me, thanks to Mickaël and Sophie to sustain me every time and for their friendship, thanks to Ange, Marine, Nadia, Lydie, Eloine, thanks to my Radia for our long discussion, for your friendship, for your love.

My final thoughts are for my family. Thank to my father and my sister for believing in me, for supporting me during threes three years and let me feel loved even when I was far away from you. Thanks to my little Niccolò for giving me some sweet moments during difficult periods. Thanks to my mother: you are not here with your body but I felt you very close to me and without your help I could not get through this period of my life.

And the last but not the least was Giuseppe, my future husband. Thank you to stay close to me even if we were far away hundreds kilometres, thank you to have been patient with me and always available to listen to me, thank you to be simply Giuseppe.