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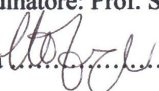
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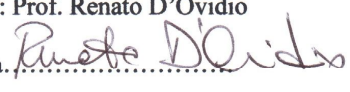
Enhancement of the wheat defence response to fungal pathogens
by limiting the degradation of the pectin component of the cell wall

BIO04

Coordinatore: Prof. Stefania Masci

Firma 

Tutor: Prof. Renato D'Ovidio

Firma 

Dottoranda: Chiara Volpi

Firma 

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RIASSUNTO

I patogeni fungini rappresentano una delle minacce più serie per la produzione agricola. La costituzione di varietà in grado di resistere all'aggressione di più patogeni rappresenta una delle strategie più promettenti nei programmi di miglioramento genetico per la resistenza. 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 incontrate dai patogeni microbici durante la colonizzazione del tessuto ospite. Per superare questo ostacolo, la maggior parte dei patogeni fungini produce una varietà 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 è controllata dall'inibitore della pectin metilesterasi (PMEI). Poiché le pectine altamente metilesterificate sono meno suscettibili all'azione di enzimi pectici come le endo-PG, l'inibizione di PME endogene operata dalle PMEI può avere un ruolo indiretto nella difesa, limitando l'azione delle PG secrete da patogeni fungini. Per verificare l'ipotesi di incrementare la resistenza del frumento modificando il grado di metilesterificazione della pectina, sono state prodotte linee di frumento duro cv. Svevo esprimenti l'inibitore della pectin metilesterasi AcPMEI (da *Actinidia chinensis*) o AtPMEI-1 (da *Arabidopsis thaliana*). Entrambi gli inibitori, AcPMEI e AtPMEI-1, conferiscono al frumento una maggiore attività inibitoria della PME endogena. Soltanto una linea esprime la AtPMEI-1 mostra una ridotta attività della PME endogena mentre 12 linee esprimenti la AcPMEI mostrano diversi livelli di riduzione dell'attività PME. Tre linee *Acpmei* sono state caratterizzate ulteriormente relativamente al livello di espressione e all'attività della proteina transgenica.

La proteina AcPMEI è risultata correttamente localizzata nell'apoplasto. Le due linee *Acpmei* con un alto livello di espressione dell'inibitore, MJ15-151 e MJ15-69, non mostrano evidenti differenze fenotipiche rispetto alle piante di controllo nonostante presentino un significativo incremento del grado di pectin metilesterificazione di circa il 20% e 32% rispettivamente e una differente distribuzione dei metilesteri. L'incremento del grado di metilazione e la distribuzione modificata dei gruppi metilici della pectina è stata correlata con una significativa riduzione dei sintomi della malattia causati dai patogeni fungini *Bipolaris sorokiniana* e *Fusarium graminearum*, che infettano rispettivamente in modo prevalente le foglie o la spiga del frumento.

Per proteggere ulteriormente la componente pectica della parete cellulare, è stato intrapreso un programma di piramidazione volto a combinare la PMEI e l'inibitore proteico della poligalatturonasi (PGIP). È stato effettuato l'incrocio tra le linee *Acpmei* e la nuova linea transgenica di frumento duro sviluppata esprimente il gene *Pvpgip2* e la progenie F₁ è stata analizzata per la presenza di entrambi i transgeni.

Infine, per selezionare ulteriori patogeni fungini da analizzare nell'incrocio *Acpmei/Pvpgip2* e nelle linee parentali, sono stati eseguiti esperimenti di infezione con il biotrofo fungino *Claviceps purpurea* su una linea transgenica stabile di frumento tenero esprimente il gene *Pvpgip2*. Una leggera, seppur significativa riduzione dei sintomi è stata riscontrata nella linea transgenica rispetto alle piante di controllo.

ABSTRACT

Fungal pathogens represent one of the most serious threats to agriculture production. The development of crop varieties that simultaneously control different pathogens is an attracting strategy to increase host plant resistance. 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). The activity of PME is regulated by specific protein inhibitors (PMEIs). Since highly methylesterified pectin can be less susceptible to hydrolysis by pectic enzymes such as fungal endopolygalacturonases (endo-PGs), the inhibition of endogenous PME by PMEI might increase pectin resistance to degradation by fungal PGs. In order to verify the possibility to improve wheat resistance by modifying the degree of pectin methylesterification, a number of wheat *Triticum durum* cv. Svevo lines expressing the pectin methylesterase inhibitors either AcpMEI (from *Actinidia chinensis*) or AtPMEI-1 (from *Arabidopsis thaliana*) has been produced. Both AcpMEI and AtPMEI-1 endow wheat with a stronger inhibitory activity of endogenous PME. Only one line expressing AtPMEI-1 shows reduced endogenous PME activity while 12 lines expressing AcpMEI show different levels of reduced PME activity. Three *Acpmei* lines were further characterized on the level of expression and the activity of the transgenic protein. AcpMEI was found to be correctly targeted to the apoplast. The two *Acpmei* lines expressing high level of the inhibitor, MJ15-151 and MJ15-69, showed no obvious phenotypic differences with the control plants but a significant increase of the degree of methylesterification (DM) of about 20% and 32%, and a different methylester distribution compared to the wild-type plants, respectively. This increased DM and modified methyl distribution of pectin was correlated with a significant reduction of disease symptoms caused by the foliar fungal pathogen *Bipolaris sorokiniana* or the floral pathogen *Fusarium graminearum*.

To further protect the cell wall pectic component, a pyramiding approach has been undertaken to combine PMEI and the polygalacturonase-inhibiting protein (PGIP). The cross between the *Acpmei* lines and a new developed durum wheat transgenic line expressing *Pvpgip2* has been performed and the F₁ progeny analyzed for the presence of both transgenes.

Finally, to select additional fungal pathogens to be tested in the cross *Acpmei/Pvpgip2* and in the parental lines, infection experiments with the biotrophic fungal pathogen *Claviceps purpurea* were performed on a stable transgenic bread wheat line expressing *Pvpgip2* and a slight, even significant, reduction of symptoms in the transgenic line compared to the control plants was observed.

1. Introduction

1.1 The Wheat

The cultivation of wheat (*Triticum* spp.) reaches far back into history. Wheat was one of the first domesticated food crops and for 8.000 years has been the basic staple food of the major civilizations of Europe, West Asia and North Africa. Today, wheat is grown on more land area than any other commercial crop with its 223 million of hectares and continues to be the most important food grain source for humans (Fig. 1.1). Its production leads all crops, including rice, maize and potatoes. World wheat production reached about 690 million of tonnes (Mt) in 2008 (Table 1.1); the ten major wheat producing countries are China (almost 110 Mt) followed by India (75.8 Mt), United States of America (55.8 Mt), Russian Federation (49.3 Mt), France (32.7 Mt), Pakistan (23.2 Mt), Germany (20.8 Mt), Canada (20 Mt), Turkey (17.2 Mt), and Argentina (16.4 Mt).

Wheat is a major diet component in a lot of countries because of the wheat plant's agronomic adaptability, ease of protein and starch digestion, ease of grain storage and ease of converting grain into flour for making edible, palatable and satisfying foods. 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 protein, when balanced by other foods that supply certain amino acids such as lysine and methionine, is an efficient source of protein. Wheat is also a popular source of animal feed, particularly in years where harvests are adversely affected by rain and significant quantities of the grain are made unsuitable for food use. Such low-grade grain is often used by the industry to make adhesives, paper additives and several other products. In addition, in the last few years there has been an increase in the use of wheat for biofuel and biodiesel production (www.nap.edu).

Tab. 1.1 Data FAO 2008 (from www.fao.org). Wheat production and wheat area harvested in different countries and in the world.

	Wheat production (tonnes)	Wheat area harvested (Ha)
World	689.945.712	223.564.097
Africa	21.128.723	9.457.290
America	118.725.565	41.416.717
Asia	280.295.319	97.498.781
Europe	248.055.737	61.596.972
Oceania	21.740.368	13.594.337

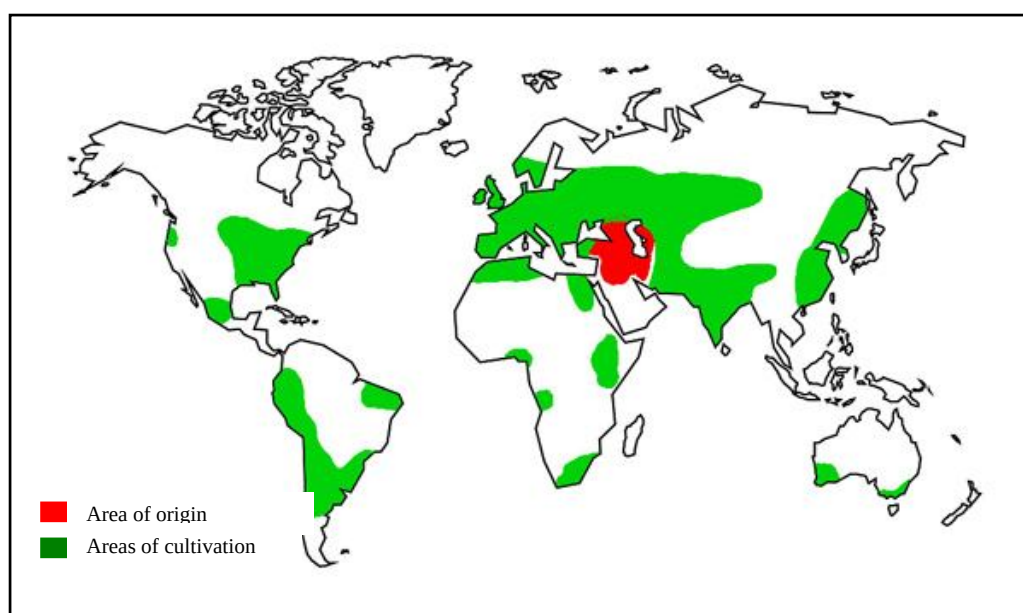


Fig. 1.1 Areas of origin and diffusion of wheat.

1.2 Origin and phylogeny of wheat

Wheat belongs to the genus *Triticum* of the family *Gramineae*.

The origin of the genus *Triticum* is found in the Middle-Eastern in the area between the Mediterranean coast and the flatlands between the Tigris and the Euphrates rivers, where

archaeological excavations demonstrated that the wheat cultivation started about 10000 years ago. From the Middle East wheat cultivation spread most likely to Egypt, where it contributed to the development of its civilization. In Western Europe, wheat was probably introduced during the Aryan populations migrations. In ancient times wheat then spread in the Mediterranean region and afterwards into Germany and Britain along with the conquest of Roman legions.

Cultivated wheat is an interesting example of natural allopolyploidy where spontaneous cross between wild species have been followed by spontaneous doubling of chromosomes to originate a fertile allopolyploid. The genus *Triticum* consists of several species, which can be divided into three basic natural groups, each distinguished by the somatic chromosome number. They form a polyploid series with diploid ($2n=2x=14$), tetraploid ($2n=4x=28$) and hexaploid ($2n=6x=42$) wheats. Wheat classification was and it is still today the object of numerous disputes and of continuous revisions; such difficulties are due mostly to the large number of species, both wild and cultivated, and to the high capacity of inter-specific crossing. Several studies were carried on using different approaches in order to discover the wild ancestors of the cultivated species and to define a classification. The first attempts were during the Roman times by Columella; however, only in the second half of the 18th century Linnaeus attributes to the genus *Triticum* first five and then seven species, on the basis of morphological characters. In 1913 Schultz still on the basis of the plants morphology proposed the subdivision 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 of species thought more or less similar, 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 wheat. The wild diploid wheats with AA genome are divided in the *T. monococcum* ssp. *boeoticum* e *T. urartu* species. These two species are recognizable because of morphological, cytogenetic and isoenzymatic differences and because of the storage proteins. The *T. monococcum* ssp. *monococcum* was most likely derived from *T. monococcum* ssp. *boeoticum*, following the domestication process. Tetraploid wheats are divided in two groups: those with AABB genome, including several subspecies of *T. turgidum* and those with AAGG genome, including some subspecies of *T. timopheevi*. Both groups show

wild forms, respectively *T. turgidum* ssp. *dicoccoides* from which eventually durum and emmer wheats derived, and *T. timopheevi* ssp. *araraticum*. Hexaploid wheats include two species: *T. aestivum* (AABBDD) with many subspecies and *T. zhukovskyi* (AABBGG). There is no knowledge of wild hexaploid wheat species. For a long time *T. monococcum* ssp. *boeoticum* was considered the donor of A genome of cultivated hexaploid and tetraploid wheats; then, thanks to the analyses with biochemical and molecular markers (Konarev, 1983; Lafiandra *et al.*, 1993; Dvorak *et al.*, 1993), it has been possible to better determine phylogeny of cultivated wheats and to identify the wild diploid species *T. urartu* as the donor of A genome (Dvorak *et al.*, 1993). The donor of D genome of cultivated hexaploid wheats has been demonstrated to be the wild species *Aegilops squarrosa* (*T. tauschii*) (Zohary *et al.*, 1962). The origin of B and G genome is more complex and it is still the subject of several wheat phylogeny studies based on cytology, nuclear and mitochondrial DNA sequences as well as chromosome rearrangement (Zohary *et al.*, 1962; Feldman M., 1966; Dvorák *et al.*, 1990). One of the most valued hypotheses picks the donor of B genome in one of the five species of the *Sitopsis* section (Sears, 1956; Dvorák and Zhang, 1990; Furuta *et al.*, 1976). With regards to G genome, Miller (1987) demonstrated the possibility that such genome was donated by *Aegilops speltoides*.

At the beginning it was thought that the A, B and D genomes were highly differentiated; then in the '50s it was discovered that it is not like that. Mostly thanks to the studies of Sears, it was determined that each chromosome of the hexaploid wheat genome has a correspondent homeologous in each of the other two genomes. Studies by Riley and Chapman (1958) showed that in the hexaploid wheat during the meiosis, each specific chromosome is paired with a specific partner (homologous pairing) and not with any of the five potential (homeologous pairing). The suppression of the homeologous pairing (which would lead to a reduced stability of the species) is maintained by the *Ph* locus located on the long arm of chromosome 5B. With the absence of the *Ph* gene or of the whole 5B chromosome there is the pairing of the homeologous chromosomes (Riley and Chapman, 1958).

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.

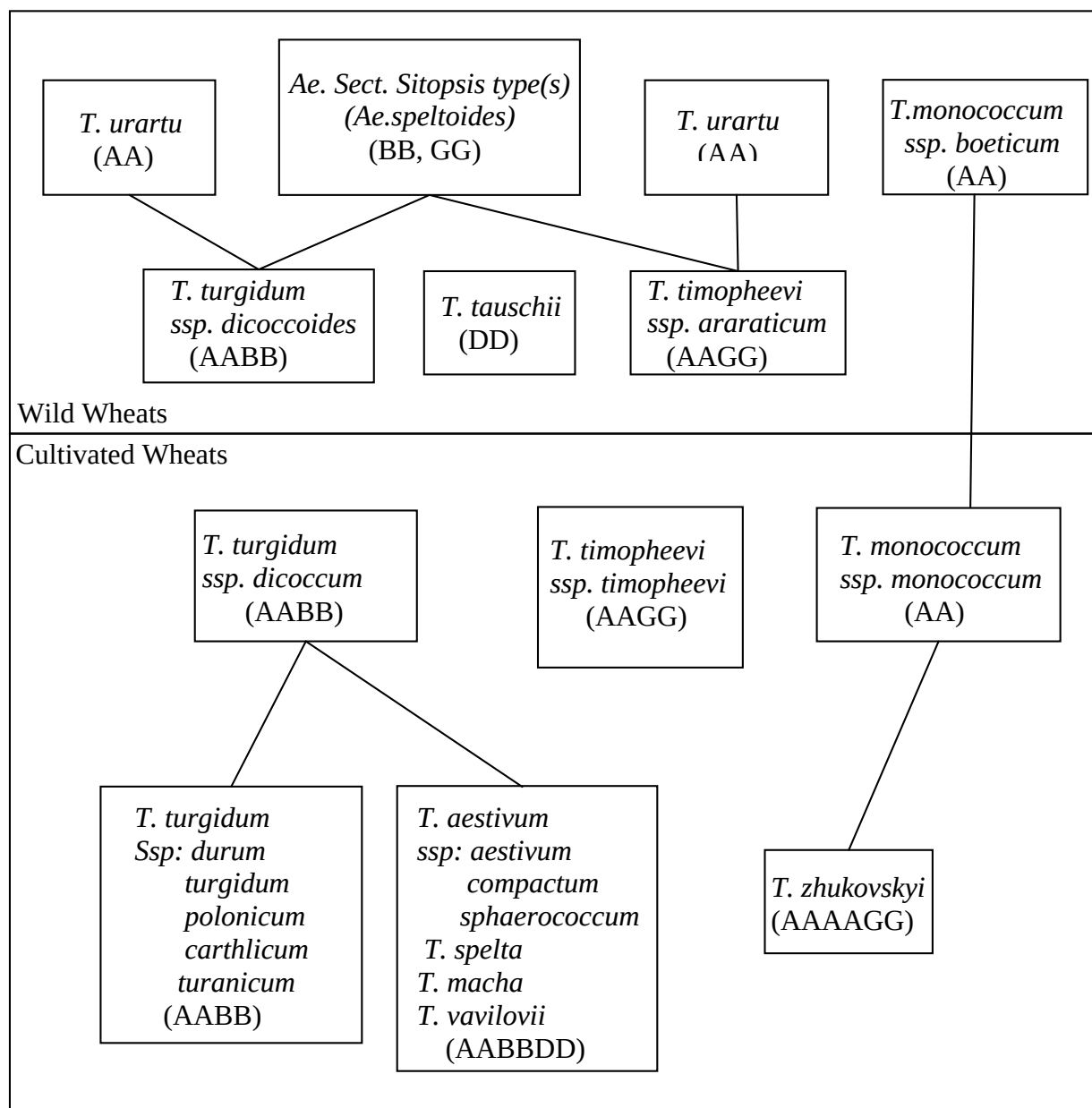


Fig. 1.2 The evolution of the polyploid wheats.

1.3 Generalities and developmental stages systems of wheat

Wheat can be grown from within the Arctic Circle to higher elevations near the equator, although the crop is most successful between the latitudes of 30° and 60°N and 27° and 40°S (Nuttonson, 1955). Wheat production in much warmer areas is also technologically feasible (Saunders and Hettel, 1994). In altitude, the crop is grown from sea level to more than 3.000 masl, and it has been reported at 4.570 masl in Tibet (Percival, 1921).

The optimum growing temperature is about 25°C, with minimum and maximum growth temperatures of 3° to 4°C and 30° to 32°C, respectively (Briggle, 1980). Wheat is adapted to a broad range of moisture conditions although most of the land area where wheat is grown receives an average of between 375 and 875 mm of annual precipitation.

Wheat is classified into spring or winter wheat traditionally referred to the season during which the crop is grown. Winter wheat development is promoted by exposure of the seedlings to temperatures in the 3-8°C range; it is usually planted in the autumn to germinate and develop into young plants that remain in the vegetative phase during the winter and resume growth in early spring. Spring wheat, instead, does not require exposure to cold temperatures for normal development and, as the name implies, is usually planted in the spring and matures in late summer.

The growth cycle of wheat is divided in different phases: 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 been proposed for describing wheat development such as the Zadoks system (Zadoks *et al.*, 1974) or the Haun (Haun J.R., 1973) and the Feekes-Large systems (Large E.C., 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. In this system, the length of each emerging leaf is expressed as a fraction of the length of the preceding fully emerged leaf; for example, a 3.2 indicates that three leaves are fully emerged, and a fourth leaf has emerged two-tenths of the length of the third.

The Zadoks system is the most widely accepted system 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 three leaves are at 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 1.2).

Table 1.2. 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 The plant immune system

Plants, unlike mammals, lack mobile defender cells and a somatic adaptive immune system. Nevertheless, each plant cell possess an innate immunity (Dangl and Jones, 2001).

The plant immune system is composed by two branches originally defined as basal or horizontal disease resistance and resistance (R) gene-based or vertical disease resistance; these two forms are now called PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) (Chisholm *et al.*, 2006; Jones and Dangl, 2006).

PTI uses transmembrane pattern recognition receptors (PRRs) that respond to slowly evolving microbial-associated molecular patterns (MAMPs, originally called PAMPs: pathogen-associated molecular patterns) and damage-associated molecular patterns (DAMPs originally called endogenous elicitors) (Boller and Felix, 2009). Bacterial flagellin is a good example of MAMP recognized by many plant species (Felix *et al.*, 1999).

ETI is based on the highly specific interaction of pathogens effectors and the products of plant *R* genes according to the gene-for-gene theory annotated by Harold Flor in the mid-20th century. Based on this theory (Flor, 1971), the plant *R* gene and the pathogen “avirulence gene” (effector gene) must have matched specificity for that *R* gene to confer resistance which is manifested as a vigorous type of defence reaction called hypersensitive response, characterized by rapid apoptotic cell death and local necrosis. Conversely, in the absence of a cognate *R* effector association, the pathogen alludes detection by the host plant, resulting in pathogen proliferation within the plant cell and the onset of disease (Chisholm *et al.*, 2006).

So far, numerous *R* genes have been cloned from a wide range of plant species. Despite the broad spectrum of resistance imparted by *R* proteins, these gene products can be divided into two main classes according to their domain organization: the nucleotide binding leucine-rich repeat (NB-LRR) and the extracellular LRR (eLRR) resistance proteins (Dangle and Jones, 2001). The first one is the most abundant and members can possess amino-terminal coiled-coil (CC) or Toll-interleukin-1 receptor (TIR) domains. Regarding eLRRs, three subclasses have been classified based on their domain structure: RLPs (receptor-like proteins; extracellular LRR and transmembrane [TM] domain), RLK (extracellular LRR, TM domain, and cytoplasmic kinase) and PGIP (polygalacturonase-inhibiting protein; cell wall LRR) (Fritz-Laylin *et al.*, 2005) (Fig. 1.3).

Only in a few cases a direct binding between *R* genes and their corresponding pathogen effectors has been demonstrated. It is now clear in fact, that *R* proteins recognize pathogen effectors

indirectly by detecting the alteration that the effector has caused to a host protein consistent with the “guard hypothesis” (Dangl and Jones, 2001; van der Biezen and Jones, 1998). This hypothesis implies that an effector, acting as a virulence factor, has a target(s) in the host and by manipulating or altering this target(s) the effector contributes to pathogen success in susceptible host genotypes; in addition, effector perturbation of a host target generates a ‘pathogen-induced modified-self’ molecular pattern, which activates the corresponding NB-LRR protein, leading to ETI. Important consequences of this model supported by evidences, are that enzymatic functions of multiple effectors could target the same host proteins. Host plants have evolved mechanisms to monitor common host targets rather than develop receptors for every possible effector. By monitoring for perturbations, R proteins indirectly detect the enzymatic activity of multiple effectors (van der Biezen and Jones, 1998).

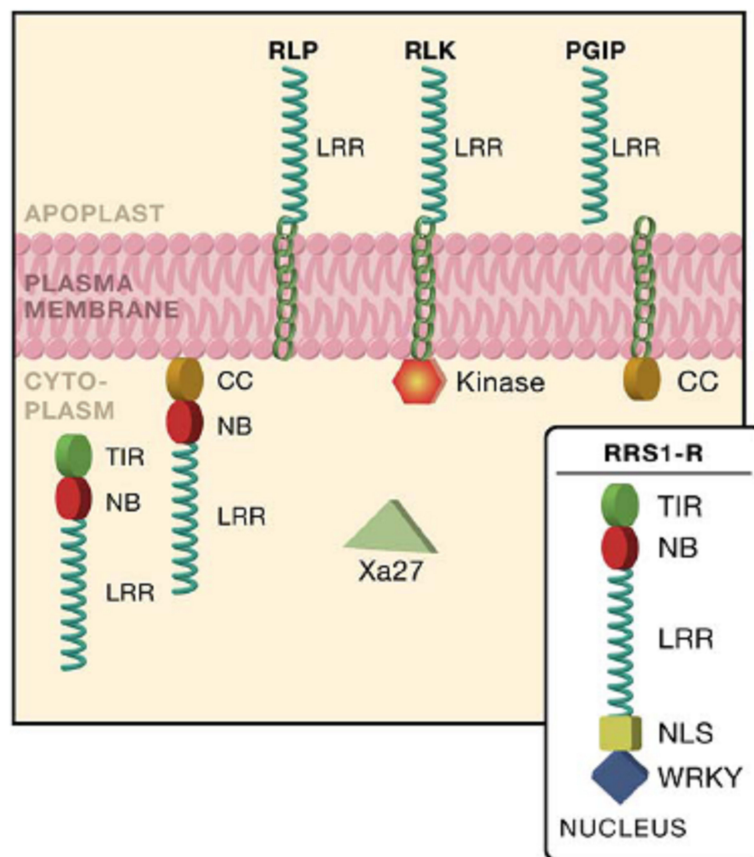


Fig. 1.3 Classes of Resistance Proteins. R proteins are divided into two main classes according to their domain organization: the nucleotide binding leucine-rich repeat (NB-LRR) and the extracellular LRR (eLRR) resistance proteins. The first one is the most abundant and members can possess amino-terminal coiled-coil (CC) or Toll-interleukin-1 receptor (TIR) domains. The RRS1-R protein is a novel member of the NB-LRR class. Three subclasses of eLRRs have been classified based on their domain structure: RLPs, RLKs, and PGIPs. Recently, a novel R protein, Xa27, was identified (from Chisholm *et al.*, 2006).

Based on the “zig-zag” model proposed by Jones and Dangle (2006), the immune system can be represented in four phases. In phase 1, PAMPs (or MAMPs) are recognized by PRRs, resulting in PAMP-triggered immunity (PTI) which usually halts infection before the microbe gains a hold in the plant. In phase 2, successful pathogens use effectors that contribute to pathogen virulence and interfere with PTI. This results in effector-triggered susceptibility (ETS). In phase 3, one of the NB-LRR specifically recognizes a given effector by resulting in effector-triggered immunity (ETI). In phase 4, natural selection drives pathogens to avoid ETI either by shedding or diversifying the recognized effector gene, or by acquiring additional effectors that suppress ETI. Natural selection favours new *R* specificities that can recognize one of the newly acquired effectors and the ETI can be triggered again (Fig. 1.4).

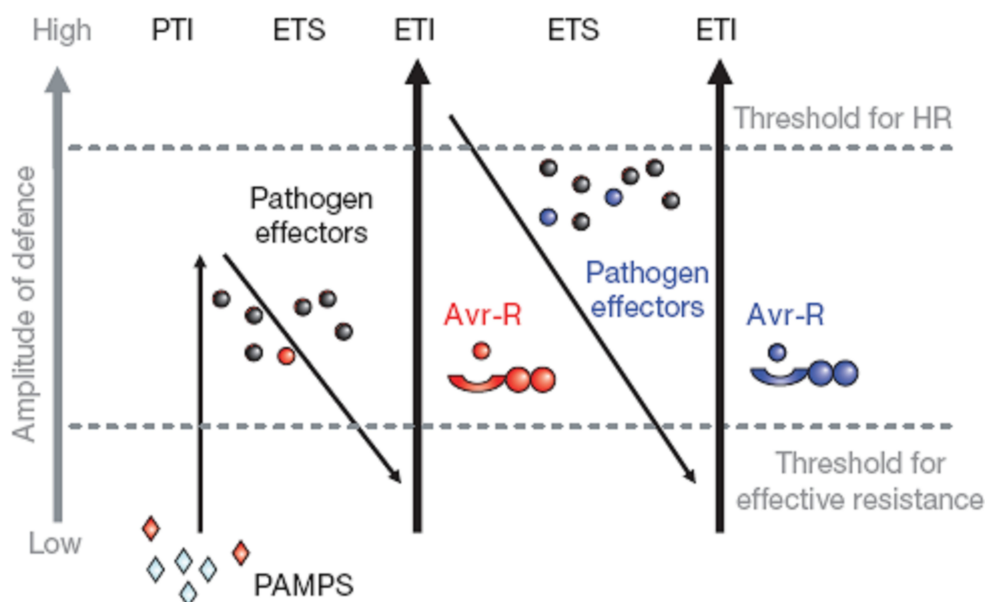


Fig. 1.4 The “zig-zag” model proposed by Jones and Dangle (2006) illustrating the plant immune system. In phase 1, PAMPs/MAMPs (red diamonds) are recognized by PRRs, resulting in PAMP-triggered immunity (PTI). In phase 2, successful pathogens use effectors that contribute to pathogen virulence and interfere with PTI resulting in effector-triggered susceptibility (ETS). In phase 3, one of the NB-LRR specifically recognizes a given effector (indicated in red) activating effector-triggered immunity (ETI), an amplified version of PTI that often passes a threshold for induction of hypersensitive cell death (HR). In phase 4, natural selection drives pathogens to avoid ETI, by acquiring additional effectors through horizontal gene flow (in blue), that suppress ETI. Natural selection favours new *R* specificities that can recognize one of the newly acquired effectors resulting again in ETI (from Jones and Dangle, 2006).

1.5 Common mechanisms of plant disease resistance

Disease outcome is determined by the so called disease triangle, a three-way interaction between the pathogen, the plant and the environmental conditions.

Plants exhibit a number of resistance mechanisms that can be grouped into constitutive or preformed and inducible and range from morphological to structural and chemical defences.

Preformed or passive defences are the first obstacle that a pathogen has to overcome to establish a disease. The typical constitutive defences are morphological, structural and chemical barriers (Ferreira *et al.*, 2007). Some of the most important structural barriers are plant cell walls and plant cuticle, containing a number of compounds including waxes, suberin, lignin and cellulose. In addition, plants constitutively produce a vast array of secondary metabolites and antifungal proteins, many of which can act as antimicrobial compounds during defence against microorganisms. Some of these are components of plant exudates and possess antifungal activity that prevent or limit spore germination of fungal pathogens or the growth of germ tube (Agrios, 1997). Examples of secondary metabolites are phenolics, saponins, terpenoids and steroids. The preformed compounds can be directly toxic, or they can occur as conjugates such as glycosides that are not toxic per se, but become poisonous following disruption of the conjugate (Agrios, 1997).

Plants also possess inhibitors of hydrolytic enzymes produced by the pathogens and detoxifying enzymes that break down pathogen-derived toxins, as well as hydrolytic enzymes like glucanases and chitinases which are able to degrade the fungal cell wall contributing to the resistance (Lawrence *et al.*, 2000).

Besides constitutive defences, an important plant strategy is to initiate defences in response to pathogen attack (Karban and Baldwin, 1997). The activation of infection-induced responses consists of three phases:

- 1) Recognition (plant perceives the presence of a pathogen)
- 2) Signal transmission (plant diffuses the signal into the cells located in the infection site)
- 3) Induction (plant induces an array of modifications both localized in the infection site and systemic, which work all together to block the infection).

Thus, inducible resistance mechanisms are active and energy-requiring systems; they are considered the second obstacle an invading pathogen has to face when attempting infection.

After the recognition event, active defence mechanisms mainly involve the oxidative burst, localized cell death, accumulation of phytoalexins, synthesis of pathogenesis-related (PR) proteins (antimicrobial enzymes such as chitinases and beta-glucanases or antimicrobial proteins

such as defensins, thionins), cell wall strengthening proteins such as hydroxyproline-rich glycoproteins, and enhanced transcription of genes encoding enzymes involved in the flow of carbon from the primary to the secondary metabolism of plants, such as peroxidases, lipoxygenases, superoxide dismutases and phenylalanine ammonia lyase (PAL) (Montesinos, 2000).

A significant role in inducible disease resistance is also played by plant cytoskeleton. Loss of non-host resistance against several non-host pathogens has been reported after disruption of plant actin microfilaments suggesting their involvement in defence against fungal penetration (Kobayashi *et al.*, 1992).

The inducible responses are turned on systematically in the plant in response to attempted infection. First, in the localized response, a form of programmed cell death, the hypersensitive response (HR), is induced in the infection sites causing a spatially confined necrosis. In the HR the plant cells in the spot of infection react to the invading pathogen by a kind of programmed cell death consisting of electrolyte leakage from the cytoplasm and oxidative burst (Montesinos, 2000). As a result, the pathogen remains confined to necrotic lesions near the site of infection.

A set of defence genes is expressed by the plant within a few minutes of the localized necrosis, both locally, at the point of infection, and systemically, throughout the rest of the plant (Antoniw *et al.*, 1980; van Loon, 1985). Thus, local HR often triggers a systemic signal which spreads from the place of plant-pathogen interaction throughout the plant, leading to systemic acquired resistance (SAR). In recent years, SAR and induced systemic resistance (ISR) have received much attention (Conrath *et al.*, 2006). SAR confers long-lasting, enhanced resistance against subsequent infections by a broad spectrum of pathogens (Durrant and Dong, 2004; Ryals *et al.*, 1996; Somssich, 2003; Sticher *et al.*, 1997); the development of SAR, contrary to HR, is slow and gradual (Scheel, 1998). In this systemic defence, the signal is mediated by an interacting set of endogenous defence signalling molecules, including salicylic acid (SA), ethylene (ET), nitric oxide (NO) or reactive oxygen species (ROS) (Baker *et al.*, 1997; Beckers and Spoel, 2006; Montesano *et al.*, 2003). These messengers interact with specific binding proteins, which are involved in the transcriptional activation of pathogen-responsive genes. The increased resistance of non-infected plant tissues to subsequent infections by the same or even unrelated pathogens is thought to be due to these SAR genes (Maleck and Dietrich, 1999; Montesano *et al.*, 2003).

ROS, such as superoxide anions, hydroxyl radicals and hydrogen peroxide, play an important role in plant defence during plant-pathogen interactions (Wu *et al.*, 1997). ROS are directly toxic to microbial pathogens; they participate in signalling the onset of other defence responses and they catalyse oxidative cross-linking of the cell wall at the site of attempted infection

(Nurnberger *et al.*, 2004). Thus, hydrogen peroxide is produced by plant cells in response to infection (Baker and Orlandi, 1995), triggering the HR (Tenhaken *et al.*, 1995), strengthening cell walls (Brisson *et al.*, 1994) and enhancing lignin formation (Wu *et al.*, 1995). In addition, H₂O₂ directly inhibits pathogen growth (Wu *et al.*, 1995) and induces the synthesis of PR proteins, phytoalexins, SA and ethylene (Chamnongpol *et al.*, 1998; Mehdy, 1994; Wu *et al.*, 1997).

Phytoalexins are low-molecular-mass secondary metabolites of non-proteinaceous nature which are produced by a broad range of plant species. They exhibit antimicrobial and antifungal activities and display an enormous chemical diversity. Phytoalexins are synthesized through complex biochemical pathways such as the shikimic acid pathway (Dixon *et al.*, 1996) and are induced by pathogen infection and elicitors (Grayer and Kokubun, 2001; Hammerschmidt, 1999).

1.6 Plant cell wall modifications

After a pathogen attack, the infection-induced responses involve not only chemical but also structural inductive mechanisms, mostly related to the plant cell wall. More than 80 years ago it was recognized that modification of the plant cell wall is a potential resistance mechanism (Young, 1926). Cell wall fortification during infection is achieved by lignification, accumulation of cell wall strengthening proteins (hydroxyproline-rich glycoproteins) and other chemical modifications of plant cells around the sites of infection, like callose deposition in cell wall appositions (papillae), just below penetration sites. Nevertheless, formation of papillae is usually accompanied by the induction of co-localized chemical responses (Bestwick *et al.*, 1998; Matern *et al.*, 1995; McLusky *et al.*, 1999; Nicholson and Hammerschmidt, 1992; Schmelzer, 2002; Thordal-Christensen *et al.*, 1997).

Besides structural modifications, in the cell wall are localized proteins which are able to counteract the hydrolytic activity of the enzymes secreted by pathogen invaders during penetration of host tissue. Among these, polygalacturonase-inhibiting protein (PGIP) inhibits and modulates the endo-polygalacturonase (endo-PG) activity of fungal pathogens (De Lorenzo *et al.*, 2001).

1.7 Plant cell wall structure and composition

Plant cell walls are the key determinants of overall plant form, plant growth and development and the responses of plants to environmental and pathogen-induced stresses. The strength, flexibility, texture and overall shape of higher plant depend on the cumulative properties of walls which surround individual cells (Carpita and Gibeaut, 1993).

Plant cell wall is a complex, diverse and dynamic structure, changing throughout the processes of cell division, growth and differentiation; it is responsible for cell adhesion, is involved in cell-cell communication and is selectively permeable. Each type of cell is distinguished from the others by the chemistry and the organization of their walls; even adjacent cells of the same type can differ for some components according to their history of exposure to environmental conditions. Thus, the composition of walls varies on the basis of the growth phase, cell type, cell position and local region within the wall (Carpita *et al.*, 2001).

Besides this, walls provide also a physical barrier between pathogens and their internal contents of plant cell. The high molecular weight polysaccharides, which are the principal components of cell walls, form a cross-linked network that resist physical penetration (Carpita and McCann, 2000). In addition, cell walls are dynamic reservoirs of antimicrobial proteins and secondary metabolites that inhibit the growth of many pathogens (Darvill and Albersheim, 1984; Thomma *et al.*, 2002). Thus, the wall is essential for plant development and survival.

Growing cells possess primary walls that need to be both mechanically stable and sufficiently extensible to permit cell expansion while avoiding the rupture of cells under turgor pressure. After the cessation of cell growth, a secondary cell wall is usually deposited between the primary cell wall and the plasma membrane conferring mechanical stability upon specialized cell types such as xylem elements and sclerenchyma cells. These secondary walls contain cellulose and matrix phase polysaccharides, such as heteroxylans and heteromannans, in a highly ordered pattern to form a rigid complex and are often impregnated with lignins.

The primary cell wall is composed of cellulose microfibrils embedded in a highly hydrated polysaccharide matrix, of which hemicelluloses and pectins are the two major classes of polysaccharides (Fig. 1.5). Hemicelluloses and pectins are synthesized within the Golgi cisternae, whereas cellulose is generated at the plasma membrane.

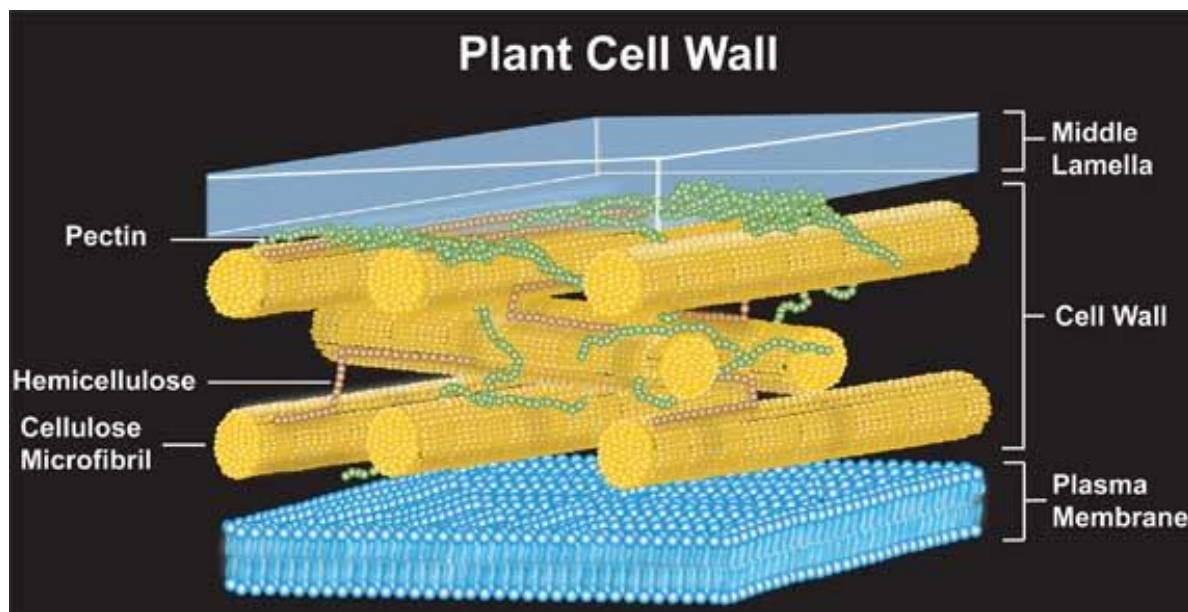


Fig. 1.5 Cell wall structure (from Sigma Aldrich web site).

Cellulose is present in the form of layers of long fibrils surrounding the plant cells. The cellulose fibrils are composed of about 30 to 36 chains of β -1,4-linked glucose that are hydrogen bonded to form an insoluble and inelastic crystalline material.

Hemicelluloses display a β -(1,4)-linked backbone. They include xyloglucans, xylans, mannans and glucomannans, and β -(1 3,1 4)-glucans. These types of hemicelluloses are present in cell walls of all plants, except for β -(1 3,1 4)-glucans, which are restricted to Poales and a few other groups. The most important role of hemicelluloses is their contribution to strengthening the cell wall by the interaction with cellulose. Cellulose microfibrils are, in fact, bonded and tethered together by cross-linking hemicellulose molecules (predominantly xyloglucan polysaccharides in dicotyledons and glucuronoarabinoxylans in the Poaceae and other monocots) (Carpita and Gibeaut, 1993; Carpita, 1996).

The load-bearing cellulose network is embedded in a more soluble matrix of polysaccharides in which pectin is the most abundant class of macromolecules.

Proteins are also present in the wall; cell wall proteins (CWP) range from structural that are strongly or covalently attached to the polysaccharides to loosely attached or soluble proteins. 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) (Jonson *et al.*, 2003).

The wall also contains other families of proteins which include enzymes such as hydrolases, proteases, glycosidases, peroxidases and esterases, expansins and wall-associated kinases (Carpita and Gibeaut, 1993).

1.8 Pectins

Pectin is one of the major components of primary cell walls and is also present in the middle lamellae between primary cell walls where it functions in regulating intercellular adhesion. Pectin is greatly reduced or absent in non-extendable secondary cell walls and is the only major class of plant polysaccharide to be largely restricted to primary cell walls.

Pectin is involved in plant growth, morphogenesis, organogenesis, development, cell-cell adhesion, defence, leaf abscission, fruit maturation and dehiscence, seed hydration, and ion binding (Ridley *et al.*, 2001; Willats *et al.*, 2001a).

Pectins of higher plants form the structurally most complex family of polysaccharides in nature. Five classes of pectinaceous polysaccharides can be distinguished: homogalacturonan (HG), xylogalacturonan (XGA), apiogalacturonan (AP), and rhamnogalacturonan I (RG-I) and II (RG-II). All of these polysaccharides are built on a HG backbone except for RG-I which, instead, is built on a polymer of galacturonic acid (GalA) and rhamnose (Rha) disaccharide subunits. HG is the most abundant pectic polysaccharide, constituting 65% of total pectin. It consists of a linear α -1,4-linked GalA homopolymer with a typical degree of polymerization of about 100 (Willats *et al.*, 2001a). Depending on the plant source, HG can also be acetylated and methyl-esterified in specific carbons, C2-C3 and C6 respectively (Fig. 1.6).

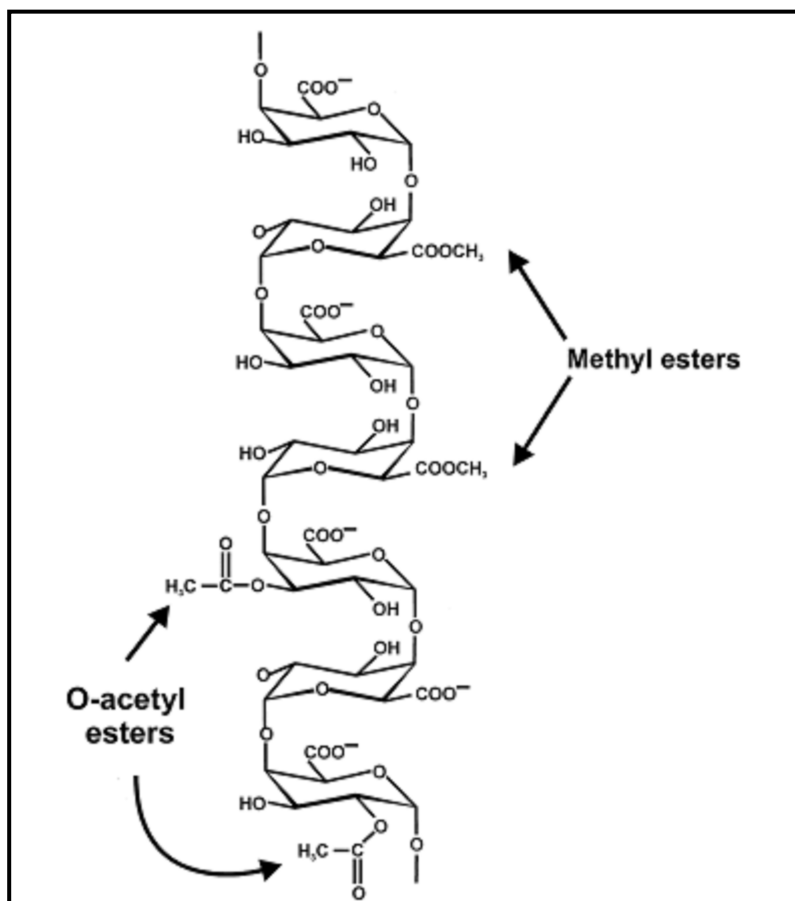


Fig. 1.6 The primary structure of homogalacturonan (from Ridley *et al.*, 2001).

HG is not distributed uniformly across the cell after its secretion and different methyl-esters patterns can be visualized in distinct region of the cell wall using specific antibodies (Willats *et al.*, 2001a). In particular, a stretch of a minimum of nine un-methyl-esterified galacturonic acid residues can form Ca^{2+} linkages promoting the formation of the so-called ‘egg-box’ or “junction zones” (Liners *et al.*, 1989). In these structures, carboxyl groups of two GalA residues belonging to different HG chains form a negatively charged pocket that can accommodate a Ca^{2+} cation. It is assumed that the presence of these ‘egg-box’ structure can induce gel formation and thus strengthen the wall. HG can be also cross-linked to other cell wall polysaccharides or to other pectin chains by uronyl esters (Brown and Fry, 1993).

GalA of HG may be substituted at C-3 with residues of xylose resulting in the xylogalacturonan (XGA) that appears to be quite widespread (Le Goff *et al.*, 2001). Substitution of GalA with apiose at C-2 or C-3 results, instead, in the formation of apiogalacturonan (AP).

Despite its name, RG-II is not structurally related to RG-I but has a HG backbone with complex side chains attached to the GalA residues (Ridley *et al.*, 2001). RG-II has a backbone of around 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 are rare 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). RG-II can dimerize by means of borate ester links through apiosyl residues of the side chains of two contiguous polymers, forming a borate-diol ester (Kobayashi *et al.*, 1996; O'Neill *et al.*, 1996; Ishii *et al.*, 1999).

While the chain lengths of the various domains can vary considerably and the sugar composition of RG-I can also be highly heterogeneous, RG-II appears to have a highly conserved structure.

Pectins are assumed to be 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 (Goldberg *et al.*, 1996). 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.

The macromolecular structure of various pectins is still unclear although the fine structure of single pectic constituents is largely known.

For many decades HG was considered the backbone of pectin, linked to RG-I, but another model also suggested that HG could form side chains of RG-I (Vincken *et al.*, 2003) (Fig. 1.7). However, the recent publication of the work of Coenen *et al.* (2007) questioned this model. Resolving the macromolecular structure of pectin is an important point because HG as backbone or as side chains will not contribute in the same way to the 3-D structure of the pectin matrix and will consequently affect cell wall structure and properties differently.

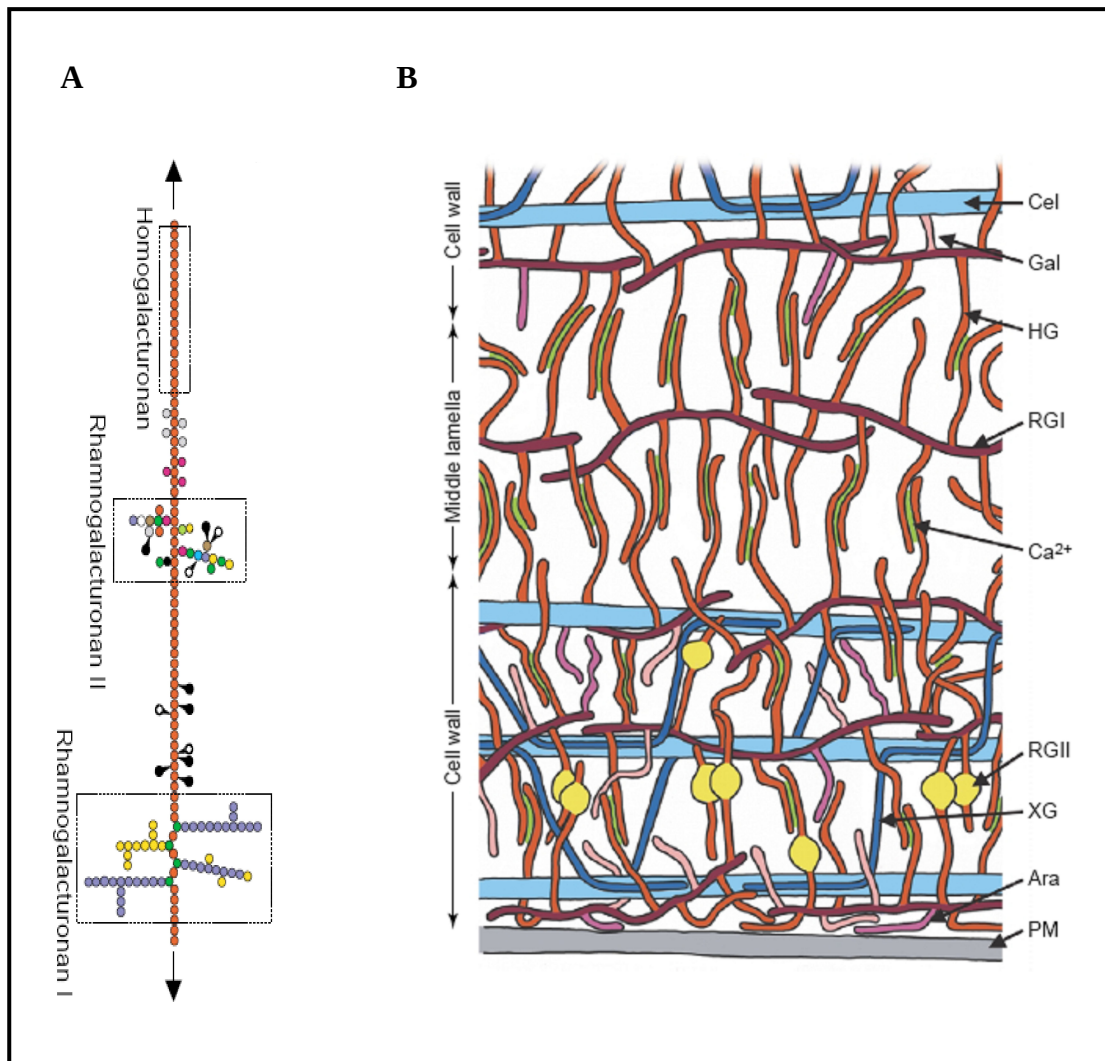


Fig. 1.7 Representations of the conventional (**A**) and the alternative model (**B**) of pectin structure (from Willats *et al.*, 2006 and Vorwerk *et al.*, 2004).

1.9 Grass cell wall: composition and differences with dicot cell wall

Whereas the overall architectures of grass and dicot cell walls are similar in the sense that they both consist of a network of cellulose fibers surrounded by a matrix of non-cellulosic polysaccharides, they differ considerably in terms of the major structural polysaccharides present, the cross-linking of the polysaccharides and the abundance of proteins and phenolic compounds (Vogel, 2008).

Grasses, in fact, as part of commelinoid monocots, contain a type II primary cell wall, differently than dicots which instead contain type I primary cell wall.

The polysaccharides composition between them vary as follow; a typical dicot wall contains ~ 30% of cellulose, 30% of hemicellulose and 35% of pectin while a typical grass species contains

~ 25% of cellulose, 55% of hemicellulose and only 2-10% of pectin (Table 1.3) (Cosgrove, 1997).

Table 1.3

Approximate composition (% dry weight) of typical dicot and grass primary and secondary cell walls

	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

The major hemicellulose in grass cell wall is the glucuronoarabinoxylan (GAX), a β 1,4-linked xylose backbone with single arabinose and glucuronic acid side chains primarily attached at the O-3 and O-2 positions, respectively (Fig. 1.8 A). In the grass primary cell walls, it replaces xyloglucan (XyG) (Fig. 1.8 C) in cross-linking cellulose microfibrils. In addition, the cell wall of grasses contains significant quantities of mixed linkage glucans (MLG, also known as β -glucans) which are unbranched homopolymers of glucose unusual because they contain both β 1,3- β 1,4-linkages (Fig. 1.8 B). MLG are unique to the cell wall of grasses (family Poaceae) and a few related families from the order Poales (Trethewey *et al.*, 2005). Recently, it has been demonstrated that members of the grass specific cellulose synthase-like *CsIF* gene family are involved in their synthesis. MLG act as storage carbohydrates and it has been suggested that they play a role in cell expansion (Buckeridge *et al.*, 2004).

Another unusual feature of grass primary and secondary cell walls is the presence of significant quantities of the hydroxycinnamates ferulic acid (up to 4%) and *p*-coumaric acid (up to 3%) (Hatfield *et al.*, 1999) which exist as unbound acids or ester- and ether-linked to the arabinosyl units of GAX and to various positions in lignin (Ishii, 1997; Ralph *et al.*, 2004).

Regarding cell wall proteins, they are much less abundant in the grasses than in dicots.

Moreover, the secondary cell walls of grasses can not be ignored since comprise at least 50% of the cell wall mass in both leaves and stems (Jung, 2003). They are composed of cellulose, GAX, and lignin, this last comprising a substantial portion ($\sim 20\%$) of the wall.

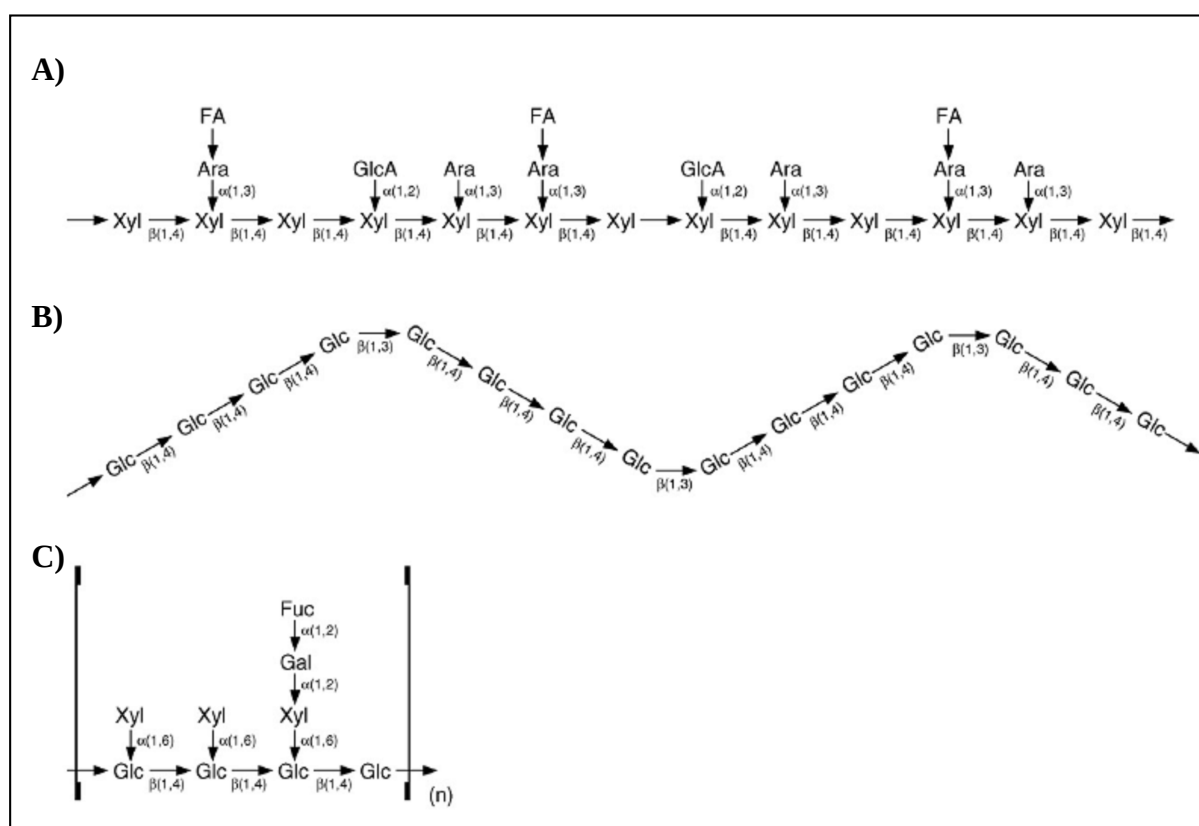


Fig. 1.8 Hemicellulose structures. (**A**) GAX consists of a $\beta(1,4)$ linked xylose (Xyl) backbone substituted with arabinose (Ara) (mainly attached at the O-3 position) and less frequently with glucuronic acid (GlcA) (mainly attached at the O-2 position) in a non-repeating fashion.. Ferulic acid (FA) is attached to the Ara side chains through various linkages. (**B**) MLG consists of an unbranched polymer of glucose (Glc) in which variable length stretches of $\beta(1,4)$ linked Glc units are interrupted with single $\beta(1,3)$ linked Glc units. The $\beta(1,3)$ linkages cause the polymer to bend. (**C**) XyG is composed of a $\beta(1,4)$ linked Glc backbone substituted in a repeating pattern of four Glc units. The nature of the side chains varies depending on the species. One typical repeat unit containing Xyl, Gal, and fucose (Fuc) is shown (from Vogel, 2008).

1.10 Pectinolytic enzymes produced by pathogens: close-up view of fungal polygalacturonases (PGs)

Pectin degrading enzymes are the first class of CWDE (cell wall degrading enzymes) produced by pathogens during plant infection, accounting for the rapid and extensive degradation of cell wall and cell death (Alghisi and Favaron, 1995). They include exo- and endopolygalacturonases, pectin methylesterases, pectin lyases and pectate lyases.

Pectin lyases (PNLs; EC 4.2.2.10) are produced by bacteria and fungi and catalyse β -elimination cleavage of methylesterified polygalacturonic acid in methylesterified pectin rather than pectate, which is, instead, the preferred substrate of pectate lyases (PeLs; EC 4.2.2.2). While pectate lyases have been shown to be important factors of virulence (Akimitsu *et al.*, 2004), little information is available on the role of PNLs during pathogenicity, except for a study reporting PNL activity in mallow leaves during the biotrophic and necrotrophic phases of infection (Wei *et al.*, 2002).

Pectin methylesterases (PMEs; EC 3.1.1.11) from microbial pathogens catalyse the de-esterification of methylesterified D- galacturonate (GalA) residues in pectic polysaccharides of the plant cell wall producing regions of polygalacturonic acid which can then be depolymerised by the polygalacturonase and pectate lyase (Fries *et al.*, 2007).

Polygalacturonases (PGs) are the most extensive studied pectinases. They are distinguished in exo - (exo-PGs; EC 3.2.1.67) and endopolygalacturonases (endo-PGs; EC 3.2.1.15); endo-PGs cleave the α - (1,4)-galacturonan linkages between non-methylated D-galacturonic acid residues while, exo-PGs catalyze the same hydrolytic cleavage but at the non-reducing end of chain.

Based on their sequences and their structurally related catalytic folding, endo-PGs have been classified in family 28 of glycoside hydrolases, GH28 (Coutinho and Henrissat, 1999). They are produced by bacteria, fungi, nematodes and insects (Jaubert *et al.*, 2002; Girard and Jouanin, 1999). Endo-PGs are secreted at the early stages of plant infection causing cell separation and maceration of host tissue; their action is a pre-requisite for a further degradation of the cell wall by other degrading enzymes (De Lorenzo *et al.*, 2001). To accommodate pathogenesis in a variety of different environmental conditions and on various hosts, fungi evolved PG isoenzymes differing in terms of sequence, stability, specific activity, pH optimum and substrate preference.

The importance of PG in the pathogenesis has been demonstrated for several fungi, such as *Botrytis cinerea*, *Aspergillus flavus*, *Alternaria citri*, *Claviceps purpurea* and *Sclerotinia sclerotiorum* (ten Have *et al.*, 1998; Oeser *et al.*, 2002; Isshiky *et al.*, 2001; Shieh *et al.*, 1997; Li *et al.*, 2004), and bacteria, such as *Agrobacterium tumefaciens* and *Ralstonia solanacearum*

(Tans-Kersten *et al.*, 2001; Rodriguez Palenzuela *et al.*, 1991). PGs from salivary glands of phytophagous insects are considered a main cause of plant damage (Girard and Jouanin, 1999; Boyd *et al.*, 2002).

The degradation of pectin chains by PGs, not only loosens the primary cell wall allowing the cellulose-hemicellulose network to become more accessible for digestion, but also leads to the release of cell wall pectin-derived oligosaccharides, the oligogalacturonides (OGs), which in turn act as elicitors of a variety of plant defences (Cervone *et al.*, 1989; D'Ovidio *et al.*, 2004a). It is thought, in fact, that during fungal attack, the action of single-attack and processive fungal endoPGs in the presence of plant-derived PGIPs (polygalacturonase-inhibiting proteins) generates these long OGs elicitors of defence responses. In the presence of PGIP, the enzymatic activity of endoPG *in vitro* is reduced in such a way that the balance between release of elicitor-active OGs and depolymerization of the active OGs, into inactive molecules, is altered and the accumulation of elicitor-active molecules is favoured (Cervone *et al.*, 1986).

It has been also proposed the capacity of PGs to elicit defence responses unrelated to their enzymatic activity for the *B. cinerea*-grapevine interaction (Poinssot *et al.*, 2003). However, the enzymatic activity of *Colletotrichum lindemuthianum* PG has been shown to be essential for activating defence responses in tobacco leaving this subject open to controversy (Boudart *et al.*, 2003).

1.11 Plant pectinolytic enzymes: close-up view of plant pectin methylesterases (PMEs)

The pectinolytic enzymes produced by pathogens are also present in plants. They display, in some cases, different structures compared to the ones secreted by pathogens and play multiple roles both in plant growth and defence. Among these, pectin methylesterases (PMEs) catalyze the demethylesterification of homogalacturonan (HG), the main pectic cell wall component. As previously specified, HG is synthesized in the Golgi apparatus, methylesterified in the *medial* Golgi by pectin methyltransferases and secreted into 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 (Fig. 1.9) (Moustacas *et al.*, 1991).

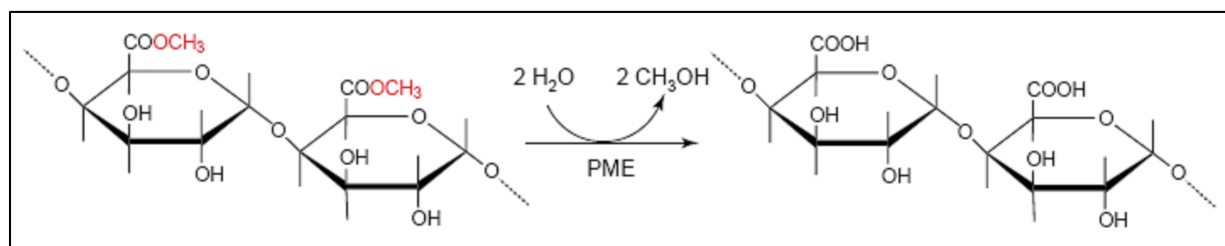


Fig. 1.9 Demethylesterification of pectins by pectin methylesterase, PME (from Micheli, 2001).

PMEs belong to class 8 (CE8) of the carbohydrate esterase (CAZy, <http://www.cazy.org/fam/CE8.html>) (Henrissat, 1991). Plants PME s belong to large multigene families. For instance, in *Arabidopsis thaliana*, 66 ORFs have been annotated as putative full-length PMEs (Coutinho *et al.*, 2003). 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 in *Oryza sativa* (35 ORFs) (<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.

It has been found that plant and microbial PMEs share five segments of high sequence similarity (GxYxE, QAVAL, QDTL, DFIFG, LGRP W) representing the PME signature patterns, although they cluster in independent clades (Markovic and Janecek, 2004).

Higher plant PMEs are frequently organized in pre-pro-proteins in which the pre-region, 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).

Based on their primary structure and the presence or absence of the PRO region, PME can be distinguished 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. 1.10) (Micheli, 2001; Tian *et al.*, 2006). The group 1 PMEs have a structure close to that of the PMEs identified in phytopathogenic organisms (bacteria and fungi).

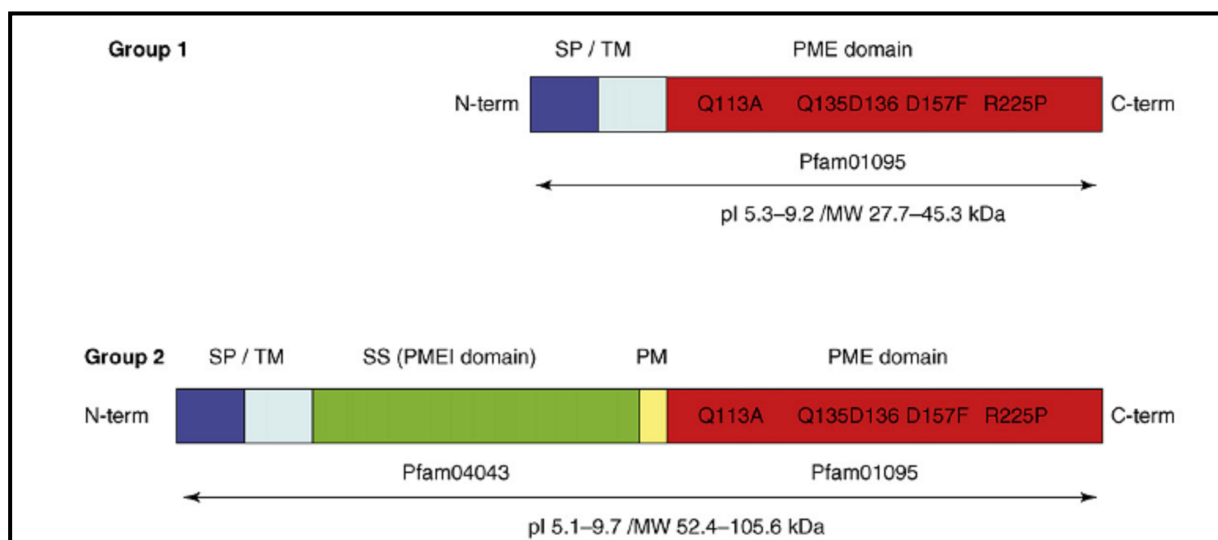


Fig. 1.10 Pectin methylesterase (PME) structural motifs. Group 1 and group 2 PMEs possess a conserved PME domain (Pfam01095) with characteristic, highly conserved amino acid fragments. Amino acids involved in the catalytic site are highlighted (Q113, Q135, D136, D157, R225; according to the carrot PME numbering). Group 2 PMEs possess a PRO region, which shares similarities with the PME 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 yield 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.*, 2009a); 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 times of active homogalacturonan synthesis and methylesterification which also take place in the Golgi apparatus (Fig. 1.11) (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 (Bosch *et al.*, 2005).

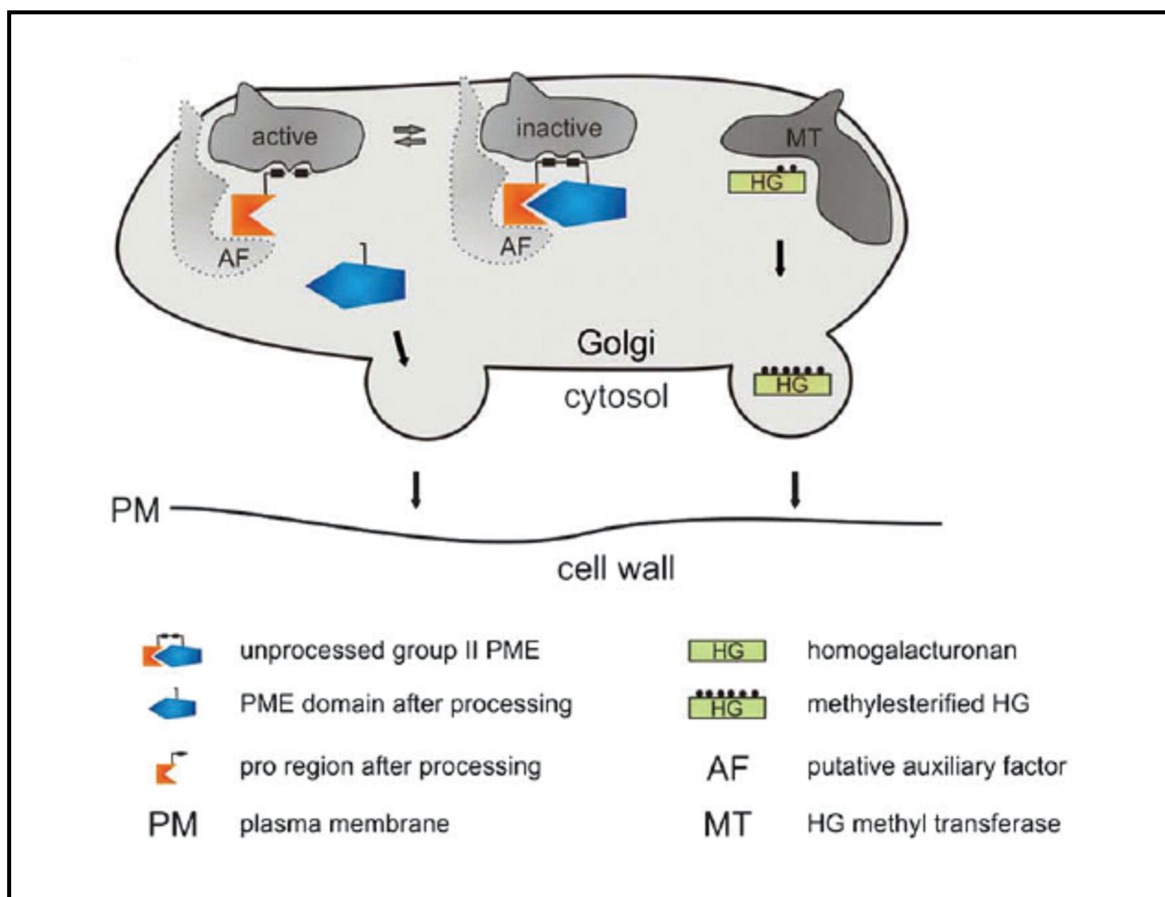


Fig. 1.11 Hypothetical model of Group II PME processing. Unprocessed group II PMEs are retained in the Golgi apparatus, possibly by either the inactive protease or auxiliary factors. Upon processing, the liberated PME domain is free to travel to the cell wall with the secretory flow (from Wolf *et al.*, 2009b).

After their secretion into the cell wall, mature PMEs could exhibit three different modes of action: (I) a single-chain mechanism where the enzyme converts all substrate sites on the polymeric chain, (II) a multiple-chain mechanism where the enzyme catalyzes only one reaction and then dissociates from the substrate and, (III) a multiple-attack mechanism where the enzyme catalyses a number of reaction cycles before the enzyme-polysaccharide complex dissociates. Single-chain and multiple-attack mechanisms have been proposed for plant and bacterial PMEs (Dongowski and Bock, 1984; Kohn *et al.*, 1985; Christensen *et al.*, 1998); in contrast with a multiple-chain mechanism proposed for fungal PMEs (Limberg *et al.*, 2000; van Alebeek *et al.*, 2003; Duvetter *et al.*, 2006).

PMEs can act on homogalacturonan randomly or linearly. When they act randomly, the demethylesterification releases protons that promote the action of endopolygalacturonases (Moustakas *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 PME's contributes to cell wall rigidification (Fig. 1.12).

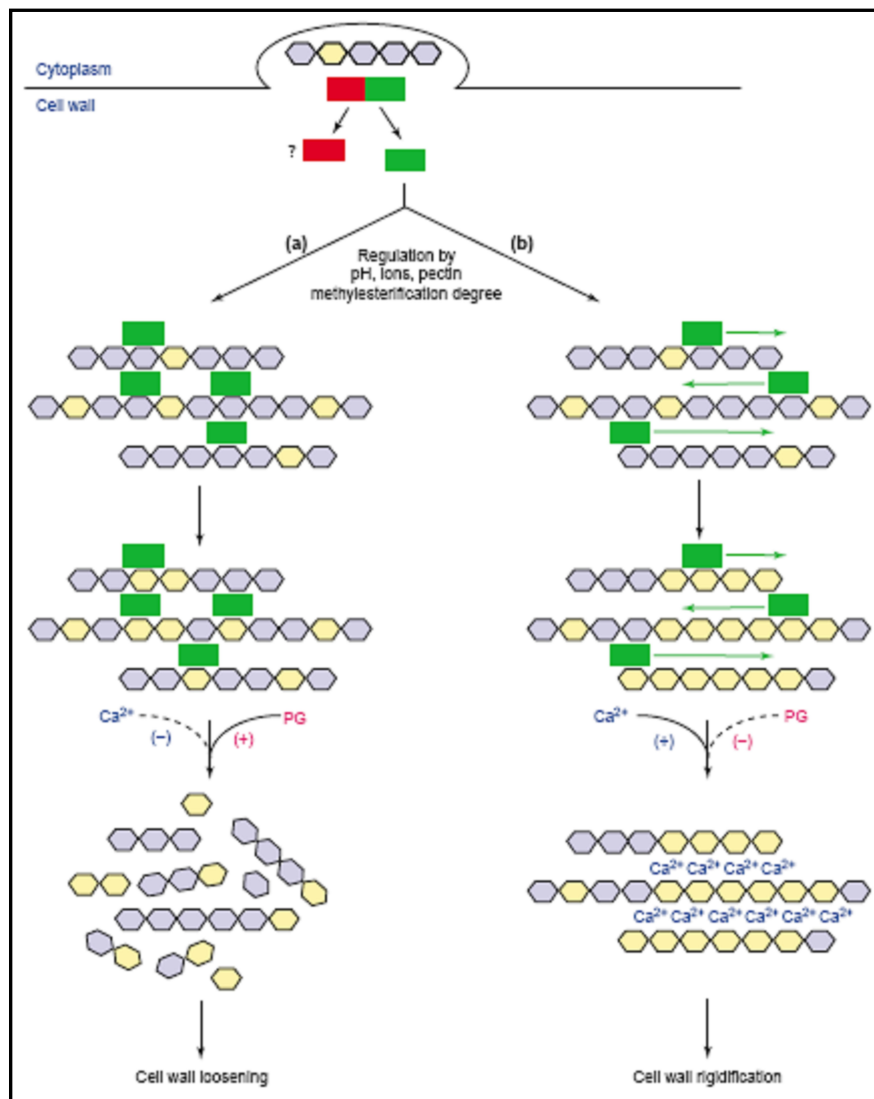


Fig. 1.12 Mode of action of pectin methylesterases (PMEs). Mature PMEs (green) can act randomly (a), promoting the action of pH-dependent cell wall hydrolases such as Endopolygalacturonases (PG) and contributing to cell wall loosening, or can act linearly (b), giving rise to blocks of free carboxyl groups 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 PME's were thought to be essentially confined to fungi, the simplest hypothesis was that random demethylesterification is produced by acidic fungal PME's, whereas linear demethylesterification was determined by alkaline plant and bacterial PME's. However, other studies provided evidences that the mode of action of PME's on HG is influenced by a wide range of 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). In Arabidopsis, recent data showed that one heterologously expressed PME isoform was likely to have a blockwise pattern of de-methyl esterification (Dedeurwaerder *et al.*, 2008). The difficulty of expressing PMEs in heterologous system and the potential redundancy of isoforms are the main reasons for the current lack of information concerning the precise characterization of the biochemical activity of PMEs.

Three three-dimensional structures have been solved so far: a bacterial PME from *Erwinia chrysanthemi* (Jenkins *et al.*, 2001) and two plant PMEs, from *Daucus carota* (Johansson *et al.*, 2002) and from *Solanum Lycopersicon* (Di Matteo *et al.*, 2005). In addition, the 3D structure of PME from *Erwinia chrysanthemi* in complex with pectin has been also determined (Fries *et al.*, 2007). The enzyme folds into a right handed parallel β -helix, typical of pectic enzymes (Jenkins and Pickersgill, 2001). Both of plant PMEs show similar folding topology to PME from *Erwinia chrysanthemi*, although turns that protrude out of the β -helix in proximity of the substrate cleft are much longer in the bacterial enzyme making its putative active site cleft deeper and narrower than that of plant PMEs (Fig. 1.13).

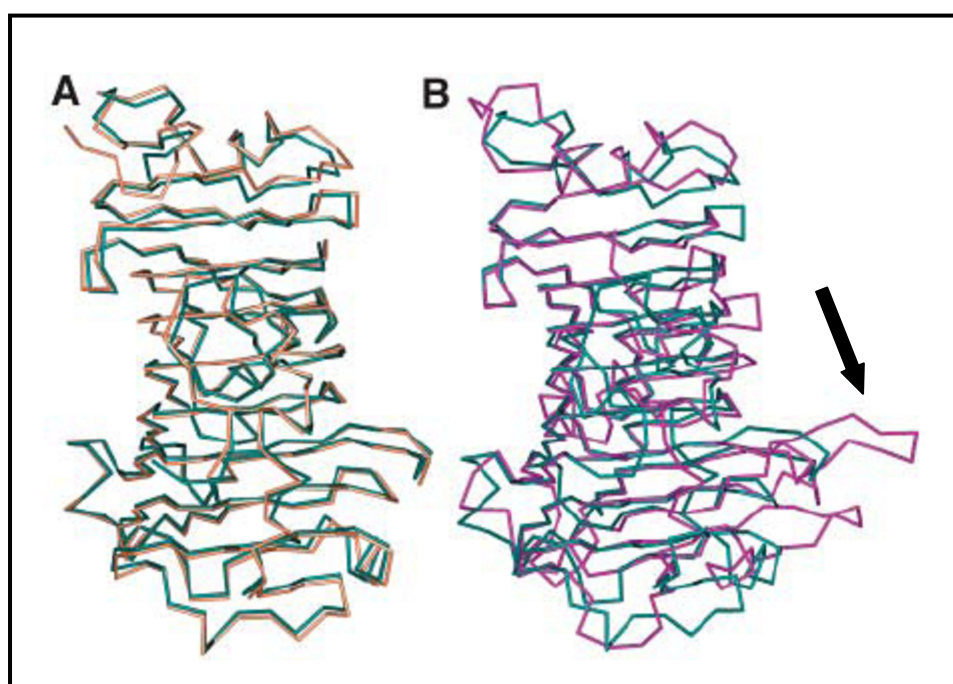


Fig. 1.13 Comparison of the known structures of PMEs. (**A**) Overlay of the C α trace of PME from tomato (green) and PME from carrot (orange). Structures are almost completely superimposable. (**B**) Superimposition of PME from tomato (green) and PME from *E. chrysanthemi* (violet). Although the β -helices 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).

1.11.1 PME_s and plant growth

The multiple roles of PME_s in plants can be illustrated by their expression in different organs and tissues. Various experiments have demonstrated that PME_s are involved, directly and indirectly, in diverse physiological processes associated with both vegetative and reproductive plant development. PME_s participate to 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: a study using an antisense transgenic plant transformed with a PME gene (*rcpme1*) demonstrated that PME activity increases during separation of the border cells of the root cap of pea, and is correlated with an increase in the amount of acidic pectin and a decrease in cell wall pH (Stephenson and Hawes, 1994). Analysis of transgenic plants also showed that *rcpme1* 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). Transgenic potato plants constitutively expressing a *Petunia inflata* 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 in *Solanum tuberosum* after silencing of an ubiquitously expressed PME (Pest2) (Pilling *et al.*, 2004).

PME activity can also modulate the sensitivity to aluminium, a toxic metal in soils that inhibits plant root elongation, (Schmohl *et al.*, 2000). It was shown, in fact, that the overexpression of PME in potato plants leads to a higher aluminium sensitivity compared to wild type plants.

- Fruit ripening: the role of PME in fruit ripening has been intensively studied in tomato in an attempt 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 lowered PME activity by the insertion of antisense cDNA of a fruit-specific *PME2*, were produced (Tieman *et al.*, 1992; Hall *et al.*, 1999). They 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.

- Microsporogenesis and pollen tube growth: advances in understanding of PME functions in reproductive development have mainly concerned their roles in pollen formation and pollen tube growth. Although the pollen-specific PME isoform VGD1 (At2g47040) is among the most abundant mRNAs in the Arabidopsis pollen transcriptome (Pina *et al.*, 2005), disruption of the gene encoding VGD1 caused only 18% reduction in total PME activity (Jiang *et al.*, 2005). This relatively small reduction in activity led to unstable pollen tubes when germinated *in vitro* and a decreased fertilization *in vivo*. The mutant showed also smaller siliques with fewer seeds. Interestingly, the knockout phenotype could be complemented with an isoform (At2g47030) closely related to VGD1 but not with a construct encoding another, slightly more distant isoform (At3g62170), indicating that different PME isoforms may display different activities and are not redundant (Jiang *et al.*, 2005). Similar, albeit weaker, effects on pollen tube growth have been observed with the NtPPME1 and AtPPME1 mutants in tobacco and Arabidopsis, respectively (Bosch and Hepler, 2006; Tian *et al.*, 2006). Mutation of QUARTET1 (QRT1), a PME expressed in Arabidopsis pollen and anthers leads to impaired pollen tetrad separation during flower development. QRT1 might act in tandem with QRT3, a polygalacturonase, to degrade demethyl-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). Studies in tobacco suggested that tight control of the degree of pectin esterification at the apex might control oscillatory pollen tube growth (Bosch *et al.*, 2005; Bosch and Hepler, 2006).

- PME as a methanol source: in a study, a close correlation was reported between PME activity and levels of methanol in fruit tissues, indicating that PME is on the primary biosynthetic pathway for methanol production in tomato fruit. Because methanol oxidation to CO₂ could result in the incorporation of methanol carbon into metabolites via the Calvin-Benson cycle, a role of PME, albeit indirect, in the photosynthetic metabolism of the plant is likely (Frenkel *et al.*, 1998).

- 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 is also essential for the extensive pectin modifications that occur during secondary wall deposition in xylem cells mediated by pectate lyases and polygalacturonases (Hertzberg *et al.*, 2001). Moreover, pectin has been shown to interact with lignin monomers and to affect lignin polymerization *in vitro* (Lairez *et al.*, 2005) suggesting that PME has a role in regulating such interactions *in vivo*.

- PME and phyllotaxis: plant organs are produced from meristems in a characteristic pattern. Even if this pattern, referred to as phyllotaxis, is thought to be generated by local gradients of auxin (Jonsson *et al.*, 2006), a key role for the mechanical properties of the cell walls in the control of organ outgrowth has been proposed (Fleming *et al.*, 1997). Recently, a study demonstrated that PME activity could induce primordia formation and override primordia patterning in *Arabidopsis* inflorescence meristems. Conversely, 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). This study leaves a question mark whether the impact of PME activity and its inhibitor (PMEI) on primordial formation occurs through direct effect on the mechanical properties of the cell wall or indirect effect through the release of OG and/or regulation of the efflux of auxin.

1.11.2 PMEs and plant defences

Because cell wall constitutes a physical barrier between the environment and the internal contents of plant cells, its modifications are often associated with plant defence responses (Vorwerk *et al.*, 2004). *Arabidopsis* microarray database revealed that the expression levels of about 75% of predicted PME sequences vary in response to biotic and abiotic stresses, such as 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 are not able to cleave highly methyl-esterified pectin. It has been also provided the evidence that the disruption of *pmea* gene in the bacterium *Erwinia chrysantemi*, reduces strongly its virulence on

Saintpaulia ionanta (Boccaro and Chatain, 1989; Beaulieu *et al.*, 1993). AtPME3 from *Arabidopsis* seems to interact with the cellulose binding protein of the parasitic nematode *Heterodera schachtii* and enhances plant susceptibility to this pathogen (Hewezi *et al.*, 2008). In addition, The functional PME from the necrotrophic *Botrytis cinerea* has been shown to be an important determinant of its pathogenicity (Valette-Collet *et al.*, 2003).

- PME and viruses: it has been reported that PME is a host receptor for the tobacco mosaic virus (TMV) movement protein. Indeed, 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). Even if it is now clear that (i) PMEs are associated with cell wall-embedded plasmodesmata, (ii) MPs colocalize with plasmodesmata and (iii) PME-like proteins bind to MPs in cell wall extracts, the mechanism allowing PMEs to facilitate viral movement remains unclear. PMEs are also thought to play a role in systemic movement of the tobamoviruses. When the spreading virions reach the vascular tissue system, they enter it and are systematically transported throughout the plant in a PME-independent manner. Phloem unloading is instead partly PME-dependent and systemic infection by viruses in tobacco can be delayed by reductions in PME expression and activity. Accordingly, silencing of PME expression led to diminished systemic movement of TMV (Chen and Citovsky, 2003). Unfortunately, a plant protection strategy based on this approach might only delay infection but not fully suppress it. These results indicate that cell wall PMEs are only one of the targets for virus-induced infection. It has been shown that co-agroinjection of functional proPME (type I proteins) cDNA and a viral vector stimulates plant virus defence via virus-induced gene silencing mechanisms that repress virus reproduction in tobacco (Dorokhov *et al.*, 2006a). The roles of PME in viral infections are still unclear, but tight regulation of different tissue-specific isoforms, and the balance between PME and MP levels, is likely to be involved.

- PME and plant-herbivore interaction: 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 MeOH emissions when compared with non-induced plants (Penñelas *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).

1.11.3 Regulation of PME activity

PME activity is regulated in part by the differential expression of isoforms in specific tissues and developmental stages, and 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. 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 reaction. In turn, the pH decrease activates glycosidases and glycosyl-transferases, involved in cell wall extension and building up. The consequent dilution of negative charges and increase of local pH result in reactivation 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).

1.12 Proteinaceous inhibitors of pectinolytic enzymes

A number of proteinaceous inhibitors against almost all the classes of pectic enzymes have been discovered in plants, such as polygalacturonase-inhibiting protein (PGIP), pectin methylesterase inhibitor (PMEI) and pectin lyase inhibitor protein (PNLIP). This last has been found in sugar beet and inhibits a PNL from *Rhizoctonia solani*, which is a fungal pathogen of sugar beet, and other fungal PNLs from *Phoma betae* and *Aspergillus japonicus* (Bugbee, 1993).

1.12.1 Polygalacturonase inhibiting protein (PGIP)

Polygalacturonase-inhibiting proteins (PGIPs) are the best characterized plant inhibitors, firstly described almost 40 years ago and now proposed to form part of the plant immune system (Federici *et al.*, 2006; Di Matteo *et al.*, 2006). PGIPs are glycoproteins located in plant cell walls of monocot and dicot plants, such as raspberry, tomato, pear, apple, grape, soybean, bean, Arabidopsis, rice, wheat, onion and leek. They specifically inhibit fungal PGs (Cook *et al.*, 1999) and PGs produced by phytophagous insects such as *Lygus rugulipennis* and *Adelphocoris*

lineolatus (D'Ovidio *et al.*, 2004b) while they are ineffective against other pectic enzymes of either microbial or plant origin (Cervone *et al.*, 1990). PGIP limits fungal colonization by inhibiting PG activity and also by modulating their activity favouring the release of oligogalacturonides (OGs) with a degree of polymerization between 10 and 15 which act as active elicitors of a wide number of defence responses, such as the accumulation of phytoalexins, the synthesis of lignin, the expression of β -1,3-glucanase and proteinase inhibitor I and the production of reactive oxygen species (Ridley *et al.*, 2001).

PGIPs or PGIP-like proteins are also involved in flower development (Gamboa *et al.*, 2001; Jang *et al.*, 2003) or possess anti-freezing properties (Worrall *et al.*, 1998).

PGIP are organized into multigene families, clustered in specific chromosomal regions. For example, the *Arabidopsis* *pgip* gene family comprises two tandemly duplicated genes, which encode functionally redundant proteins that are up-regulated through different signal transduction pathways in response to fungal infection (Ferrari *et al.*, 2003). In *Phaseolus vulgaris*, instead, the *pgip* gene family, spanning a region of 50Kbp, is composed by four genes, each with different recognition capabilities; they display arrangements and similarities suggesting that they derive from a common ancestor as a result of a sequence of duplication-divergence-duplication events (D'Ovidio *et al.*, 2004b).

Expression of *pgip* genes is spatially and temporally regulated during development and in response to several stress stimuli, both biotic and abiotic, such as pathogen infection, wounding, salicylic acid, low temperature, jasmonate and OGs (D'Ovidio *et al.*, 2004a; Ferrari *et al.*, 2003; D'Ovidio *et al.*, 2006; Reymond *et al.*, 2000).

Pgip genes encode for isoforms with similar structures but different specificities (D'Ovidio *et al.*, 2004a; De Lorenzo *et al.*, 2001).

Not only do PGIPs from different plant sources differ in their inhibitory activities but also PGIPs from a single plant is often capable of inhibiting PGs from different fungi or different PGs from the same fungus, although the strength of the inhibition can differ (De Lorenzo *et al.*, 2001). In addition, the type of inhibition can be either competitive, non-competitive or mixed, indicating again the versatility of PGIPs to evolve different recognition specificities (Sicilia *et al.*, 2005); for example, PGIP2 from *Phaseolus vulgaris* (PvPGIP2) acts competitively against PG of *Fusarium moniliforme* (FmPG; renamed *Fusarium phyllophilum*-Fp- in Mariotti *et al.*, 2008) (Federici *et al.*, 2001), non-competitively against PGII of *Aspergillus niger* (AnPGII) (King *et al.*, 2002) and has a mixed mode of inhibition against PG1 of *Botrytis cinerea* (BcPG1) (Sicilia *et al.*, 2005).

PGIPs, like many resistance (*R*) genes, belong to the superfamily of leucine-rich repeat (LRR) proteins of the extracytoplasmic type. The LRR is a versatile structural motif widespread among microorganisms and animals, responsible for protein-protein interactions (Shanmugam, 2005).

The typical structure of PGIPs comprises a signal peptide for translocation into the ER and a mature polypeptide of 292-315 amino acids displaying several potential glycosylation sites. The mature PGIPs typically contain 10 imperfect LRR, each derived from modifications of a 24-amino acid leucine-rich peptide. The LRR element matches the consensus sequence **LxxLxLxx**NxLT/SGxIPxxLxxLxx, where residues indicated in bold form a β -strand/ β -turn structure, responsible for binding PGs (De Lorenzo *et al.*, 1994).

The crystal structure of PvPGIP2, which is the most wide-spectrum and efficient inhibitor of fungal PGs so far characterized, has been determined at 1.7 Å resolution (Di Matteo *et al.*, 2003) providing the first structure of a plant LRR protein. PvPGIP2 shows a curved and elongated shape, more twisted than other LRR proteins (Fig. 1.14). A long parallel β -sheet (B1) occupies the concave inner side of the structure, corresponding to the β -sheet originally predicted by modelling studies, where the residues determining the affinity and specificity of PvPGIP2 are known to reside (Leckie *et al.*, 1999). An additional extended parallel β -sheet, absent in the majority of other LRR proteins, characterizes the structure and places the fold of PvPGIP2 between the typical LRR structure and the β -helical architecture found in pectate lyases and PGs (Jenkins and Pickersgill, 2001). The second β -sheet may contribute to the formation of an additional surface for interactions with other ligands.

The structure of PvPGIP2 reveals also the presence of a negative pocket, formed by three aspartic residues highly conserved on all PGIPs, which is thought to accommodate the positive charged residues Arg 267 and Lys 269 on the surface of the PG of *F. phyllophilum*, thus covering its active site and preventing access to the substrate. The residue Gln224 of PvPGIP2, which is crucial for the specificity of the inhibitor towards FpPG, is adjacent to the negative pocket putatively involved in PG binding and may interact with an unidentified partner residue of FpPG to correctly lock Arg 267 and Lys 269 into the negative pocket. In PvPGIP1, which is unable to interact with FpPG, this role may not be fulfilled by the corresponding Lys224 (Leckie *et al.*, 1999).

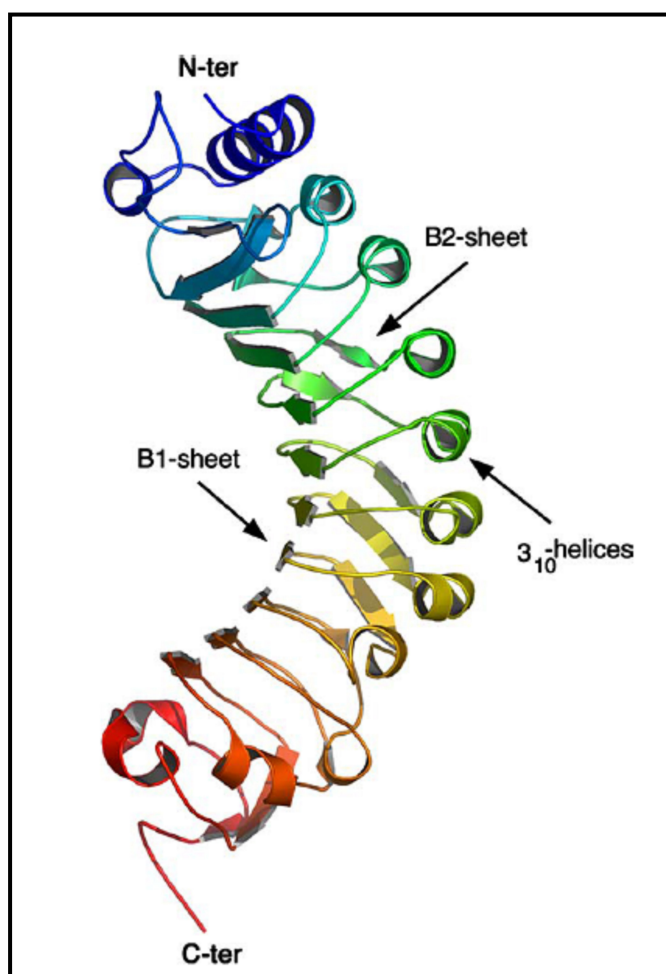


Fig. 1.14 Ribbon representation of the crystal structure of PGIP2 from *P. vulgaris* (from Di Matteo *et al.*, 2006).

PGIPs also show the capability to interact with stretches of unesterified HG of the cell wall. Four positive residues in the PvPGIP2 were found to be responsible for this binding. It has been suggested that PGIP interacts with pectin and PGs through overlapping although not identical regions; in fact, PGs display *in vitro* the binding of PGIP to pectin (Spadoni *et al.*, 2006). Spadoni *et al.* also proposed that stretches of unmethylated pectin should be the anchorage place of PGIP in the wall in physiological conditions, protecting in this way pectin from the degrading activity of fungal PGs.

Regarding PGIP activity in monocots, until a few years ago the only information available were on pectin-rich species such as *Allium cepa* (Favaron *et al.*, 1993) and *A. porrum* (Favaron, 2001). Lately, PGIPs have been characterized in wheat, although their N-terminal sequences (Lin and Li, 2002; Kemp *et al.*, 2003) do not show any similarity with the typical PGIP sequences determined from purified proteins or deduced from gene sequences (De Lorenzo *et al.*, 2001). Moreover, a rice *pgip* gene has been reported to be involved in flower development (Jang *et al.*,

2003). In addition, four rice (*Oriza sativa* L.) and three wheat (*Triticum aestivum* L.) *pgip* genes were recently identified (Janni *et al.*, 2006; Di Giovanni *et al.*, 2008). The OsPGIP1 in rice, although lacking an entire LRR repeat, is an active inhibitor of fungal PGs indicating that the genetic events that can generate variability at the *pgip* locus are not only limited to substitutions or small insertions/deletions, as so far reported, but can also involve variation in the number of LRRs. In wheat, each genome contains a single copy of *pgip* gene, in particular, *Tapgip1* is localized on chromosome 7B, *Tapgip2* on chromosome 7D and *Tapgip3* on chromosome 7A. This last has been found to be inactivated by the insertion of a LTR *copia* retrotranspon within the fourth LRR (Di Giovanni *et al.*, 2008).

1.12.2 Role of PGIPs in plant defence

An indication of the involvement of *pgip* genes in plant defence responses came from the observation that the expression of some *pgip* genes is induced in response to several stress stimuli, such as wounding, pathogen infection, and elicitor treatments (De Lorenzo *et al.*, 2001). Then, a direct evidence of the involvement of PGIP in plant defence has been provided by using transgenic plants transformed with *pgip* genes. In these experiments tomato and grapevine plants overexpressing a pear PGIP showed reduced disease symptoms following the infection with *Botrytis cinerea* (Powell *et al.*, 2000; Agüero *et al.*, 2005); Arabidopsis plants transformed with two endogenous *pgip* genes, *Atpgip1* and *Atpgip2* (Ferrari *et al.*, 2003) and tobacco plants overexpressing the bean PvPGIP2 resulted in enhanced resistance to *B. cinerea* (Manfredini *et al.*, 2005). Arabidopsis plants with antisense expression of *pgip* genes showed a reduced inhibitory activity in response to abiotic stress and were more susceptible to *B. cinerea* (Ferrari *et al.*, 2006).

The effectiveness of PGIP in limiting host tissue colonization has been demonstrated not only in dicot plants but also in monocot plants. In fact, transgenic wheat plants expressing PvPGIP2 showed a significant reduction of symptoms following the infection of *Bipolaris sorokiniana* (Janni *et al.*, 2008) or *Fusarium graminearum* (Janni *et al.*, 2007) suggesting that pectin hydrolysis is an important step for fungal penetration of monocot grass plants in spite of a low pectin content in their cell wall.

Furthermore, because of their ability to limit the rate of PG-derived OGs, PGIPs are included among the microbe-detecting molecules that are employed by the plant immune system (Federici *et al.*, 2006).

1.12.3 Pectin methylesterase inhibitors (PMEIs)

Molecules of different nature from different species has been reported to exhibit PME inhibitory activity. The first PME inhibitor was isolated in the sap extract of potato tuber during purification of a PME isozyme (Ricard and Noat, 1986). The inhibitor, purified by ion-exchange and gel-filtration chromatography was extremely thermostable and showed an estimated molecular weight of about 158–232 kDa. The purified inhibitor was demonstrated to be mainly composed by uronic acid and neutral sugars while no amino acids were detected. In addition, it was deactivated by the pectic enzymes polygalacturonase (PG) and pectate lyase suggesting a pectic configuration. The inhibitor was active against PME from potato and from different plant species, with an uncompetitive mode of action, whereas it did not affect bacterial and fungal PMEs. An inhibitory activity was also found in banana fruit (*Musa sapientum* L.) (Wu *et al.*, 2002) especially in the rubbery fruit even if the chemical nature of this inhibitor was not elucidated. This inhibitory molecule was active against the banana PME and the pea pod (*Pisum sativum*) PME and was extremely thermostable. A highly thermostable inhibitory activity, consisting of polypeptides with molecular weights ranging from 3.5 to 4.5 kDa, was also found in intact jelly fig (*Ficus awkeotsang*) achenes (Jiang *et al.*, 2002).

To date, four proteinaceous PME inhibitors (PMEIs) have been identified and characterized: AcPMEI from kiwi (*Actinidia chinensis*) (Balestrieri *et al.*, 1990), AtPMEI-1 and AtPMEI-2 from *Arabidopsis thaliana* (Wolf *et al.*, 2003; Raiola *et al.*, 2004) and CaPMEI1 from pepper (*Capsicum annuum*) (An *et al.*, 2008). AcPMEI (accession number P83326) was the first proteinaceous PME inhibitor discovered by Balestrieri *et al.* in 1990 during a study aimed at the characterization of PME from kiwi fruit. The inhibitor was purified to homogeneity by a single-step procedure based on affinity chromatography from fully ripe kiwi fruit. The complete amino acids sequence of the natural PMEI purified from kiwi fruit was obtained by direct protein sequencing (Camardella *et al.*, 2000): it 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). This hypothesis has been confirmed by the isolation of two entire cDNAs and one fragment coding for PMEI isoforms from *Actinidia deliciosa* (accession numbers: AB091088, AB091089, AB091090) (Irifune *et al.*, 2004). These cDNA sequences show an N-terminal extension of 32 amino acid

residues, which corresponds to the signal peptide required for the extracellular targeting of the protein.

AcPMEI was found to be specific for pectin methylesterase (PME) because it was effective in inhibiting PME but ineffective against other polysaccharide-degrading enzymes, such as polygalacturonase and amylase and completely aspecific as far as the pectin methylesterase source is concerned. AcPMEI is indeed active against several plant PMEs (from kiwi, orange, apple, tomato, apricot, carrot, potato and banana) whereas it does not inhibit fungal and bacterial PMEs (Balestrieri *et al.*, 1990). The inhibition mode of AcPMEI on kiwi, tomato and carrot PMEs was found to be competitive, while the inhibition of banana or strawberry PMEs is with a non competitive mode (Ly-Nguyen *et al.*, 2004). The inhibition occurs through the formation of a reversible 1:1 complex. The stability of the complex between AcPMEI and 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). A dissociation constant value of 53 nM was calculated at pH 7 for the interaction between AcPMEI and PME-1 from tomato, the 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).

Analysis of genomic data showed that PMEIs (like PMEs) belong to large multi gene families; for instance, in *Arabidopsis thaliana* 69 ORFs have been annotated as putative full-length PMEIs, while in monocots, such as *Oryza sativa* and *Brachypodium distachyon*, 25 and 38 putative PMEIs were respectively annotated.

In *Arabidopsis thaliana*, two PMEI genes, named *Atpmei-1* (At1g48020) and *Atpmei-2* (At3g17220), were characterized (Wolf *et al.*, 2003; Raiola *et al.*, 2004). The intronless *Atpmei-1* and *Atpmei-2* share 64% identity at the nucleotide level. At the amino acid level, the two encoded proteins AtPMEI-1 and AtPMEI-2 share about 38% identity with AcPMEI and 45% identity with each other. Removal of the predicted N-terminal signal peptide generates mature proteins of 151 amino acids (AtPMEI-1: molecular mass of 16.266 Da, predicted pI =7.7) and

148 amino acids (AtPMEI-2: molecular mass of 15.615 Da, predicted pI = 9.0). Although they both present potential N-glycosylation sites, only AtPMEI-2 showed to be glycosylated *in vivo*, while AtPMEI-1, like AcPMEI, is not glycosylated (Raiola *et al.*, 2004). 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, expressed in *Pichia pastoris* and purified to homogeneity, were assayed against various PMEs confirming the results already obtained with AcPMEI. Indeed, they were found to be 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).

Surface Plasmonic Resonance (SPR) studies on the kinetic parameters of interaction between the inhibitors from Arabidopsis and PME-1 from tomato revealed that AtPMEI-2 has a stronger affinity for tomato PME with 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 transcripts level of *Atpmei-1* and 2 in different tissues revealed very low expression of both isoforms in most tissues except for flowers. The high expression in flowers was followed by root tissue for AtPMEI-1 and by sink leaves for AtPMEI-2 with AtPMEI-2 showing a broader expression range than AtPMEI-1. The analysis of Arabidopsis transformants containing the gene GUS 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 their involvement in a post-translational regulation of pollen and flower PMEs.

Very recently, a PME inhibitor from pepper (*Capsicum annuum*), named CaPMEI1 (AF477956), has been isolated and functionally characterized in pepper leaves infected by *Xanthomonas campestris* pv. *vesicatoria* (Xcv). 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 isoelectric point (pI) of 6.51; the four Cys residues typical of PMEIs are also conserved. RNA analysis, performed to examine the organ-specific expression of CaPMEI1 in pepper plants, 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).

1.12.4 Structural analysis of PMEI and PME-PMEI complex

To date, 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 ($\alpha 1$, $\alpha 2$, $\alpha 3$ and $\alpha 4$) aligned in an antiparallel manner in a classical up-and-down four-helical bundle (Fig. 1.15).

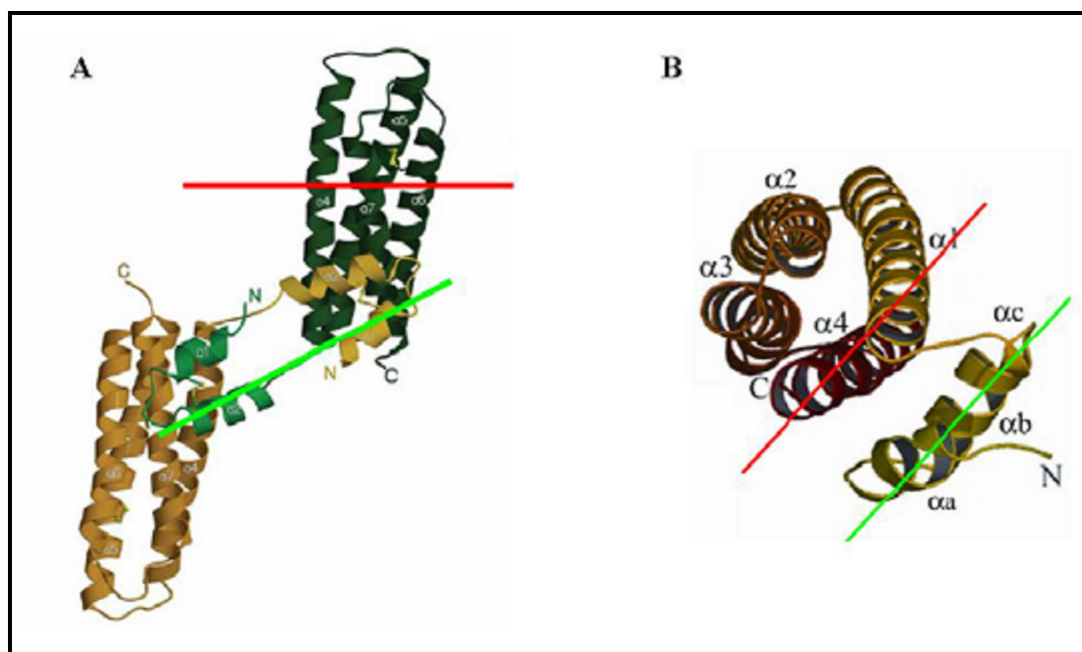


Fig. 1.15 (**A**) Ribbon representation of the AtPMEI-1 dimer with the respective molecules shown in green and yellow. (**B**) Ribbon representation of the AcPMEI monomer. Red and green lines show core plan orientation and N-terminal extension orientation, respectively (from Hothorn *et al.*, 2004).

The interior of the bundle is stabilized by hydrophobic interactions and by a disulphide bridge which connects helices $\alpha 2$ and $\alpha 3$. The N-terminal region is composed of three short and distorted helices (αa , αb and αc) and extends outside the central domain. Although both inhibitors are quite similar in the bundle, significant differences are located in the N-terminal region; in fact, in AtPMEI-1 the N-terminal region, composed of three short and distorted helices, extends outside the central domain forming an hairpin whose particular orientation allows the extensive contacts with the α -hairpin of a neighbouring molecule forming in solution an active dimer (Hothorn *et al.*, 2004). In addition, Pro-28 of AtPMEI-1, 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, is replaced by a Lys 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).

PMEIs show structural similarity with the PME pro-region; in fact, even if the sequences are quite variable, they maintain the conservation of the four Cys residues favouring the hypothesis that the PRO-region has an inhibitory function towards its own PME. This suggests that PMEI could be evolved following a genome rearrangement that occurred during evolution producing the physical separation of the inhibitory domain initially linked to the catalytic domain (Giovane *et al.*, 2004).

PMEIs also share several structural properties with the invertase inhibitors (INH), in particular the presence of a domain characterized by four conserved cysteine residues that form two disulfide bonds. Indeed, according to the Pfam protein domain database (<http://pfam.wustl.edu/>), PMEIs belong to the family PF04043 of invertase/pectin methylesterase inhibitors (INH)/PMEIs (Rausch and Greiner, 2004). The elucidation of the 3D-structure of an INH from tobacco (Nt-CIF), together with the 3D-structure of PMEI revealed that both inhibitors exhibit an almost identical fold but recognize different target enzymes (Hothorn *et al.*, 2004) (Fig. 1.16).

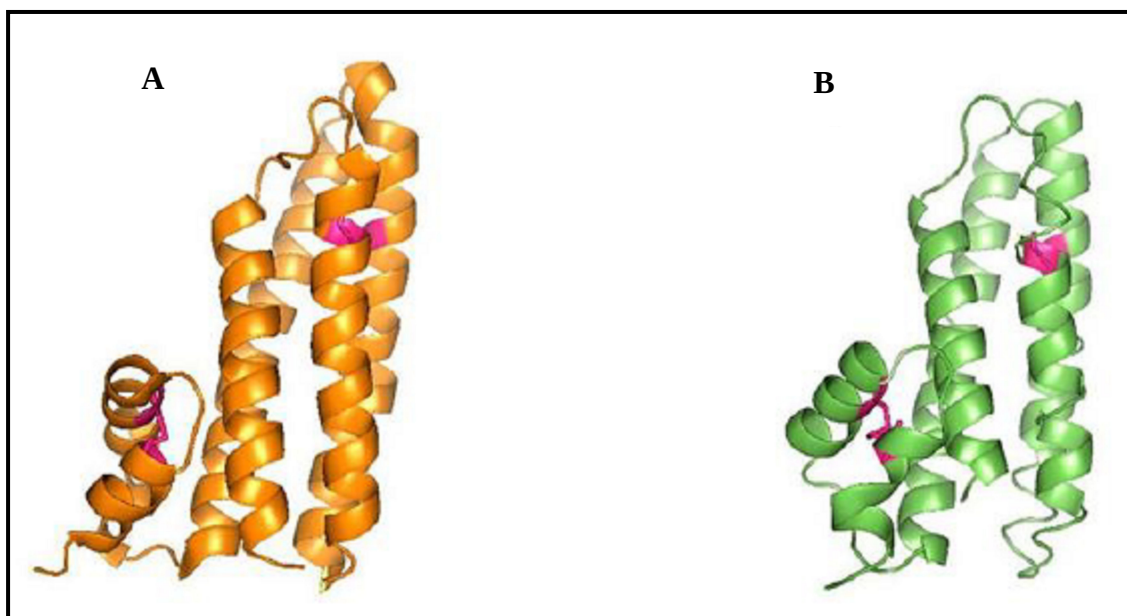


Fig. 1.16 Ribbon representation of (**A**) NtCIF and (**B**) AcPMEI. Disulfide bridges are in pink (from Hothorn *et al.*, 2004 and Di Matteo *et al.*, 2005).

The three-dimensional structure of the complex PME-PMEI has been also solved. AcPMEI interact with PME from tomato forming a stoichiometric 1:1 complex in which the inhibitor covers the shallow cleft of the enzyme where the putative active site is located, confirming the observed competitive mode of inhibition. The four-helix bundle of PMEI packs roughly perpendicular to the parallel β -helix of PME, and the three helices α_2 , α_3 and α_4 , but not α_1 , interact with the enzyme in proximity of the putative active site (Fig. 1.17). The relative position of α_2 and α_3 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 even if it was proposed to be crucial for the interaction between AtPMEI-1 and PME from carrot. 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. The structure of the PME-PMEI complex provides a possible explanation for the lack of inhibition of PMEIs on PMEs from the bacterium *E. crysanthemii* and the fungus *Aspergillus aculeatus* and also why there is no interaction between INH and PMEs (Raiola *et al.*, 2004; Giovane *et al.*, 2004). In *E. crysanthemii*, the putative binding site cleft is much deeper than in plant-derived PMEs.

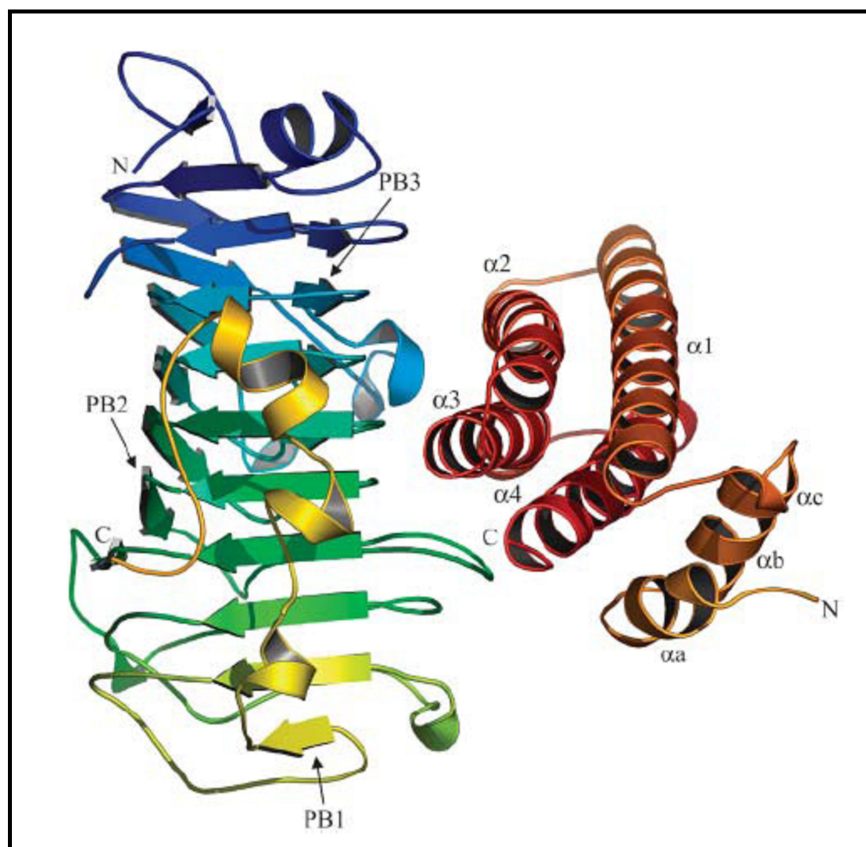


Fig. 1.17 Ribbon representation of the PME-PMEI complex. The enzyme (PME-1 from tomato) is shown in green-blue on the left side. The inhibitor (AcPMEI) is represented in yellow-red on the right side; the α -helices of the four-helix bundle are indicated as $\alpha 1$ to $\alpha 4$, whereas helices of the N-terminal region are named αa , αb , and αc (from Di Matteo *et al.*, 2005).

It is likely 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).

Regarding instead the absence of interaction of INH with PME, a possible explanation provided by the structural view of the PME-PMEI complex, is the lack in INH of some residues important for the formation of the complex as well as the distortion of $\alpha 2$ helix of INH compared with that of PME (Di Matteo *et al.*, 2005).

1.12.5 Physiological role of PMEIs

Different PME and PMEI isoforms are highly expressed in pollen and pollen tubes, consistent with the proposed key role of a dynamic pectin metabolism in the polar growth of this cell type (Pina *et al.*, 2005). Recently, it was demonstrated that the PME isoform AtPPME1 (At1g69940) and the PMEI isoform AtPMEI-2 (At3g17220), 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 patterning. Whether the impact of PME/PMEI activity on primordial formation occurs through direct effect on the mechanical properties of the cell wall or indirect effect through the release of OG and/or regulation of the efflux of auxin remain still a question.

1.12.6 Role of PMEIs and pectin composition in plant defence

Although the specificity of PMEI to plant PMEs suggests their involvement in physiological processes, some evidences also indicate a role in plant defences.

In Arabidopsis, the overexpression of the endogenous PMEI (AtPMEI-1 and AtPMEI-2) caused a reduced PME activity and an increased content in methylated pectin. These transgenic lines not only showed an altered growth (longer roots and bigger rosettes with respect to the wild type

plants) but were also found to be more resistant to the necrotrophic fungus *Botrytis cinerea* (Lionetti *et al.*, 2007).

In pepper, the recent characterization of the *pmei* gene *Capmei1* revealed that pathogens and abiotic stresses can induce the expression of this gene. In addition, the purified recombinant CaPMEI1 protein not only inhibited PME, but also exhibited antifungal activity against some plant pathogenic fungi such as *F. oxysporum* f.sp. *matthiole*, *A. brassicicola* and *B. cinerea* (An *et al.*, 2008).

Virus-induced gene silencing of CaPMEI1 in pepper conferred enhanced susceptibility to *Xanthomonas campestris* pv. *vesicatoria* (Xcv), accompanied by suppressed expression of some defence-related genes, such as PR1 and PR10. Moreover, transgenic Arabidopsis lines overexpressing CaPMEI1 exhibited enhanced resistance to *Pseudomonas syringae* pv. *tomato*, mannitol and methyl viologen, but not to the biotrophic pathogen *Hyaloperonospora parasitica*. Based on these results, the authors suggested that CaPMEI1 may be involved in basal disease resistance, as well as in drought and oxidative stress tolerance in plants. The same authors recently identified the promoter of the *Capmei1* gene from pepper genomic DNA. The activity of the -958-bp CaPMEI1 promoter was analyzed in GUS-fused constructs not only in tobacco, using an *Agrobacterium*-mediated transient expression assay, but also in Arabidopsis plants transformed using the full-length CaPMEI1 promoter-GUS construct. Analysis of the CaPMEI1 promoter reinforced the hypothesis of the CaPMEI1 involvement in regulating the plant defence responses to pathogen infection, as well as to ethylene and methyl jasmonate treatment (An *et al.*, 2009)

Because PME is involved in the systemic spread of *Tobacco mosaic virus* through the plant, acting as a cell-wall receptor of the virus movement protein, PMEI might be involved in a defence mechanism against virus propagation by forming a high-affinity PME-PMEI complex (Chen and Citovsky, 2003).

Regarding the bacterial and fungal infections, it is known that pectin degrading enzymes are produced by pathogens to macerate and spread into the plant tissue. Highly methylesterified pectins are less susceptible to the action of PG and pectate lyase (Willats *et al.*, 2001b). By inhibiting the endogenous PME and maintaining the pectin in a highly methylated form, PMEI might indirectly play a role in plant defence against pathogen attack by limiting the action of microbial PGs (Juge, 2006). Indeed, the resistance of the Arabidopsis transgenic plants expressing AtPMEI-1 and AtPMEI-2 to the infection of *B. cinerea* was related to the impaired ability of the fungus to grow on methylesterified pectins (Lionetti *et al.*, 2007). The importance of the pectin composition in plant resistance has been demonstrated in other 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. They include bean, potato, tomato and wheat. 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*, and susceptible potato cultivars possess an additional PME isoform (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. When bean cell walls were digested with an endo-polygalacturonase from that fungus, the fragments recovered from resistant cultivars proved to exhibit higher elicitor activities than the fragments from a susceptible cultivar (Boudart *et al.*, 1998). In wheat, differences in methyl ester distribution of homogalacturonan have been found in near-isogenic lines resistant and susceptible to the fungal pathogen *Puccinia graminis* f. sp. *tritici*. In particular a more blockwise distribution of methyl esters in the homogalacturonan of susceptible wheat cultivars was found compared with a more random distribution in the near-isogenic resistant lines (Wietholter *et al.*, 2003). The authors also proposed a differential enzymatic breakdown of wheat pectins upon rust invasion leading to the generation of suppressor-active pectic oligomers in susceptible plants only.

The involvement of pectin in plant defences also includes the generation of oligogalacturonides (OGs) which are active-elicitors produced by pectate lyase and polygalacturonase-mediated breakdown of homogalacturonan. The induction of plant defences by OGs has been widely studied and their efficacy has been shown to depend upon the degree of polymerization which has to be between 10 and 15 units (Cervone *et al.*, 1989). Moreover, it has been recently demonstrated in strawberry that activity of OGs requires demethylesterification by a pectin methylesterase (Osorio *et al.*, 2008).

1.13 Aim of the work

Several evidences have been reported on the importance of the structure and composition of pectin in plant resistance in different plant-pathogen systems, including wheat. In addition, the recent demonstration that transgenic wheat plants expressing a polygalacturonase inhibitor (PvPGIP2) showed a significant reduction of symptoms following the infection with *Bipolaris sorokiniana* (Janni *et al.*, 2008) or *Fusarium graminearum* (Janni *et al.*, 2007) suggests that pectin hydrolysis is an important step for fungal penetration of monocot grass plants in spite of a low pectin content in their cell wall.

Aim of this work is to enhance wheat defence responses against fungal pathogens by limiting the degradation of the pectin component of the cell wall. To obtain that, wheat plants expressing pectin methylesterase inhibitors (AtPMEI-1 or AcPMEI) have been produced, characterized and tested against fungal pathogens, including *F. graminearum*, the causal agent of Fusarium Head Blight (FHB), one of the most important wheat disease worldwide. These transgenic *Acpmei* plants have been also crossed with transgenic plants expressing PvPGIP2, a polygalacturonase inhibiting protein particularly effective in the inhibition of fungal polygalacturonases.

2. Materials and Methods

2.1 Construction of the pUBI::Acpmei and pUBI::Atpmei-1 vectors

To assemble the monocot expression vectors pUBI::Atpmei-1 and pUBI::Acpmei, the coding region of *Atpmei-1* or *Acpmei*, this last with the *Pvpgip1* signal peptide, 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* genes were generated by PCR amplification using the forward and reverse primers Atpmei1F1Bam/Atpmei1R1Bam for *Atpmei-1* and AcpmeiF1Bam/AcpmeiR1Bam for *Acpmei*. PCR reactions were performed in a reaction volume of 25µl with 10ng of plasmid DNA (pAcpmei or pAtpmei-1), 2,5 units of FastStart High Fidelity PCR system (Roche Diagnostics, Monza, Italy), 1x Taq PCR buffer, 50ng 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 5.248-bp plasmid, pUBI::Atpmei-1 or a 5.236-bp plasmid, pUBI::Acpmei. 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 *pmei* and the insertion sites were confirmed by nucleic acid sequencing.

2.2 Production of transgenic wheat plants

2.2.1 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 Phytigel 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/l; L-asparagine 0,15g/l 0,25% v/v di phytigel 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/l; L-asparagine 0,15g/l 0,25% v/v di phytigel 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/l 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

2.2.2 Embryos isolation

Caryopses 10 to 18 days post anthesis were collected from *Triticum durum* cv. Svevo wheat plants, (Zadoks stage 72) grown in a climate chamber or in the field, and surface-sterilized in 70% ethanol containing 50µl/100ml of Tween 20, by sonication 6 times for 20 sec plus one time for 10 sec. Caryopses were then sterilized with 70% ethanol for 5 min and 20% sodium hypochlorite for 15 min, followed by three changes of sterile distilled water. Immature embryos, 0,5 to 1 mm long, were aseptically removed under a stereo microscope (Fig. 2.1a), placed with

the scutella exposed on the dissecting medium (Fig. 2.1b) and stored in the dark at 23°C for 5 days for the callus induction (Fig. 2.1c).

Among the explants, 0,5-1 mm immature embryos were found to be the ones with the highest transformation frequency and the lower number of chimeric transformants.

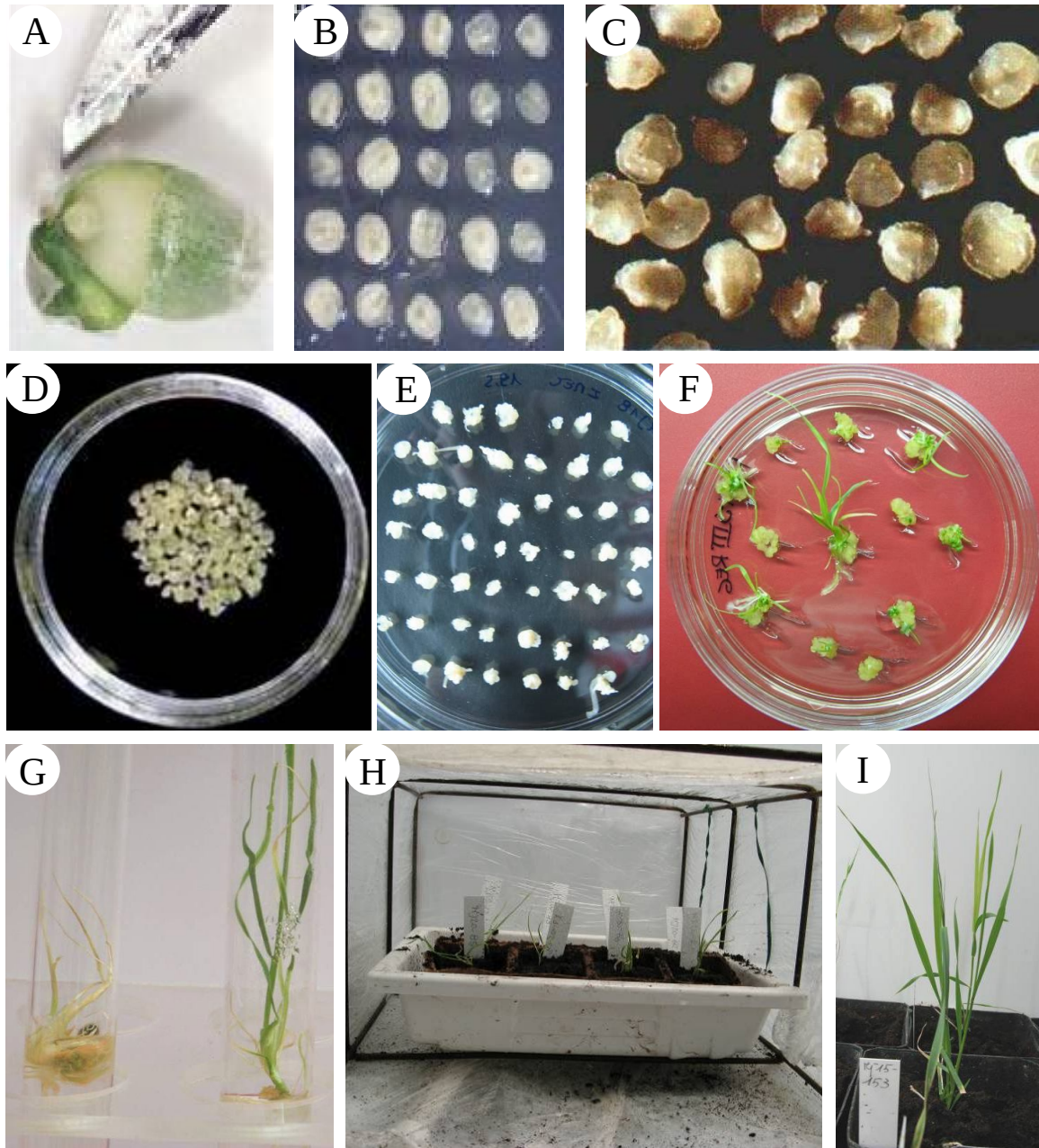


Fig. 2.1 (**A**) Isolation of immature embryo from wheat caryopsis. (**B**) Immature scutella plated for bombardment. (**C**) Formation of embryogenic callus. (**D**) calli in the bombardment medium, (**E**) Bombarded calli in the recovery medium. (**F**) Regeneration of plantlet on selective medium, (**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 Huw D. Jones and Peter R. Shewry; C image is from Weeks *et al.* 1993.

2.2.3 Coating gold particles with DNA, bombardment and plant regeneration

Before the bombardment the coating of gold particles with DNA was performed.

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, spun and the ethanol removed. The microprojectiles were then washed with cold sterile water (200µl), spun and the supernatant discarded.

At this point, the gold particles were coated with plasmid DNA. For the transformation with *pmei* genes, the plasmid pUBI::BAR (5.505 bp), carrying the *bar* gene that confers resistance to the bialaphos herbicide, was co-bombarded with pUBI::Atpmei-1 (5.248 bp) or pUBI::Acpmei (5.236 bp) in a 1:3 molar ratio. Instead, for the transformation experiments with *Pvpgip2* gene, the plasmid pUBI::BAR was co-bombarded with pUBISR2 (5.719 bp) with the same ratio as above. 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): $\text{bp gene/bp marker} \times 15\mu\text{g} \times (1/\text{plasmid concentration}) \times 3$

Marker plasmid (µl): $15\mu\text{g} \times (1/\text{plasmid concentration})$

The microprojectiles pellet was resuspended in a solution containing 2.5M CaCl₂ (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. 2.2).

Immediately after the bombardment, calli were kept in the bombardment medium (Fig. 2.1d) and stored in the dark at 23°C for 24 hours; they were, then, maintained in the recovery medium for 4 weeks transferring them to a fresh medium at 2-week intervals (Fig. 2.1e). For the regeneration, calli were transferred to the regeneration medium containing the Bialaphos herbicide for 6 weeks at 27°C with 16-h light period (43µE/m²) transferring them to a fresh medium at 2-week

intervals. At this stage, new shoots come from the calli resistant to the herbicide, usually starting from the third week (Fig. 2.1f).

Shoots were then transferred to Pyrex tubes containing the rooting medium, which is without 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 bialaphos-containing medium were defined as resistant (Fig. 2.1g, right).

Sensitive plantlets initiated root formation, but the primary root soon stopped growing and put out only a few short lateral roots (Fig. 2.1g, left). Aerial portions of sensitive plantlets exhibited yellow necrosis and reduced vigour within 1 week, whereas resistant plantlets thrived in the rooting media.

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. 2.1h). After that, plants were transferred to bigger pots; these primary regenerants are called T₀ plants (Fig. 2.1i).

Some of the lines showed normal levels of fertility and seed set. However, most of them showed reduced seed set compared with non-transformed Svevo plants and some of them were sterile.

The total process starting from the excision of the embryos up to the anthesis of regenerated T₀ plants, lasts about 168 days. The presence of the transgene in the regenerated plants were checked by PCR.

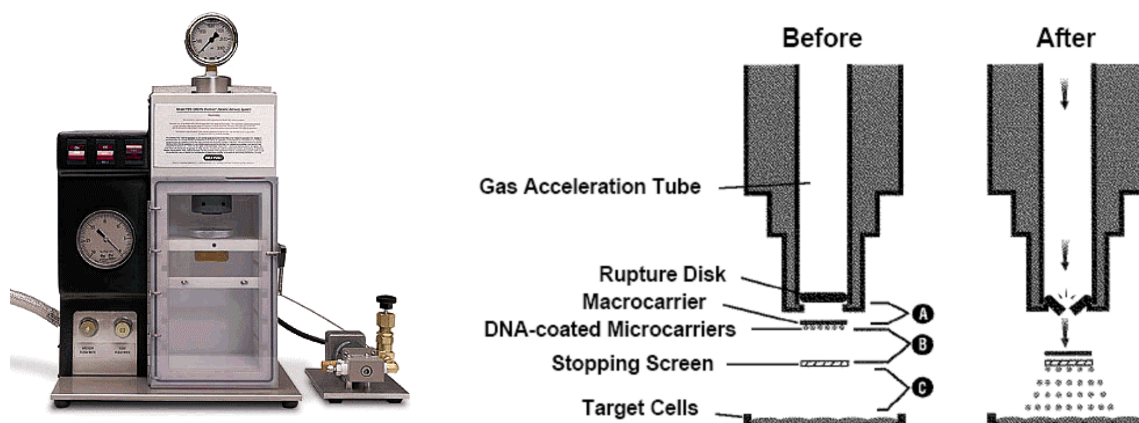


Fig. 2.2 (Left) The Biolistic PDS-1000/He instrument. (Right) The Biolistic bombardment process. The Biolistic PDS-1000/He system uses high pressure helium, released by a rupture disk, and partial vacuum to propel a macrocarrier sheet loaded with microscopic gold microcarriers toward target cells at 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).

2.3 Control of transgenic plants

Before putting on germination, seeds were analyzed for the presence of the transgene (*Atpmei-1* or *Acpmei* or *Pvpgip2*) 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 1cm²).

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. Specific oligonucleotides, designed for each transgene (*Atpmei1F3/Atpmei1R4* for *Atpmei-1*, *AcpmeiF4/AcpmeiR4* for *Acpmei* and 615F/853R for

Pvpgip2) were used. Control DNAs included untransformed *T. durum* cv. Svevo (negative) and the vectors pUBI::Atpmei-1 or pUBI::Acpmei or pUBISR2 (positive).

2.4 Nucleic acids extraction and analysis

2.4.1 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.

2.4.2 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 mortar and pestle in liquid nitrogen. 450µl of RLT buffer (containing dithiothreitol, DTT) were added to the tissue powder and incubated at 56°C for 3 min to help the disruption of the tissue. 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, 500µl 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-freeTM” kit (Ambion) and following the manufacturer’s instructions.

2.4.3 Isolation of plasmid DNA

DH5 α 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 ampicillin (0,1mg/ml) 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 150 μ l of SET (50mM Tris-HCl pH 8; 50mM EDTA pH 8; 20% Sucrose) using a vortex. 350 μ l of lysis buffer (0,2M NaOH; 1% SDS) were added and, after mixing gently, tubes were left on ice for 10 min. 250 μ l 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 20 μ l of sterile water and treated with RNase (2 μ l of 1mg/ml) at 37°C for 20 min. The average yield using this method was about 2 μ g per 1,5ml of bacterial culture.

2.4.4 Midi-and Maxi preparation of plasmid DNA

Plasmid DNA was extracted using the “PureLinkTM HiPure Plasmid Filter Purification” kit from Invitrogen, following the manufacturer’s instructions.

2.4.5 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 0,5 μ g/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.

2.4.6 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.

2.4.7 Gel extraction and purification of DNA fragments

For the purification of DNA fragments from agarose gels a “QIAquick PCR purification “kit (Qiagen) was used according to the procedures specified from the manufacturer.

2.4.8 Digestion with restriction enzymes

All the restriction enzymes were purchased from Roche Diagnostics 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. Digestion of genomic DNA was performed in a appropriate reaction buffer using 5-10µg of DNA in a final volume of 40µl.

2.4.9 Southern blotting

Genomic DNA (8 to 10µg) was cleaved with *SphI* or *BamHI* restriction enzyme, 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.

2.4.10 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 at 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 MgCl₂).

The filter was put into a plastic bag and 1ml of CSPD (Disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]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.

2.4.11 Labelling of the *Acpmei*/probe by PCR

The coding region of *Acpmei* was 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 H₂O, 10µl of 10x buffer, 10mM dATP, dCTP, dGTP, 1mM dTTP, 0,1mM DIG-11dUTP-alkali labile (Roche Diagnostics) 100mM primers *Acpmei*F4/*Acpmei*R4, 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). 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.

2.4.12 qPCR and Real time two-step RT-PCR

RNA of *Acpmei* transgenic lines was used to generate first strand cDNA following the manufacturer's instructions reported in "QuantiTect Reverse Transcription" kit (Qiagen).

Genomic DNA or cDNA was real-time quantified by using the "QuantiTect SYBR® Green PCR Master Mix" (Qiagen) in a MyCycler™ thermal cycler (Bio-Rad Life Science, Segrate, Italy). Both reactions, qPCR and qRT-PCR, were performed in a total volume of 20µl using 1µl of DNA (or cDNA) and oligonucleotide primers specific for *Acpmei* (0,5µM each).

Amplification conditions were:

Cycle 1 (1X)	50°C for 2 min
Cycle 2 (1X)	95°C for 5 min
Cycle 3 (40X)	95°C for 30 sec 60°C for 40 sec 72°C for 30 sec

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).

2.4.13 Specific oligonucleotide design

Specific primers were designed for *Atpmei-1* and *Acpmei*, using the function primer design of the DNAMAN software (Lynnon Biosoft, Quebec, Canada). A list of the oligonucleotides used in PCR reactions with the *Atpmei-1*, *Acpmei*, *Pvpgip2*, *bar* and *actin* genes is reported in Table 2.2.

Table 2.2 Oligonucleotides used for the screening and the characterization of transgenic lines.

Primer name	Sequence 5'-3'	Utilization
Atpmei1F1Bam	ATATGGATCCATGGCTGCGAATCTAAGG	Cloning <i>Atpmei-1</i> in the pAHC17 vector
Atpmei1R1Bam	CCGTGGATCCTTAATTACGTGGTAACATGTTA	Cloning <i>Atpmei-1</i> in the pAHC17 vector
AcpmeiF1Bam	GAGTGGATCCATGTCTTCAAGCTTAAGCATAA	Cloning <i>Acpmei</i> in the pAHC17 vector
AcpmeiR1Bam	GCGAGGATCCTTAAGAACCTGGAAGAAGAT	Cloning <i>Acpmei</i> in the pAHC17 vector
Atpmei1F3	ATGGCTGCGAATCTAAGGAAC	Screening T plants
Atpmei1R4	GTATCAGCTCCATCCAATGC	Screening T plants
AcpmeiF4	CTTGTATCTTTGAGAACTGCAC	Screening T plants, RT-PCR, qPCR, probe amplification
AcpmeiR4	TGAGTTGGAATATTTGGTGGAC	Screening T plants, probe amplification
AcpmeiR2	CCAAGAGAATCAATAGCATCAGC	RT-PCR, qPCR
BAR1	CATCGAGACAAGCACGGTCA	Screening marker gene
BAR2	GAAACCCACGTCATGCCAGT	Screening marker gene
615F	CCTCACCGGGAAGATTCCG	Screening T plants and crosses
853R	TTAGCTGCGTCAGTCCCTGC	Screening T plants and crosses
77F (Actin)	TCCTGTGTTGCTGACTGAGG	Reference gene in RT-PCR, qPCR
312R (Actin)	GGTCCAAACGAAGGATAGCA	Reference gene in RT-PCR, qPCR

2.5 Protein extraction and analysis

2.5.1 Total protein extraction and Bradford assay

Crude protein extracts from transgenic and control plants were obtained from fresh or frozen leaves (at 80°C) using a mortar 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 10.000g. 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.

2.5.2 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. For each samples, 10µg of protein extract were loaded into the gel that was run at a constant voltage of 200V until the bromophenol blue dye front disappeared. When necessary, protein separation was improved by stopping the run 5 minutes after the dye run off.

2.5.3 Silver staining

After SDS-polyacrylamide gel electrophoresis, proteins were silver stained. They were first 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% AgNO₃, 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.

2.5.4 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 that recognizes an N-terminal peptide of mature bean PGIP (Bergmann *et al.*, 1994) or a specific polyclonal antibody raised against AcPMEI was 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.

2.5.5 Densitometry analysis

The bands detected by chemiluminescent assay were analysed by densitometry using "Kodak Digital Science 1D" program which evaluates the relative amount of protein staining and quantifies the results in terms of optical density.

2.5.6 Extracellular washing fluid isolation

Extracellular fluid was extracted from hypocotyls (Zadoks stage 61) by vacuum-infiltration with acetate buffer as described by Salvi and associates (1990). The extracellular fluid had negligible glucose-6-phosphate dehydrogenase activity, verifying that it was free of cytoplasmic contamination.

2.6 Enzymatic assays

Enzymatic activity of PGs was evaluated using both a modified agarose diffusion assay (Taylor and Secor 1988), also called “Cup-plate” and by measuring the release of reducing end-groups from Na-polygalacturonate (Milner and Avigad 1967). In the first method, a solution containing PG or culture filtrate was added to 0,5cm wells on plates containing 100mM sodium acetate, pH 4.6, 0,5% polygalacturonic acid and 0,8% agarose. Plates were incubated for 19 h at 30°C, and the halo caused by enzyme activity was visualized after 15 min of treatment with 6N HCl. PG activity was expressed as agarose diffusion units, with 1 agarose diffusion unit defined as the amount of enzyme that produced a halo of 0,5cm radius (external to the inoculation well) after 19 h at 30°C. Inhibitory activity was expressed as inhibitory units, with 1 inhibitory unit defined as the amount of PGIP that reduced by 50% 1 agarose diffusion unit of PG.

In the latter method, the incubation mixture contained 200µl of 0,5% polygalacturonic acid from orange or 0,5% pectin from apple 70-75% methylesterified (Sigma-Aldrich) in 100 mM sodium acetate, pH 4.7, and bovine serum albumin at 0.1 mg/ml. After addition of the enzyme, the mixture was incubated at 30°C for 1 h. PG activity was expressed as reducing units (RU). One RU was defined as the amount of enzyme required to release reducing groups at 1 µmol/min using D-galacturonic acid as standard.

All the PGs used in the assays were kindly provided by Prof. Felice Cervone, Department of plant Biology, University of “La Sapienza, Rome, and by Prof. Francesco Favaron, Institute of Plant Pathology, University of Padova.

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 Na₂HPO₄, 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 5mm were made and the protein samples (30µl) 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 leaves carefully chosen at the same stage of development.

2.7 Cell wall analysis

2.7.1 Isolation of cell walls

Leaves were frozen in liquid nitrogen and homogenized using mortar and pestle. The ground tissue was washed twice in 70% ethanol, vortexed and pelleted by centrifugation at 10,000g for 10 min. The pellet was suspended with a chloroform:methanol mixture (1:1 v/v) and centrifuged as above. This step was repeated twice and after centrifugation, the Alcohol Insoluble Solid (AIS) fraction obtained, was treated twice with 80% acetone and air dried.

2.7.2 Determination of the Degree of Methylesterification of the cell walls

This assay is based on the principle that methanol yielded after saponification of uronosylmethyl esters is oxidized to formaldehyde and detected subsequently with a pentane-2,4-dione to form a yellow chromogen. Paired assays of uronic acid give the proportion of methylesterified uronosyl esters.

1mg of Alcohol Insoluble Solid fraction was saponified and suspended in 0,25M NaOH. The solution was incubated at room temperature for 1 h and afterwards neutralized with HCl. After centrifugation at 10,000g, aliquots of the supernatant (20µl) were loaded in microtiter plate (96-well cod.9018 from Costar) filling up with water to a total volume of 50µl. Alcohol oxidase (50µl) was added to each well (0.03 units in 0,1M sodium phosphate, pH 7.5; Sigma), and this mixture was incubated at room temperature for 15 min on a shaker. Thereafter, 100µl of a mixture containing 0,02M 2,4-pentanedione in 2M ammonium acetate and 0,05M acetic acid was added. After 10 min of incubation at 68°C, samples were cooled on ice and absorbance was measured at 412 nm in microplate reader (ETI-System Reader; Sorin Biomedica Cardio S.p.A.). The methanol content was estimated as the amount of formaldehyde produced from methanol by alcohol oxidase, according to Klavons and Bennett (1986), by comparison with a standard calibration curve.

For the determination of uronic acid content, the saponified samples were treated with 200µl of 2M TFA for 1h at 121°C in screw-cap tube properly closed. After washing three times with 2-propanol, uronic acids were quantified by colorimetry using the automated sulfamate/m-hydroxy diphenyl assay (Filisetti-Cozzi and Carpita, 1991). In this assay, the TFA hydrolysed samples were dissolved in water (100µl), vortexed and spun. An aliquot (90µl) of supernatant was taken, mixed with sulfamate reagent (10µl; 4M sulfamic acid/potassium sulfamate solution, pH1.6) and

borate buffer (600µl; 0.0125M sodium tetraborate in conc. H₂SO₄), vortexed, boiled for 5 min in a water bath and let it cool off by plunging tubes into an ice bath for about 10 min. 15µl of biphenyl solution (0,15% *m*-hydroxydiphenyl in 0,5% NaOH) were added into each tube and mixed; after a pink colour appeared (in about 10 min) the absorbance was read at 540nm (200µl in microplate reader) (ETI-System Reader; Sorin Biomedica Cardio S.p.A.).

Uronic acid concentration was estimated by comparison with a standard calibration curve using D-galacturonic acid treated like the other samples. The degree of methylesterification was expressed as the ratio of nmole methanol/nmole uronic acid (%).

2.7.3 Immunodot assay

The immunodot assay was conducted on the Chelating Agent Soluble Solids (ChASS) fractions obtained homogenizing 15mg of AIS in a buffer containing 50mM Tris-HCl and 50mM trans-1,2-cyclohexanediarnetetraacetic acid (CDTA), pH 7.2, at 80 °C. After centrifugation at 10,000×*g* for 10 min, samples were dialysed and freeze-dried. Equal amounts of ChASS fractions from each line were dissolved in water, spotted, as 1µl aliquots, in 1:3 dilution series onto a nitrocellulose membrane (Amersham, UK) and left them dry. Specific epitopes were detected using PAM1ScFv (Raiola *et al.*, 2004) and JIM5 (Willats *et al.*, 2000) monoclonal antibodies (Willats *et al.*, 1999; Clausen *et al.*, 2003). Membranes were blocked with 3% non-fat dry milk powder (Bio-Rad) in phosphate-buffered saline (PBS; Bio-Rad) for 1 h prior to incubation for 1.5 h with primary antibodies. After extensive washes in PBS, membranes were incubated with anti-rat secondary antibody conjugated to horseradish peroxidase (Amersham, UK) for JIM5, and with anti-HIS conjugated to horseradish peroxidase (Sigma) for PAM1. Membranes were washed as described above prior to detection using Luminol reagent (“Santa Cruz Technologies”).

2.8 Fungal growth on different pectic substrates

Fusarium graminearum was cultured on Szecsi solid medium [0,09% NH₄H₂PO₄, 0,2% (NH₄)₂HPO₄, 0,01% MgSO₄, 0,05% KCl, 2% Bactoagar] amended as sole carbon source, either with 1% (w/v) PGA (Sigma) or 1% (w/v) pectin from apple (Sigma-Aldrich) with 70-75% degree of methylesterification for 8 days at room temperature. The radius of the mycelium from three replicates for each medium was measured.

2.9 Infection of transgenic plants

2.9.1 Fungal cultures and plant inoculation

Fusarium graminearum (strain 3827) was cultured on SNA medium (Urban *et al.*, 2002) containing 0,1% KH₂PO₄, 0,1% KNO₃, 0,1% MgSO₄ 7 H₂O, 0,05% KCl, 0,02% glucose, 0,02% sucrose, 2% bactoagar (Difco) with 200 ppm biotin and 200 ppm thiamine (Fig. 2.3 C).

Bipolaris sorokiniana (strain 62608) and *Claviceps purpurea* (03-20-1 isolate) were cultured on potato dextrose agar (PDA; Fluka) (Fig.2.3 B and A).

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 $2,5 \times 10^4$ conidia/ml for *F. graminearum* and 3×10^5 conidia/ml for *B. sorokiniana*.

For plant inoculation with *C. purpurea*, conidia concentration in the honeydew from infected *Triticum aestivum*, cv. Glasgow was estimated by a Thoma chamber adjusting the final concentration to 1×10^5 conidia/ml.

For plant inoculation with *B. sorokiniana* and *F. graminearum*, a final concentration of 6000 conidia or 500 conidia in 20µl was respectively used. Tween 20 was added to a final concentration of 0,05%.

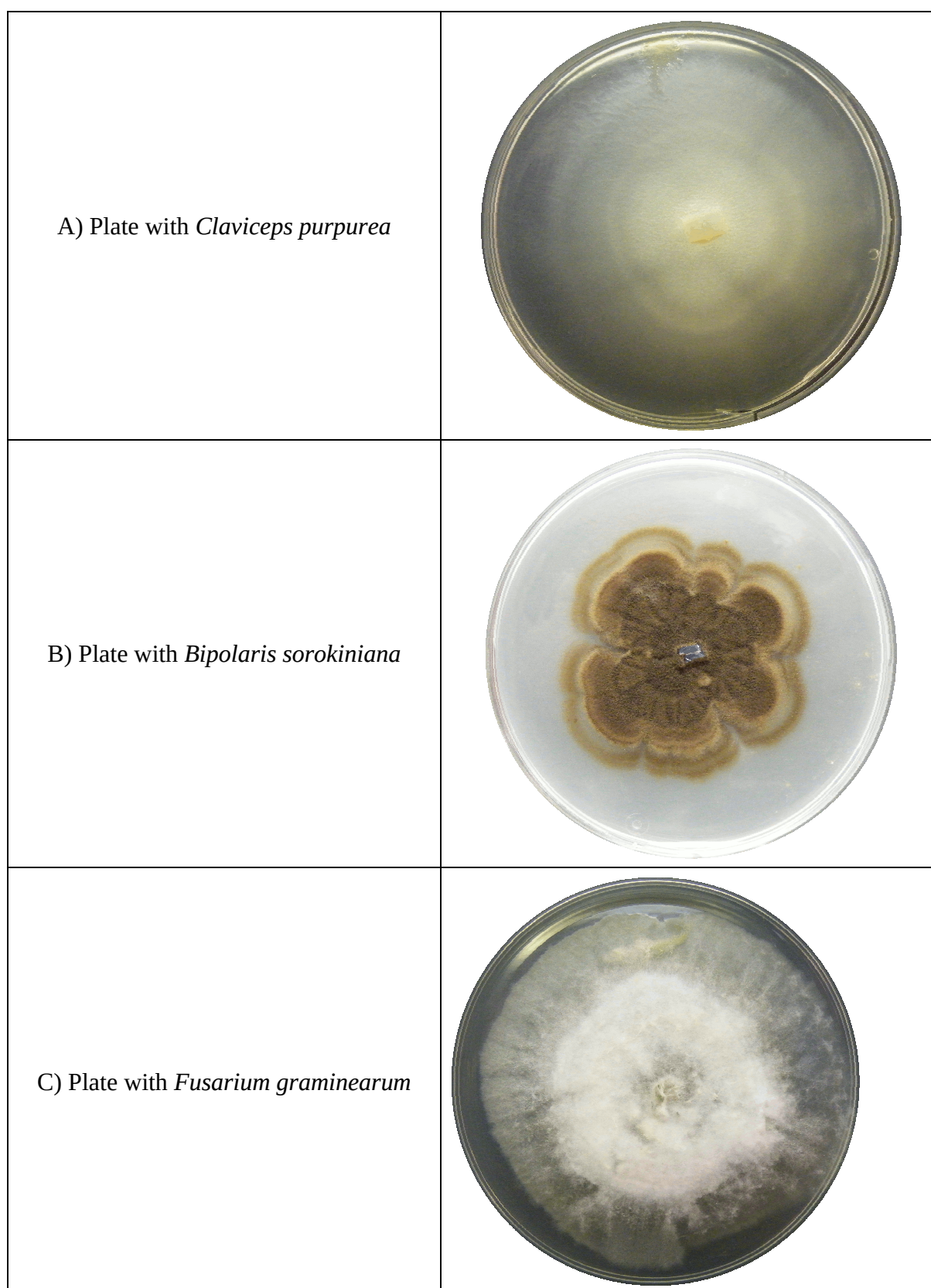


Fig. 2.3 Plates with (A) *Claviceps purpurea*, (B) *Bipolaris sorokiniana* and (C) *Fusarium graminearum* fungi.

2.9.2 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.

2.9.3 Leaf infection with *Bipolaris sorokiniana*

The upper surface of the first leaf of T₂ transgenic (*Acpmei* lines) and control wheat plants in the third leaf-emerged stage (Zadoks stage 13) was inoculated with 20µl of conidia suspension of the 62608 strain of *Bipolaris 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. The symptoms, which manifest with the appearance of reddish-brown spots of variable dimensions on the leaf surface, were visible 48 hpi. Lesion sizes were determined 72 hpi by scanning the leaf surface and calculating the square centimeters of the infected area by using the Adobe Photoshop program (Microsoft, Segrate, Italy). The average area per lesion was calculated as weighted arithmetic mean. Two separate experiments were performed using 23 plants of the MJ15-151 line with a corresponding number of wild type plants (cv. Svevo), and 15 plants of the MJ15-69 line with a similar number of wild-type plants (cv. Svevo).

2.9.4 Spike infection with *Fusarium graminearum*

At anthesis (Zadoks stage 68) plants were inoculated with 20µl of fresh conidial suspension (containing 500 conidia) between the bracts of two florets of two opposite spikelets. Only a spike was inoculated per plant. The inoculated spikes were then sealed for 2 days with a plastic bag to maintain high humidity for the fungal growth and the temperature of the climatic chamber was set at day/night of 21°C/18°C.

Disease symptoms were followed up to 18 days post infection and measured as percentage of infected spikelets per spike (Bai *et al*, 2001).

A single independent experiment with transformed (MJ15-151 and MJ15-69) and control plants (wild-type and null segregant) was performed in T₃ generation using at least 25 plants of each genotype.

2.9.5 Spike infection with *Claviceps purpurea*

This infection experiments were performed at NIAB (National Institute of agricultural and Botany, Cambridge, UK). For inoculation, honeydew containing conidia obtained from infected wheat spikes of *Triticum aestivum* cv. Glasgow was used. These inoculations were made by using the isolate 03-20-1.

The spikes were inoculated before anthesis (Zadoks stage 59), when the spikes had just completely emerged from the boot. Twenty-forty individual florets were inoculated per spike by injecting a spore suspension into the primary and secondary florets on each spikelet using a syringe and hypodermic needle to fill the florets.

A single independent experiment with 45 replicates for each transgenic (J82-23a line of *Triticum aestivum* cv. Bobwhite expressing PvPGIP2) and control plants (null segregant line) was performed.

In the infected ears of both transgenic and control plants, honeydew was detectable 8 dpi and sclerotia were formed about 20 dpi.

Ergot disease symptom was evaluated both by assessing the honeydew production on a scale from 1 to 4 (1=none, 2=honeydew confined within the glumes, 3=honeydew exuding from the florets in small drops, and 4=large drops of honeydew running down the spike) and by measuring the sclerotia weight (Menzies *et al.*, 2004).

2.9.6 Fluorescence microscopy on ovaries infected with *Claviceps purpurea*

About 50 ovaries from infected transgenic and control plants were collected up to 7 dpi, longitudinally bisected and incubated with 1M KOH overnight. After washing extensively with water, ovaries were stained with 0,05% aniline blue (m/v in 0,067M K₂HPO₄) for 2 hours at room temperature (Hood and Shew, 1996) and then observed with a microscope (Zeiss Axioscop 20) equipped for epifluorescence microscopy (mercury short arc lamp HBO 50w).

2.9.7 Statistical analysis of the data

Data were analyzed statistically applying the student's *t* test with 2 tails hypothesizing different variances between the two groups being considering.

3. Results

3.1 Transgenic wheat plants expressing pectin methylesterase inhibitors

3.1.1 Comparison between endogenous pectin methylesterase activity in Wheat and Arabidopsis

The endogenous pectin methylesterase activity in wheat (*Triticum durum* cv. Svevo) and Arabidopsis (*Arabidopsis thaliana*, Col 0) was compared by radial gel diffusion assay. Two, five and ten micrograms of total protein extracts from leaves of adult plants in late stage of flowering were loaded into the wells and the diameter of the red-stained zones, resulting from the hydrolysis of esterified pectin in the gel, was measured and compared (Fig. 3.1). Wheat showed a PME activity 30% lower than Arabidopsis.

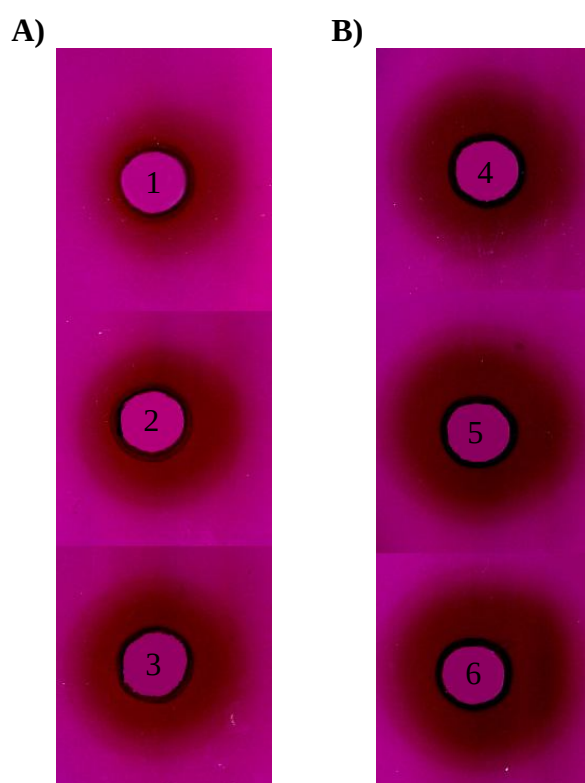


Fig. 3.1 Gel diffusion assay showing endogenous PME activity of wheat and Arabidopsis leaf total protein extract. **A)** Wheat; **B)** Arabidopsis. Total protein extracts loaded in each well were: 2 μ g (1 and 5), 5 μ g (2 and 5) and 10 μ g (3 and 6).

3.1.2 Pectin methylesterase activity in different wheat tissues

Endogenous wheat pectin methylesterase activity in different tissues such as leaf, stem, coleoptile, callus, root and spike was measured. Roots were further divided in basal and apical roots while spikes were divided in ovary, anther, glume and grain. The radial gel diffusion assay loading ten micrograms of each extract revealed PME activity in all tissues except for grain (Fig. 3.2).

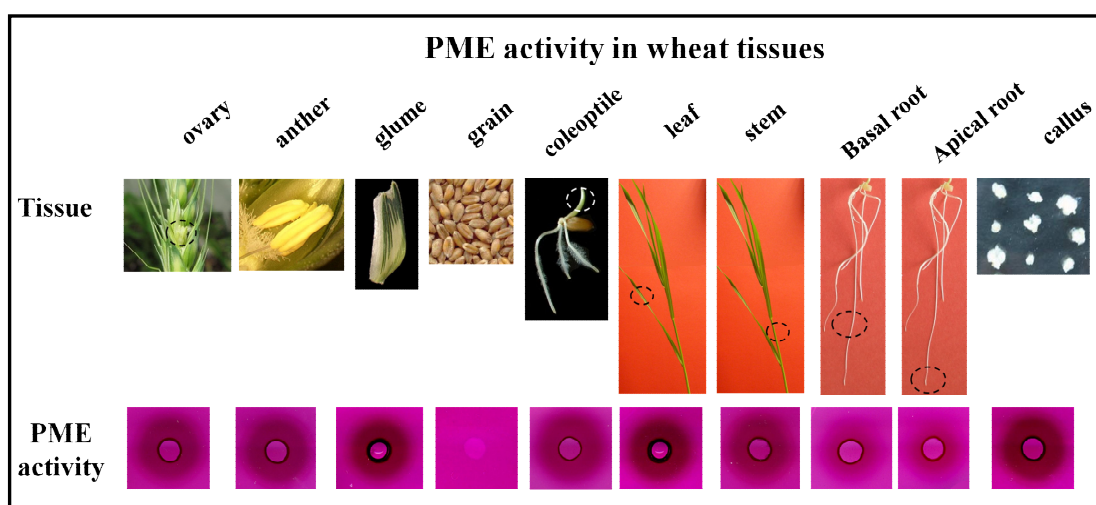


Fig. 3.2 Gel diffusion assay showing endogenous wheat pectin methylesterase activity in different tissues.

3.1.3 Inhibitory activity of PMEIs from Arabidopsis and kiwi against wheat PME

The capability of purified AtPMEI-1, AtPMEI-2 or AcPMEI to inhibit wheat PME activity was verified by radial gel diffusion assay. Nine μ g of total leaf protein extract from *Triticum durum* cv. Svevo were loaded alone or in combination with 15ng of inhibitors. Both AtPMEIs and AcPMEI recognized and inhibited wheat PME with AcPMEI showing a stronger inhibitory activity against wheat PME compared to AtPMEIs (Fig. 3.3). Based on this result, AcPMEI and AtPMEI-1 were used for the subsequent transformation experiments. AtPMEI-1 was preferred over AtPMEI-2 because is not glycosylated.

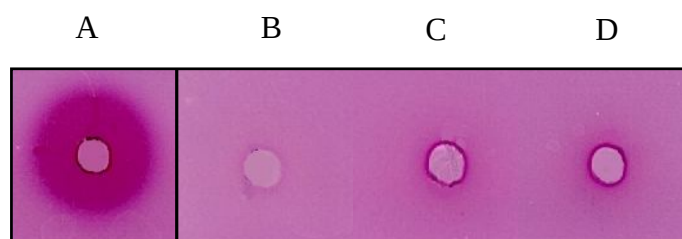


Fig. 3.3 Radial gel diffusion assay showing the capability of AtPMEI and AcpPMEI to inhibit wheat PME. Nine μ g of leaf protein extract from Svevo were loaded alone (**A**) or in combination with 15ng of AcpPMEI (**B**), AtPMEI-1 (**C**) and AtPMEI-2 (**D**).

3.1.4 Construction of the vectors for the bombardment

For the transformation of wheat plants with pectin methylesterase inhibitors, *Atpmei-1* (from *Arabidopsis thaliana*) and *Acpmei* (from *Actinidia chinensis*) genes (kindly provided by Prof. Bellincampi, La Sapienza University, Rome) were used. The complete coding region of *Atpmei-1* (accession number at1g48020), including the N- terminal signal sequence, or the coding region of *Acpmei* (accession number P83326) with the signal peptide of the polygalacturonase-inhibiting protein 1 (*Pvpgip1*) for the apoplastic targeting, was inserted into the *Bam*HI site of pAHC17 under control of the constitutive *Ubi1* promoter and nopaline synthase terminator, to generate the pUBI::*Atpmei-1* and pUBI::*Acpmei* constructs (Fig. 3.4 A and B). Another construct, pUBI::BAR, carrying the *bar* gene that confers resistance to the bialaphos herbicide, was co-transformed with each one of the above constructs to allow the selection of herbicide resistant regenerating shoots. In this construct also, the *bar* gene is under control of the *Ubi1* promoter (Fig. 3.5).

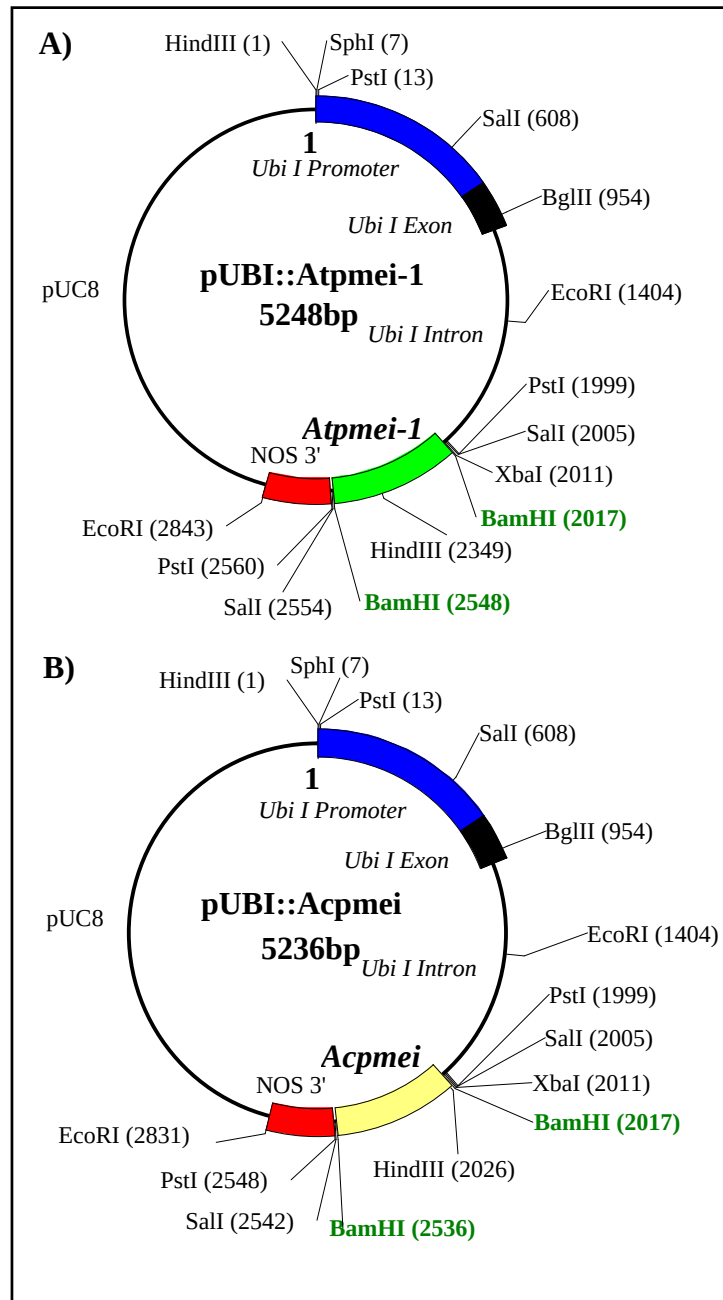


Fig. 3.4 Plasmids pUBI::Atpmei-1 (**A**) and pUBI::Acpmei (**B**) containing the *Ubi1* promoter and the *Atpmei-1* or *Acpmei* genes, used for the biolistic bombardment.

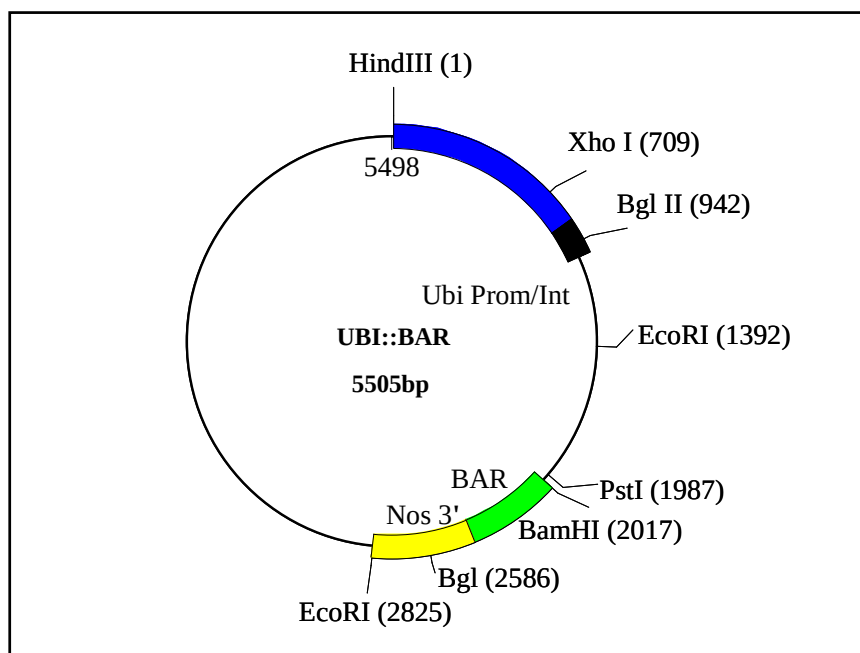


Fig. 3.5 Plasmid pUBI::BAR containing the *Ubi1* promoter and the *bar* gene, used for the biolistic bombardment.

3.1.5 Production of transgenic plants

The construct containing either the *Atpmei-1* (pUBI::Atpmei-1) or the *Acpmei* gene (pUBI::Acpmei) and the *bar* selectable marker plasmid were used to co-bombard *Triticum durum* cv. Svevo immature embryos following the procedure reported by Janni *et al*, 2008.

In total, 2133 immature wheat embryos were co-transformed using pUBI::Atpmei-1 and pUBI::BAR, while 2105 immature embryos were instead co-transformed using pUBI::Acpmei and pUBI::BAR. For each transformation with the *Atpmei-1* or *Acpmei*, two separate bombardment experiments were performed (Tables 3.1 and 3.2).

Table 3.1 Bombardment experiments with pUBI::Atpmei-1 and pUBI::BAR

cv	# bombardment	# bombarded embryos	# regenerated lines in the soil*
Svevo	MJ14	1601	40
Svevo	MJ22	532	25

* The variability of the number of regenerated lines put in the soil in the different bombardment experiments depends on the contamination events.

Table 3.2 Bombardment experiments with pUBI::Acpmei and pUBI::BAR

cv	# bombardment	# bombarded embryos	# regenerated lines in the soil
Svevo	MJ15	1916	137
Svevo	MJ21	189	2

3.1.6 Selection of transgenic plants

Forty and twenty-five independent regenerated lines were obtained from the bombardment experiments MJ14 and MJ22, respectively, using the *Atpmei-1* gene. All regenerated plants were analyzed for the presence of the transgene by PCR using specific oligonucleotides (Atpmei1F3/Atpmei1R4) which produce an amplicon of 410bp. The amplification reactions were performed on genomic DNA extracted from leaves of the transgenic lines (T_0) using the genomic DNA from cv. Svevo as negative control and the plasmid DNA pUBI::Atpmei-1 as positive control (Fig.3.6 A).

The PCR analysis from the MJ14 and MJ22 lines showed that 20 and 17 T_0 lines respectively were positive for the presence of the transgene with a transformation efficiency of 1,2% for MJ14 and 3,2% for MJ22. The presence of the marker gene was also verified by PCR using the specific primer combination BAR1/BAR2 developed on the *bar* sequence of *Streptomyces hygroscopicus* (accession number X17220) which amplifies a 406bp region of the gene (Fig. 3.6 B).

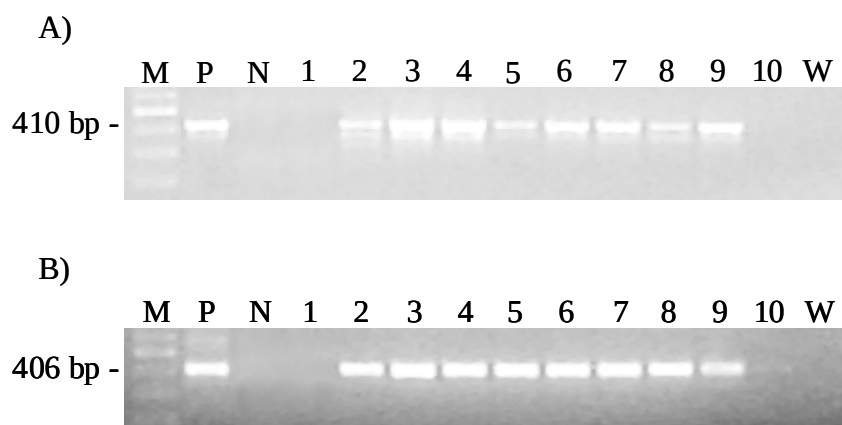


Fig. 3.6 1,5% Agarose gel of the amplification products from T₀ leaf DNA. (**A**) PCR products obtained using the specific primers for *Atpmei-1*, *Atpmei1F3/Atpmei1R4* which produce an amplicon of 410bp. (**B**) PCR products obtained using the specific primers for *bar* gene, *BAR1/BAR2* which produce an amplicon of 406bp. Samples: M) Marker GeneRuler 100 bp DNA Ladder, P) plasmid DNA (pUBI::Atpmei-1 in “A” and pUBI::BAR in “B”) used as positive control, N) Genomic DNA from *Triticum durum* cv. Svevo used as negative control, 1-10) some of the regenerated T₀ lines, W) water.

The marker transformation efficiency was 1,6% and 3% for MJ14 and MJ22, respectively.

Of the T₀ lines containing *Atpmei-1* (hereafter *Atpmei-1* lines), some were sterile and others died in the soil; the remaining 22 T₀ positive lines were self-pollinated in order to obtain the T₁ lines that were analyzed again by PCR to distinguish in the progeny the plants containing the transgene from those that had lost the transgene by segregation. Sixteen T₁ lines confirmed the presence of the *Atpmei-1* transgene (Table 3.3).

Table 3.3 *Atpmei-1* transgenic lines obtained and analyzed by PCR

# bombardment	T ₀ regenerated lines	T ₀ positive lines (by PCR)*	T ₁ lines	T ₁ positive lines (by PCR)
MJ14	40	20	11	9
MJ22	25	17	11	7

* MJ14 bombardment: of the 20 lines positive for the presence of the transgene, some were sterile and others died in the soil; the remaining 11 lines were self-pollinated in order to obtain the T₁ generation. MJ22 bombardment: of the 17 lines positive for the presence of the transgene, some were sterile and others died in the soil; the remaining 11 lines were self-pollinated in order to obtain the T₁ generation.

From the bombardment experiments MJ15 and MJ21 using the *Acpmei* gene, 137 and 2 independent regenerated lines respectively were obtained. These T₀ lines were analyzed for the presence of the transgene by PCR using the specific primers *AcpmeiF4/AcpmeiR4*, which

produce an amplicon of 407bp. A positive (plasmid DNA pUBI::Acpmei) and negative control (genomic DNA from cv. Svevo) were also included in the analysis (Fig. 3.7 A). PCR showed that 69 and 2 T₀ lines were positive for the bombardment experiments MJ15 and MJ21, respectively, with a transgene transformation efficiency of 3,6% for MJ15 and 1% for MJ21. The presence of the *bar* gene was also verified (Fig. 3.7 B) and the PCR result showed a marker transformation efficiency of 4,3% for MJ15 and 1% for MJ21.

Of the T₀ lines containing *Acpmei* (hereafter *Acpmei* lines), some were sterile and others died in the soil; the remaining 42 T₀ positive lines were self-pollinated in order to obtain the T₁ lines that were analyzed again by PCR for the segregation. Thirty-six T₁ lines confirmed the presence of the *Acpmei* transgene (Table 3.4).

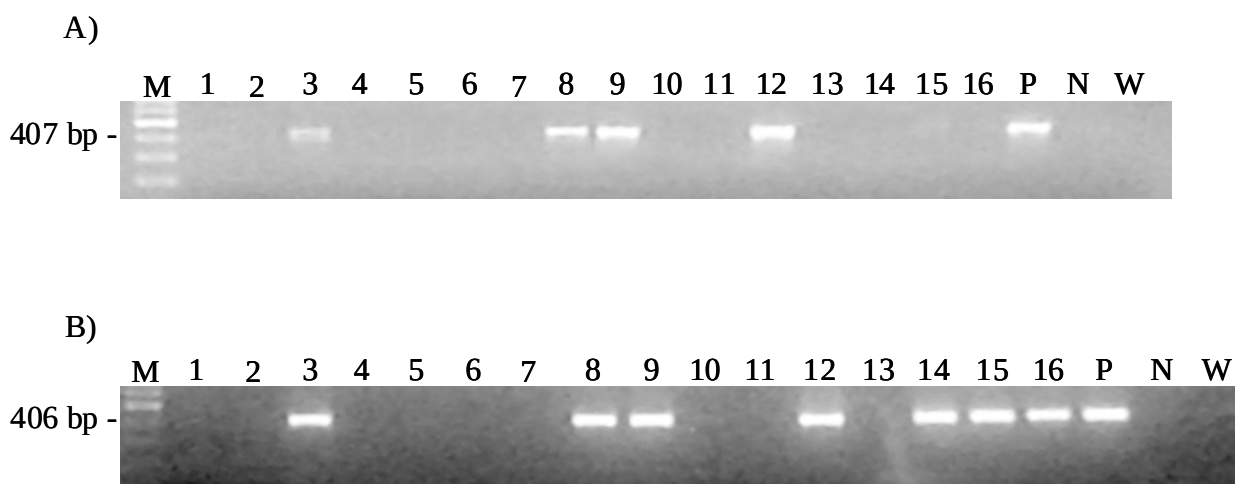


Fig. 3.7 1,5% Agarose gel of the amplification products from T₀ leaf DNA. (**A**) PCR products obtained using the specific primers for *Acpmei*, AcpmeiF4/AcpmeiR4 which produce an amplicon of 407bp. (**B**) PCR products obtained using the specific primers for *bar* gene, BAR1/BAR2 which produce an amplicon of 406bp. Samples: M) Marker GeneRuler 100 bp DNA Ladder, P) plasmid DNA (pUBI::Acpmei in “A” and pUBI::BAR in “B”) used as positive control, N) Genomic DNA from *Triticum durum* cv. Svevo used as negative control, 1-16) some of the regenerated T₀ lines, W) water.

Table 3.4 *Acpmei* transgenic lines obtained and analyzed by PCR

# bombardment	T ₀ regenerated lines	T ₀ positive lines (by PCR)*	T ₁ lines	T ₁ positive lines (by PCR)
MJ15	137	69	40	34
MJ21	2	2	2	2

* Of the 69 lines positive for the presence of the transgene, some were sterile and others died in the soil; the remaining 40 lines were self-pollinated in order to obtain the T₁ generation..

All the T₁ positive lines were analyzed for the endogenous PME activity by radial gel diffusion assay. These experiments were performed to verify the functionality of the PMEI encoded by the *Atpmei-1* or *Acpmei* transgenes. A reduced endogenous PME activity was expected in the transgenic lines as compared to the control plants because of the activity of AtPMEI or AcPMEI. Different quantities (2,5µg, 5µg, 10µg and 20µg) of total leaf protein extract from transgenic and wild-type plants were loaded onto the agarose gel containing pectin (70-75% methylesterified) from apple and the halo produced by the endogenous PME activity was measured. Relative PME activity of transgenic lines was calculated considering the halo produced by the extract of the wild-type plants as 100% of PME activity. Of the 16 *Atpmei-1* lines, only 1 line (MJ14-43) showed a slightly reduced endogenous PME activity (about 70% of the control PME activity; Fig. 3.8), whereas 12 *Acpmei* lines, out of 36 lines, showed different levels of reduced PME activity that varied from about <5% to 80% of the control PME activity (Fig. 3.9 and Fig. 3.10). In order to verify whether the reduction or the lack of the halo in the transgenic samples was due to the inhibitor activity of PMEI and not to non specific inhibitors present in the protein extract, the activity of orange peel PME was verified by adding this enzyme on boiled samples. Moreover to verify the extent of the inhibitor activity of the protein extracts, orange peel PME was added to each transgenic and control sample (Fig. 3.11).

The only *Atpmei-1* line showing reduced endogenous PME activity and all the 12 *Acpmei* lines with different levels of endogenous PME activity were self-pollinated in order to obtain T₂ progenies.

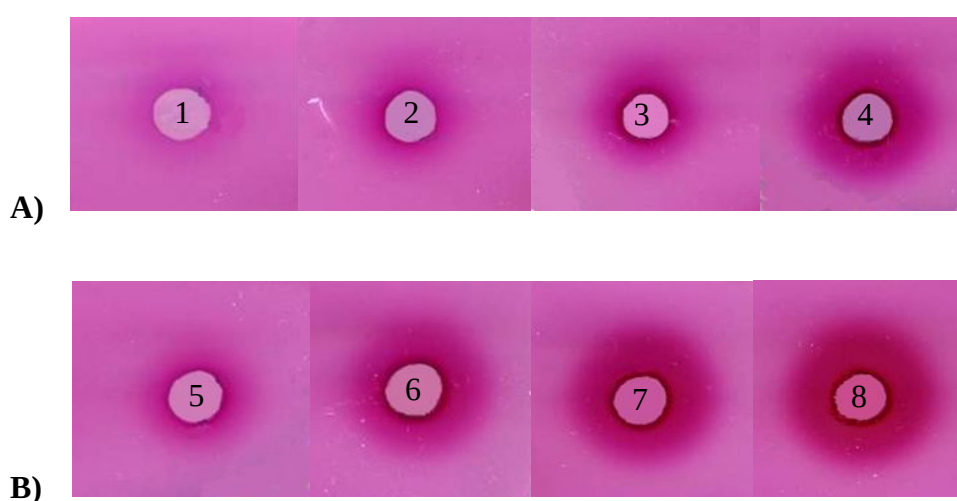


Fig. 3.8 Gel diffusion assay showing endogenous PME activity of wheat leaf total protein extract. (**A**) *Atpmei-1* line MJ14-43. (**B**) Wild type. Total protein extracts loaded in each well were: 2,5µg (1 and 5), 5µg (2 and 6), 10µg (3 and 7) and 20µg (4 and 8).

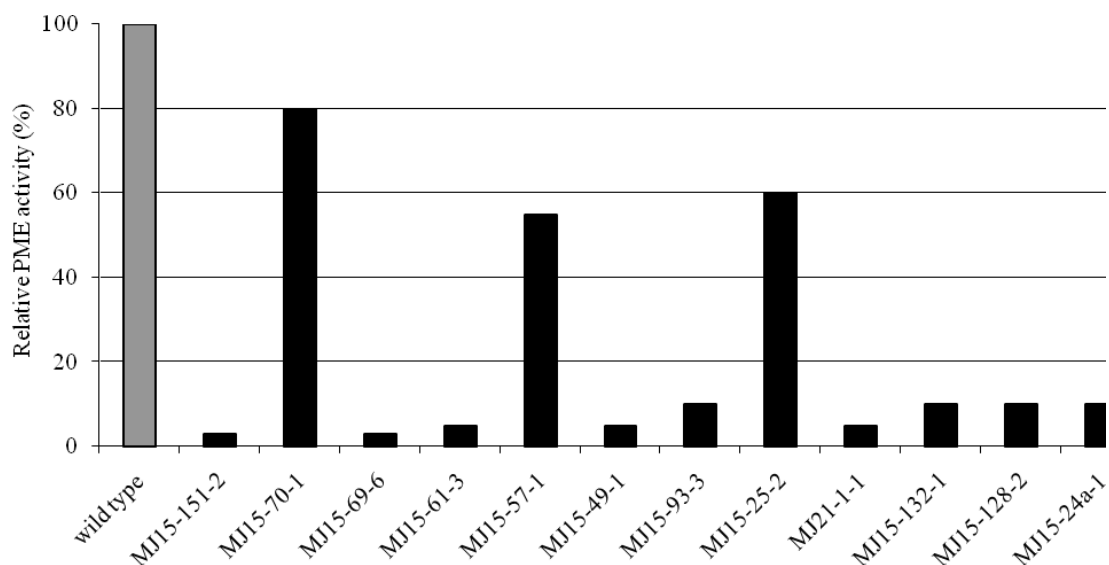


Fig. 3.9 Different levels of reduction of endogenous PME activity of the 12 *Acpmei* lines (T_1). Relative PME activity of each transgenic line is expressed as a percentage of PME activity of control plants (wild type). Data are based on the halo produced on agarose gel diffusion assay.

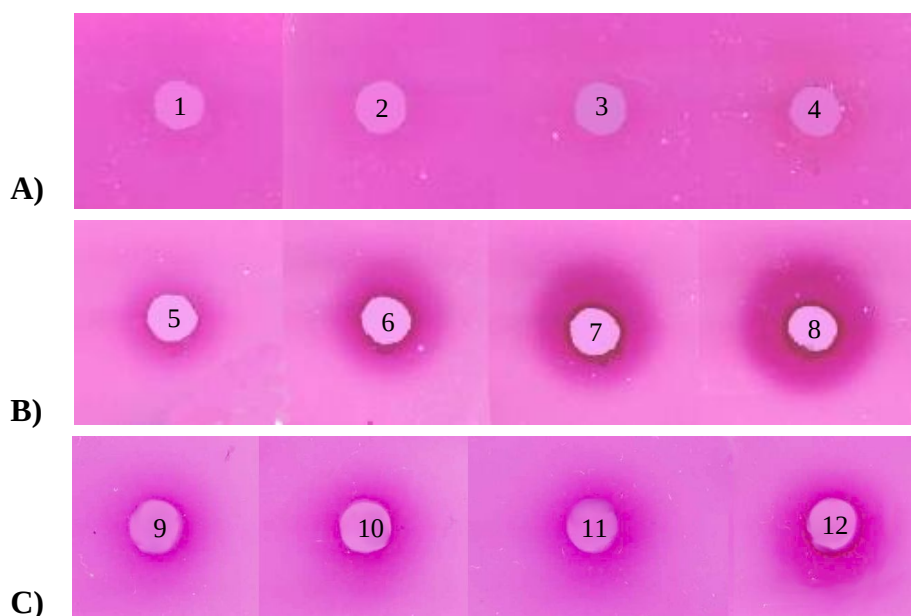


Fig. 3.10 Gel diffusion assay showing endogenous PME activity of wheat leaf total protein extract. **A)** MJ15-151; **B)** Wild type; **C)** MJ15-57. Total protein extract loaded in each well were: 2,5 μ g (1, 5 and 9), 5 μ g (2, 6 and 10), 10 μ g (3, 7 and 11) and 20 μ g (4, 8 and 12). MJ15-151 and MJ15-57 lines show about <5% and about 60% of endogenous PME activity, respectively.

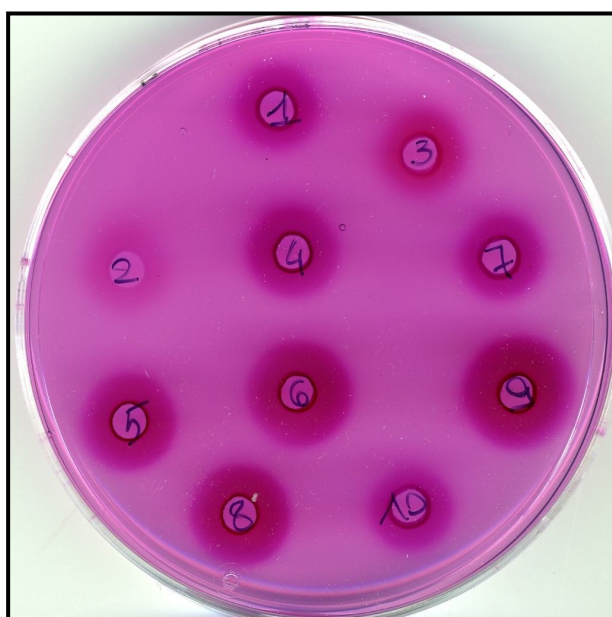


Fig. 3.11 The plate represents an example of the procedure used to screen the transgenic plants. 1) Orange peel PME (18ng); 2) MJ15-69 total protein extract (10 μ g); 3) MJ15-69 total protein extract (10 μ g) plus orange peel PME (18ng); 4) boiled MJ15-69 total protein extract (10 μ g) plus orange peel PME (18ng); 5) MJ15-47 total protein extract (10 μ g); 6) MJ15-47 total protein extract (10 μ g) plus orange peel PME (18ng); 7) boiled MJ15-47 total protein extract (10 μ g) plus orange peel PME (18ng); 8) Wild type (cv. Svevo) total protein extract (10 μ g); 9) Wild type total protein extract (10 μ g) plus orange peel PME (18ng); 10) boiled wild type total protein extract (10 μ g) plus orange peel PME (18ng). Samples 4, 7 and 10 were added to verify that the reduction or the lack of the halo in the transgenic samples was due to the inhibitor activity of PME and not to non specific inhibitors present in the protein extract, while samples 3, 6 and 9 were included to verify the extent of the inhibitor activity of the protein extracts.

3.1.7 Choice of the *Acpmei* lines for further characterization

The level of PME activity in the *Atpmei-1* line and in eight *Acpmei* lines was further verified on T₂ progenies by radial gel diffusion assay. The *Atpmei-1* line MJ14-43 confirmed the result obtained in T₁ plants with about 30% reduction of PME activity. Due to this minor effect on PME activity and to the lack of additional *Atpmei-1* lines, it was decided not to further characterize *Atpmei-1* lines.

Similarly to what observed in the *Atpmei-1* line, also the T₂ progenies of MJ15 151 and MJ15-69 lines confirmed the very low PME activity observed in T₁ generation and the MJ15-57 line confirmed a 55% reduction of PME activity. Conversely, plants from the T₂ progenies of MJ21-1, MJ15-61 and MJ15-49 lines, having a low PME activity in T₁ generation, showed variability in PME activity, with some plants confirming the strong reduction of PME activity observed in T₁ generation and other showing a varying degree of reduction of PME activity. Finally, the

MJ15-93 and MJ15-25 lines with 10% and 60% of PME activity in T₁ generation, respectively, as compared to control plants, underwent silencing in T₂ generation because they did not show any reduction of PME activity (Table 3.5).

Based on these results the three *Acpmei* lines, MJ15 151, MJ15-69 and MJ15-57, showing a stable PME activity over two generations and a different degree of reduction of PME activity were selected for the subsequent analysis.

Table 3.5 Relative PME activity of some *Acpmei* lines in T₁ and T₂ generations.

Line	Relative PME activity in T ₁ (%)	Relative PME activity in T ₂ (%)
MJ15-151	<5	<5
MJ15-69	<5	<5
MJ21-1	10	Variable
MJ15-61	10	Variable
MJ15-93	10	100
MJ15-25	60	100
MJ15-57	55	55
MJ15-49	10	Variable

3.1.8 Western blot analysis of the selected *Acpmei* lines

Ten micrograms of total leaf protein extracts were fractioned on polyacrylamide gels and transferred onto PVDF membranes. Western blot assay with a polyclonal antibody anti-AcPMEI, revealed a specific signal with a molecular mass of about 18 KDa corresponding to the transgenic protein, which is not present in the wild-type cv. Svevo (Fig. 3.12).

Western blot result confirmed radial diffusion assay data by showing that the degree of PME activity of the three transgenic lines correlates with the level of PME accumulation. In particular, the immunodecoration signal is weaker in the MJ15-57 and stronger in MJ15-69 and MJ15-151 lines (Fig. 3.12).

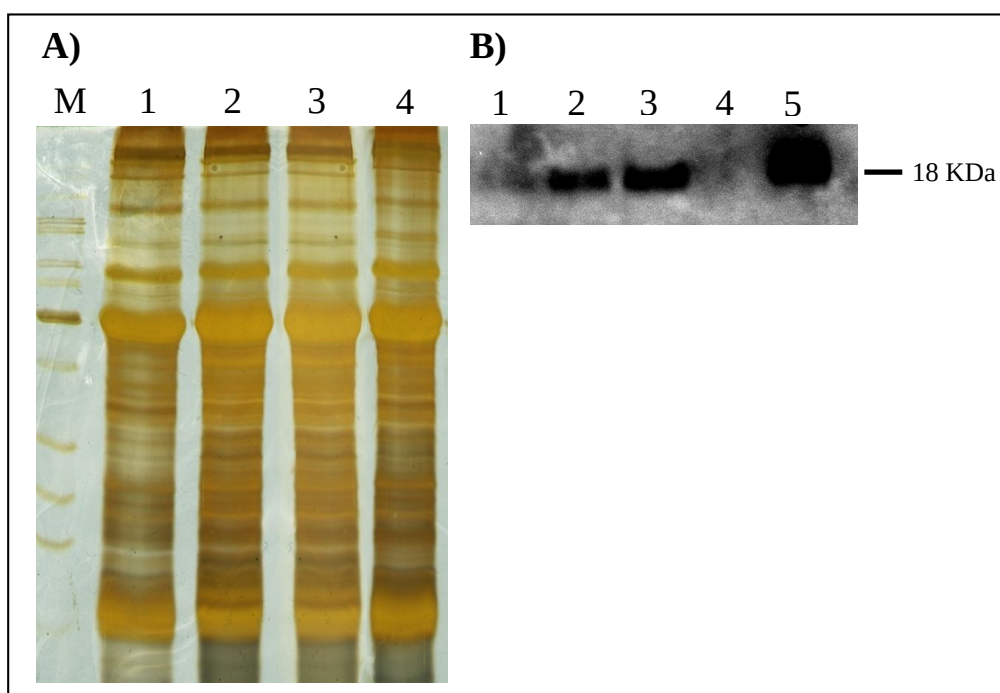


Fig. 3.12 Analysis of total protein leaf extract of the selected transgenic lines. **A)** 15% SDS-PAGE of total protein extracts of the selected *Acpmei* lines stained with silver nitrate. Ten μ g of each sample were loaded. **B)** Western blotting of total protein extracts (10 μ g) of the selected *Acpmei* lines. A polyclonal Ab anti-AcPMEI was used. M) Marker of molecular mass 10-200 KDa (Fermentas), 1) MJ15-57, 2) MJ15-151, 3) MJ15-69, 4) Wild type, 5) Purified AcPMEI (25ng).

3.1.9 Southern blot analysis of the selected *Acpmei* lines

Genomic DNA of the selected lines was analyzed by Southern blot using the *Acpmei* coding region as probe. Cleavage with *Bam*HI restriction enzyme, which causes the excision of the *Acpmei* coding region, revealed in all of the transgenic lines the expected hybridization signal of about 400bp (Fig. 3.13 A). Digestion with *Sph*I restriction enzyme, which cuts once within the pUBI::*Acpmei* construct, produced in all the transgenic lines the expected 5,2 kb hybridization fragment corresponding to entire construct. Additional hybridizing fragments were found in all of the transgenic lines suggesting different insertion sites and transgene rearrangement events in all of the transgenic lines. In particular, MJ15-151 showed the higher number of additional hybridizing fragments and MJ15-57 possesses more additional hybridizing fragments than MJ15-69. As expected, no hybridization signals were present in genomic DNA of non transformed and null segregant lines (Fig. 3.13 B).

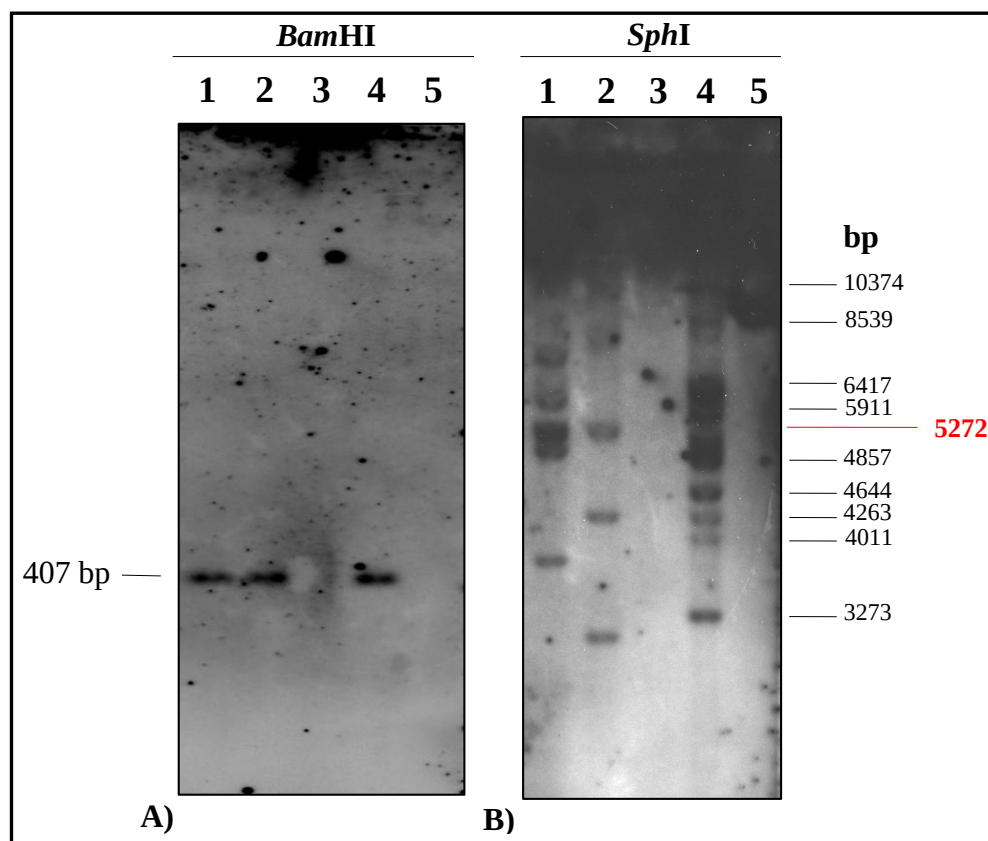


Fig. 3.13 Southern blot of the selected *Acpmei* lines. Genomic DNA (8 μ g) of T₃ transgenic lines was digested with **A)** *Bam*HI and **B)** *Sph*I and probed with digoxigenin-labelled coding region of *Acpmei*. Samples: 1) MJ15-57, 2) MJ15-69, 3) Wild type, 4) MJ15-151, 5) Null Segregant of MJ15-151.

3.1.10 Relative copy number of the transgene in the selected *Acpmei* lines

qPCR experiments were performed to quantify the relative copy number of the transgene in MJ15-57, MJ15-69 and MJ15-151 lines. Two different experiments were performed and similar results were obtained. In both experiments, relative quantification was based on the use of *actin* as housekeeping gene. Data from a single qPCR experiment are reported in figure 3.14. The results of qPCR are in agreement with those obtained by Southern blot since confirmed that MJ15-69 line contains less copies of the *Acpmei* transgene. In particular, this line possesses about 5-fold and 4-fold less copy numbers of the transgene compared to MJ15-151 and MJ15-57 lines, respectively.

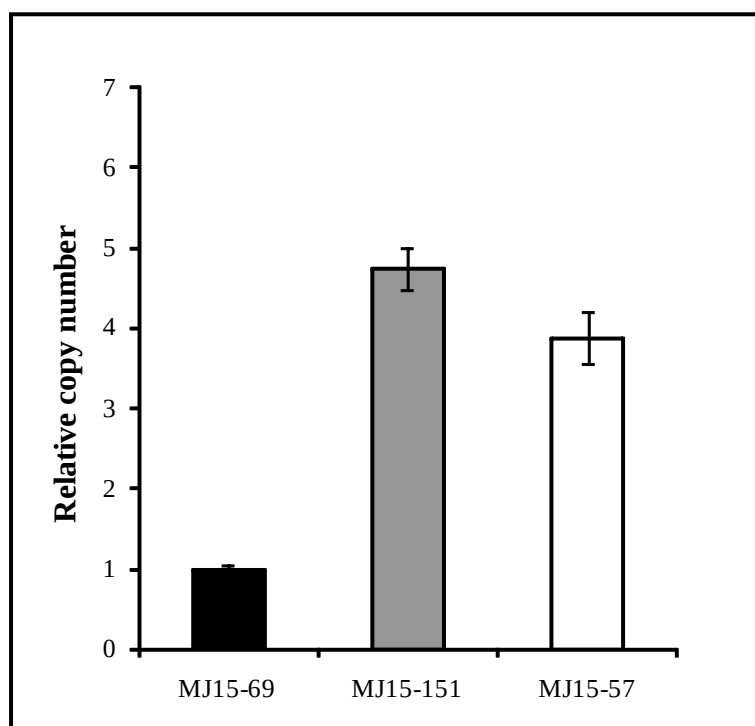


Fig. 3.14 Relative copy number of the transgene in the selected lines. Normalization was done using *actin* gene as housekeeping. A single qPCR test of two different experiments that produced similar results is reported. Bars represent standard deviation between the three replicates of each sample.

3.1.11 Transcripts and protein accumulation and AcPMEI activity in the selected lines

Accumulation of *Acpmei* transcript in MJ15-151, MJ15-69 and MJ15-57 lines was verified by qRT-PCR on cDNA. This analysis was performed twice and in both cases the transcripts level of the MJ15-151 and MJ15-69 lines was 6-fold and 4-fold higher than in MJ15-57 line, respectively (Fig. 3.15).

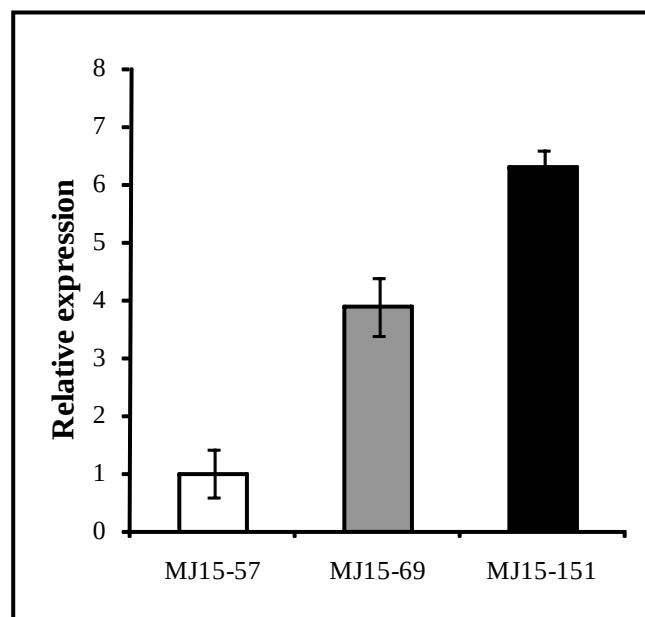


Fig. 3.15 Quantification of the *Acpmei* transcript accumulation in the selected lines. Normalization was done using *actin* gene as housekeeping. A single qRT-PCR test of two different experiments that produced similar results is reported. Bars represent standard deviation between the three replicates of each sample.

The occurrence of a lower transcript accumulation in MJ15-57 was also confirmed at protein level. Western blotting analysis of total leaf protein extract of MJ15-57 line showed a lower accumulation of AcPMEI and radial gel diffusion assay demonstrated that the endogenous PME activity of MJ15-57 was higher compared to that of MJ15-151 and MJ15-69 (Fig. 3.16 A and B). Western blot analysis showed also that the accumulation of AcPMEI was higher in MJ15-69 compared to MJ15-151 (Fig. 3.16 C). Densitometry analysis of the immunodecoration signal corresponding to AcPMEI, performed on three different quantities of total protein extracts (7,5µg, 10µg and 15µg) of each line, showed that signal intensity was 14% higher in MJ15-69 compared to MJ15-151. Similarly, a slightly higher (10%) PME activity was observed in MJ15-151. This level of accumulation and activity found in leaf tissue of the MJ15-151 and MJ15-69 lines was also detected in the spike tissue, demonstrating, as expected, that the *Ubi1* promoter regulates a constitutive expression of *Acpmei* and none tissue-specific degrading activity towards AcPMEI is activated in these tissues. (Fig. 3.17).

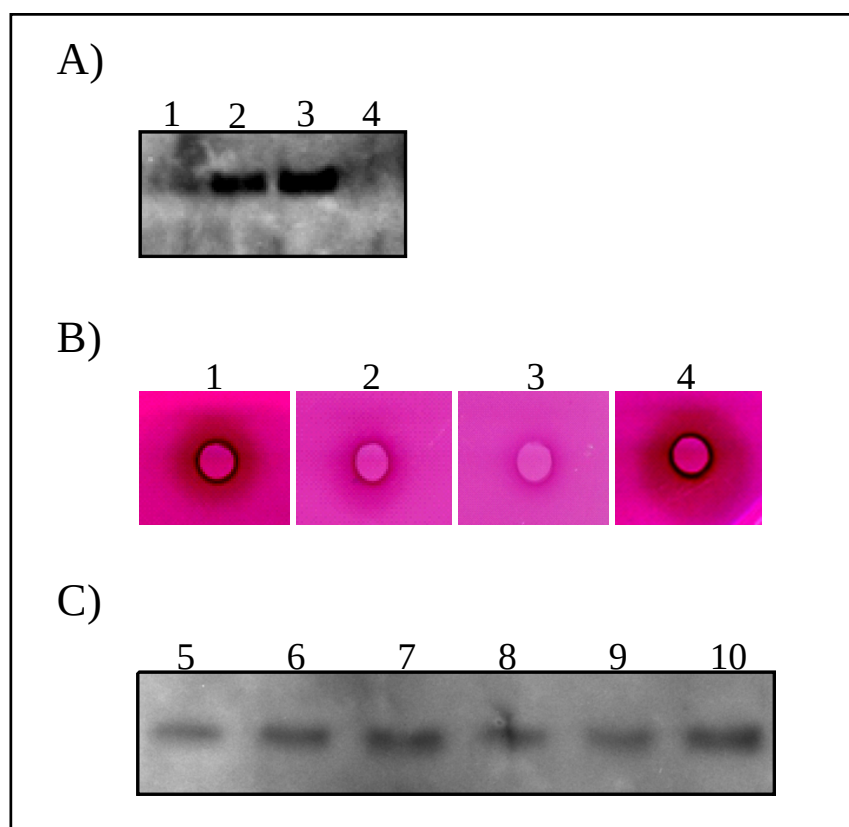


Fig. 3.16 Analysis of the protein accumulation and AcPMEI activity in the selected lines. (**A**) Western blotting on total protein extracts (10 μ g) of the selected *Acpmei* lines. A polyclonal Ab anti-AcPMEI was used. (**B**) Gel diffusion assay on total leaf protein extracts (10 μ g) of the selected *Acpmei* lines. (**C**) Western blotting on different quantities of total protein extracts of MJ15-151 and MJ15-69 lines. Samples: 1) MJ15-57, 2) MJ15-151, 3) MJ15-69, 4) Wild type, 5-7) MJ15-151 (7,5 μ g, 10 μ g and 15 μ g respectively), 8-10) MJ15-69 (7,5 μ g, 10 μ g and 15 μ g respectively).

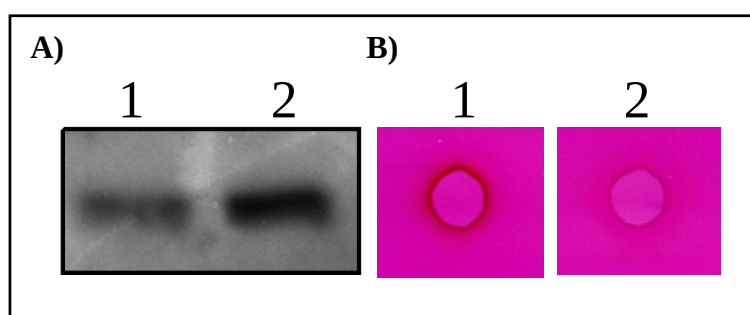


Fig. 3.17 Analysis of the protein accumulation and AcPMEI activity in the spike tissue. (**A**) Western blotting on total protein extracts from spike tissue (10 μ g) of the MJ15-151 and MJ15-69 lines. (**B**) Gel diffusion assay on total protein extracts from spike tissue (10 μ g) of the MJ15-151 and MJ15-69 lines. Samples: 1) MJ15-151, 2) MJ15-69.

3.1.12 Apoplastic localization of AcPMEI in transgenic lines

Because the construct used for the transformation contains the coding region of *Acpmei* with the *Pvpgip1* signal peptide for the apoplastic targeting, the correct localization of the protein in the extracellular fluids was verified. Hypocotyls of the transgenic wheat lines MJ15-151 and MJ15-69 were vacuum-infiltrated, as described in the Materials and Methods section, and the extracellular fluids recovered were analyzed by western blot for the presence of AcPMEI and by radial diffusion assay for the activity of the transgenic protein. Intercellular fluids showed no activity of Glucose-6-P dehydrogenase (G6PDH), an intracellular marker, indicating no detectable contamination of cytoplasmic components. As shown in Figure 3.18, the transgenic protein was detected in extracellular fluids isolated from stems of both lines; moreover, the cup-plate assay using 10µl and 30µl of the extracellular fluids showed inhibitory activity of the endogenous PME confirming that the inhibitor has been correctly targeted to the apoplast (Fig. 3.19).

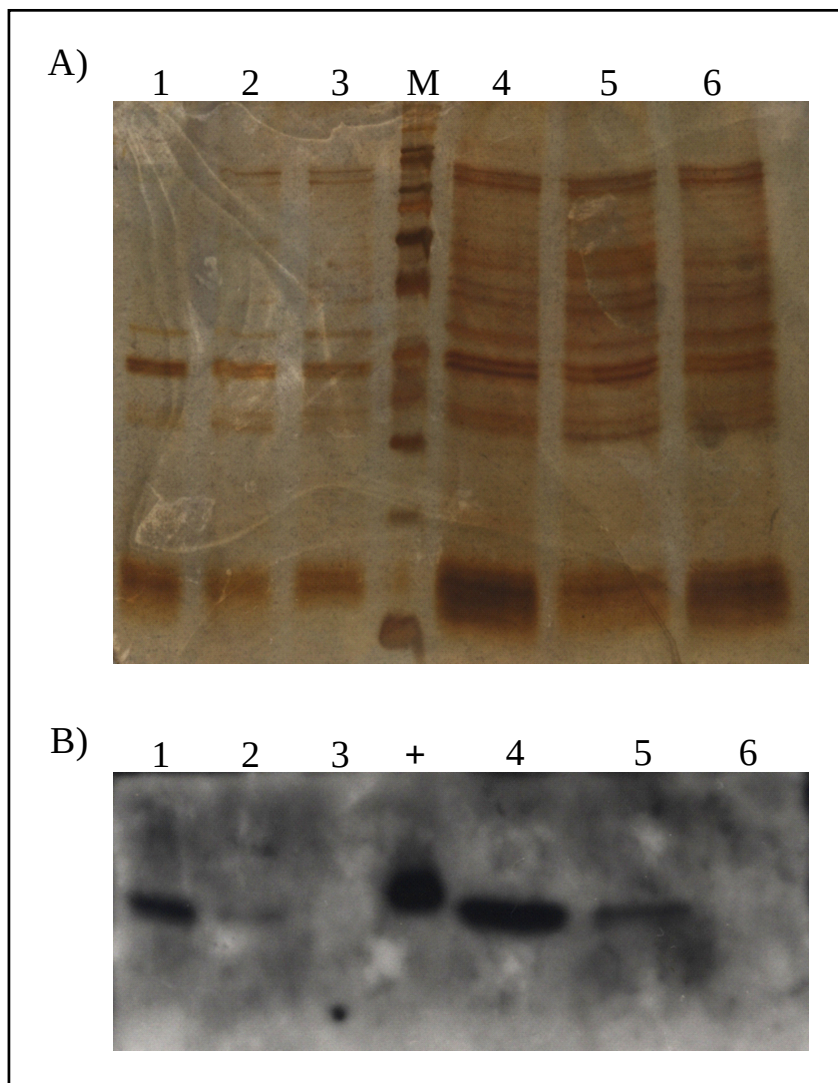


Fig. 3.18 (A) 15% SDS-PAGE of the extracellular liquid of MJ15-151 and MJ15-69 lines stained with silver nitrate. Ten and 30 µl of the extracellular protein extract were loaded for each sample. **(B)** Western blotting of the extracellular liquid of MJ15-151 and MJ15-69 lines. Ten and 30 µl of the extracellular protein extract of each sample and 25 ng of the purified AcPMEI were loaded. M) Marker of molecular mass 10-200 KDa (Fermentas), 1) MJ15-151 (10 µl), 2) MJ15-69 (10 µl), 3) Wild type (10 µl), 4) MJ15-151 (30 µl), 5) MJ15-69 (30 µl), 6) Wild type (30 µl), +) Purified AcPMEI (25 ng).

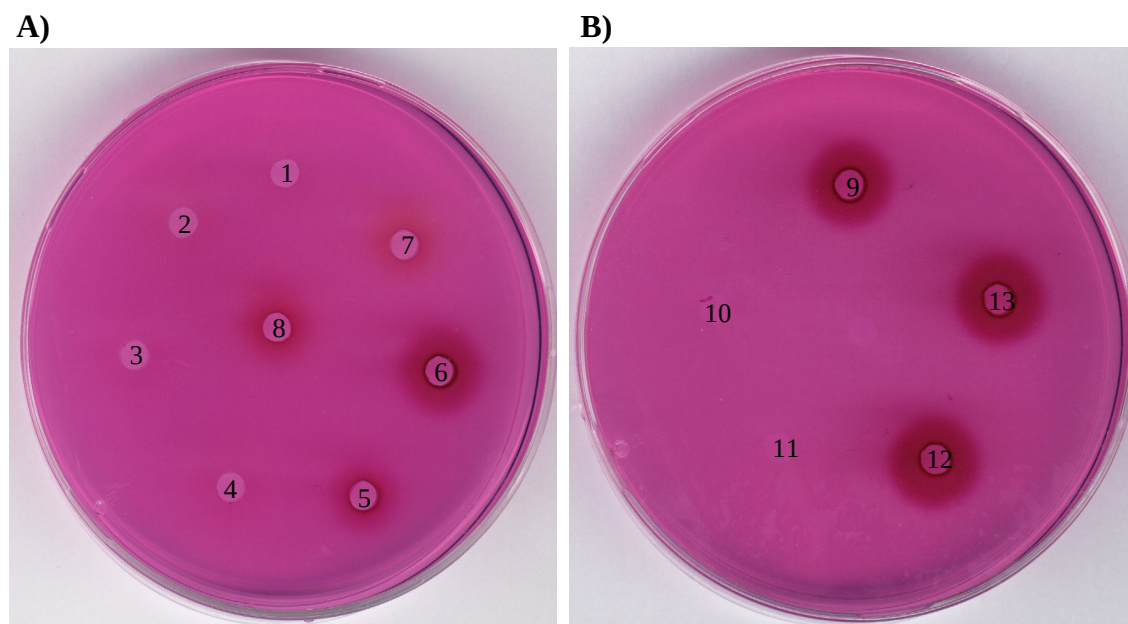


Fig. 3.19 Gel diffusion assay on extracellular fluids of transgenic and wild type plants. (**A**) Samples: 1) MJ15-151 (10 μ l), 2) MJ15-151 (30 μ l), 3) MJ15-69 (10 μ l), 4) MJ15-69 (30 μ l), 5) Wild type (10 μ l), 6) Wild type (30 μ l), 7) MJ15-151 total leaf protein extract (10 μ l), 8) MJ15-69 total leaf protein extract (10 μ l). (**B**) Samples: 9) Orange peel PME (18ng); 10) MJ15-151 extracellular fluid (10 μ l); 11) MJ15-69 extracellular fluid (10 μ l); 12) boiled MJ15-151 extracellular fluid (10 μ l) plus orange peel PME (18ng); 13) boiled MJ15-69 extracellular fluid (10 μ l) plus orange peel PME (18ng). The samples containing the boiled extract and the PME were included to exclude the presence of inhibitors into the extracellular fluid of transgenic plants that lack completely of the halo.

3.1.13 Degree of pectin methylesterification of the selected *Acpmei* lines

The degree of pectin methylesterification (DM) was determined in cell walls extracted from the selected lines and wild-type plants (WT). A null segregant line (NS) was also included in the experiment. As compared to the wild-type plants, the transgenic lines MJ15-151 and MJ15-69 showed a significant increase of DM of about 20% and 32% respectively (Fig. 3.20). The MJ15-57 line expressing a lower level of inhibitor and NS line which lost the transgene by segregation, showed no differences compared to WT. The content of uronic acids in transformed plants was not significantly different from that of WT plants (Table 3.6).

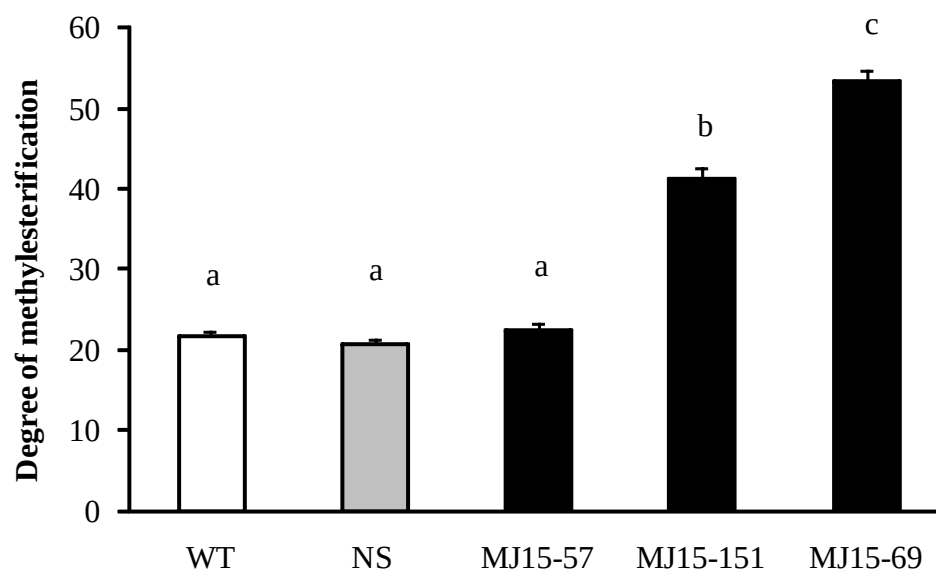


Fig. 3.20 Determination of the degree of pectin methylesterification of cell wall from *Acpmei* lines (MJ15-151, MJ15-69 and MJ15-57 lines), a null segregant line (NS) and WT. The different letters indicate data sets significantly different according to Tukey's Student range test ($P < 0.05$). Values represent the average $\pm$ standard error of three independent experiments with $n = 3$ plants each.

Table 3.6 Quantification of uronic acids content in *Acpmei* lines, NS and WT cell walls. The same letters indicate not significant differences according to Tukey's Student range test ($P < 0.05$). Data represent average $\pm$ SE of 9 biological replicates.

Line	Uronic acids ($\mu\text{moles/mg cell wall}$)
WT	0.11 ± 0.007 a
NS	0.12 ± 0.004 a
MJ15-57	0.12 ± 0.004 a
MJ15-151	0.12 ± 0.007 a
MJ15-69	0.13 ± 0.004 a

3.1.14 Immunocytochemical analysis of cell walls of *Acpmei* transformants

In order to investigate the spatial distribution of methylated and non-methylated pectic domains in cell walls of *Acpmei* and control plants (Null Segregant and wild type) an immunocytochemical analysis using monoclonal antibodies with appropriate binding specificity for different pectin substrates was performed.

The binding specificity of JIM5, JIM7 and PAM1 (Willats *et al.*, 1999; Clausen *et al.*, 2003) was first confirmed using these antibodies on a non methylated substrate, such as polygalacturonic acid, and a highly methylated substrate, such as pectin from apple (70-75 % methylated).

Using JIM5, which recognizes three or four contiguous unesterified GalA residues between two methylester groups, the signal decreased in the highly methylated pectin and using PAM1, which recognizes about 30 contiguous de-esterified residues (blockwise), the signal disappeared in the highly methylated pectin confirming their specificity of binding. JIM7 instead, which recognizes methylesterified GalA with adjacent or flanking unesterified GalA, was not able to discriminate between non methylated and highly methylated pectins and for this reason was not used in the subsequent analysis with the transgenic and control lines (Fig. 3.21).

The immunocytochemical analysis with JIM5 on the transgenic lines revealed a decreased signal on MJ15-151 and MJ15-69 lines compared to both controls, WT and NS, indicating a low level of non methylated pectins in the transgenic lines (Fig. 3.22). A reduced signal was also obtained with PAM1 on both lines compared to the WT, indicating a reduced level of de-esterified pectin stretches in the transgenic lines (Fig. 3.22).

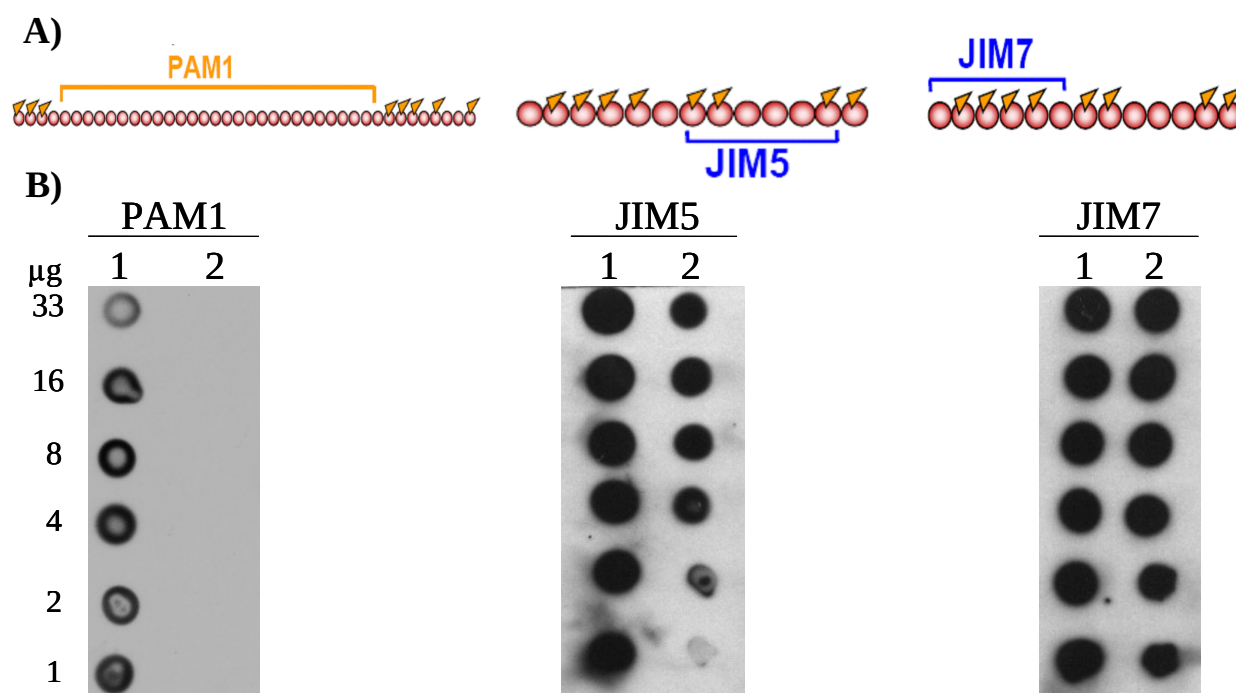


Fig. 3.21 (A) Pectic polysaccharide epitops recognized by PAM1, JIM5 and JIM7 monoclonal antibodies. About 30 contiguous de-esterified residues (blockwise), three or four contiguous unesterified GalA residues between two methylester groups and methylesterified GalA with adjacent or flanking unesterified GalA are recognized by PAM1, JIM5 and JIM7 respectively. **(B)** Immunodot analysis to check the appropriate binding specificity for different pectin substrates of PAM1, JIM5 and JIM7 monoclonal antibodies. 1) polygalacturonic acid (PGA), 2) 70-75% methylesterified pectin from apple.

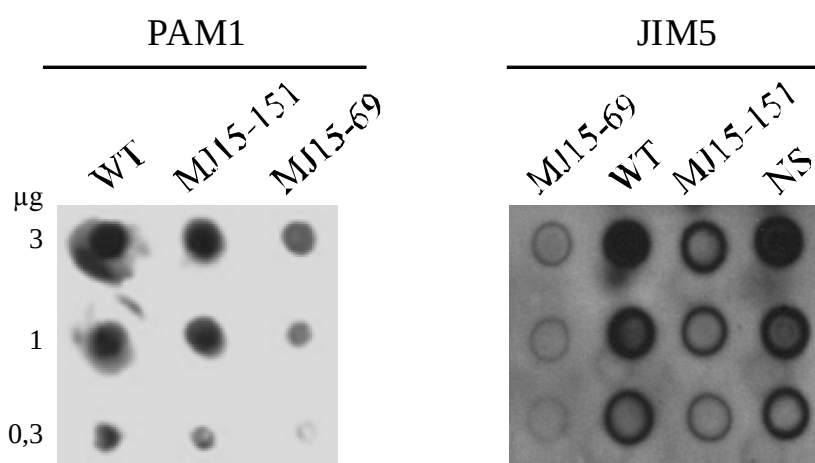


Fig. 3.22 Immunodot analysis of pectin in *Acpmei*, NS and Wild type plants. The indicated amounts of ChASS fraction were applied at a single point to a nitrocellulose membrane.

3.1.15 Phenotypic analysis of the transgenic plants

Because the degree of methylesterification in the cell walls of dicot plants can alter plant growth and roots length, a phenotypic analysis of the transgenic plants has been performed. The phenotypic features analyzed were the stem size and the boot leaf width and length at the Zadoks stage 75, the seed germination, the roots length and the number of spikelets per spike (Fig. 3.23 A, B, C and D; Fig. 3.24 A and B).

The roots length was evaluated on hydroponic wheat cultures. Roots were measured when the hypocotyls were 1,5 cm long.

As control, the Null Segregant (NS) of MJ15-151 line was used; the NS of MJ15-69 line was not included in the analysis because it was not homozygous when the analysis was performed.

The transgenic lines did not show any significant differences compared to the NS for any of the parameters analyzed.

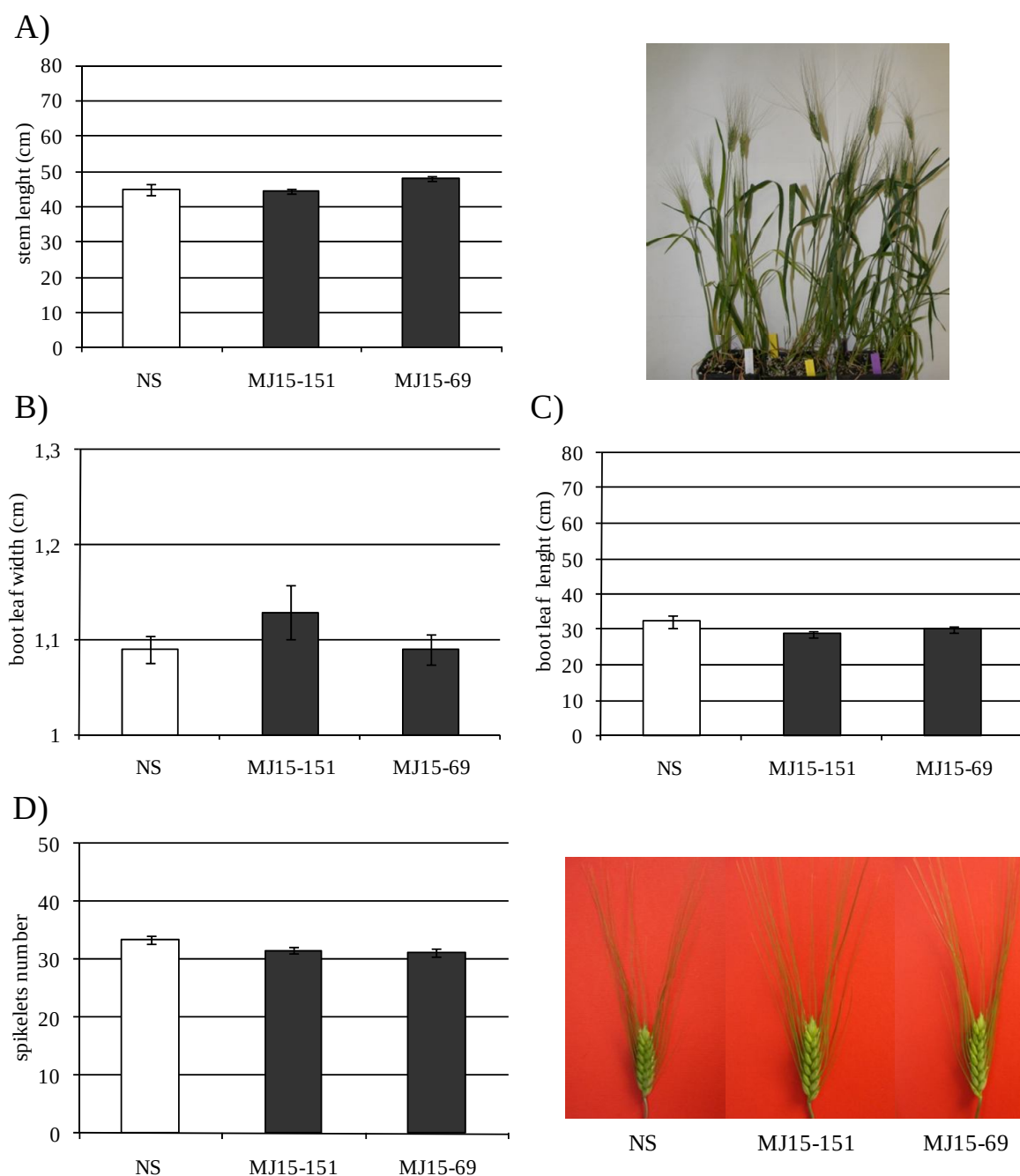


Fig. 3.23 Phenotypic analysis of transgenic (*Acpmei* lines) and control plants (Null segregant lines). No significant differences were observed between T and NS plants. (**A**)Stem length, (**B**) boot leaf width and (**C**) length measured at the Zadoks stage 75; (**D**) spikelets number. Data represent the averages and the standard errors of at least 25 plants each genotype.

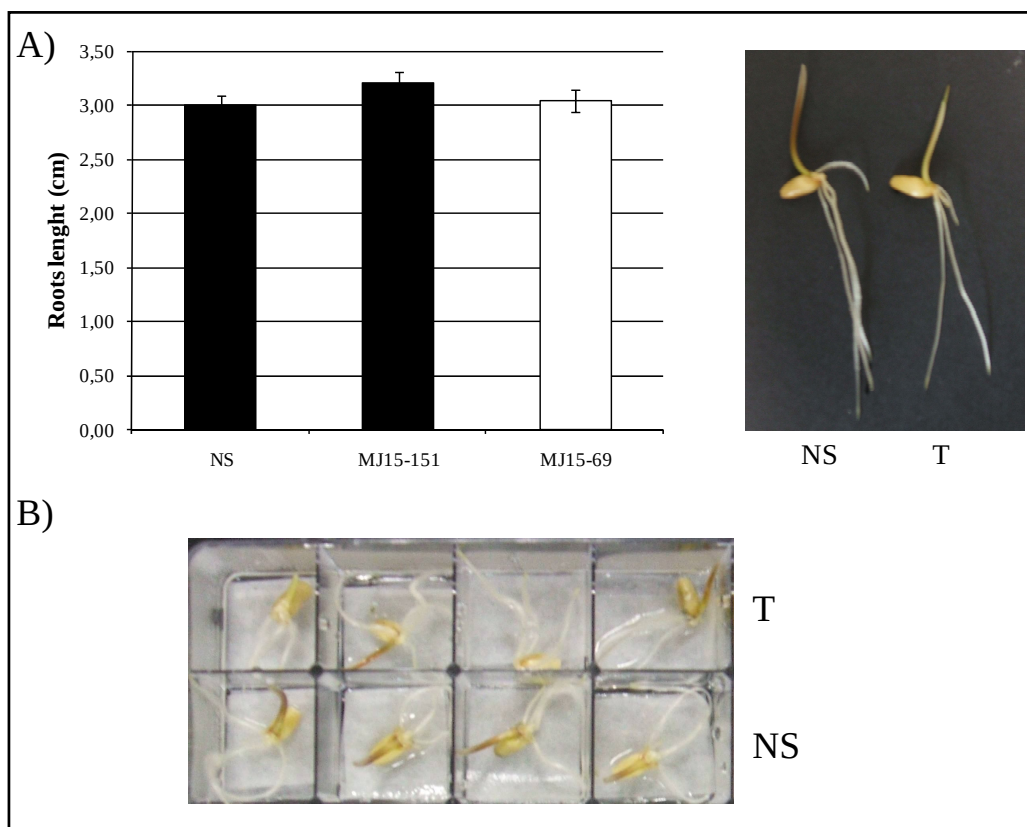


Fig. 3.24 Phenotypic analysis of transgenic (*Acpmei* lines) and control plants (Null segregant lines). No significant differences were observed between T and NS plants. (**A**) Roots length; (**B**) Seed germination. Data represent the averages and the standard errors of 20 plants each genotype.

3.1.16 *In vitro* activity of *Bipolaris sorokiniana* and *Fusarium graminearum* PGs on non- or highly-methylesterified substrate

Before performing the infection experiments with the fungal pathogen *Bipolaris sorokiniana* and *Fusarium graminearum*, it was verified whether the PGs produced by these fungi were affected by the methylation of the pectic substrate. A preliminary agar diffusion assay showed that PGs from both fungi have a reduced hydrolytic activity on a highly methylated substrate such as 70-75% methylesterified pectin from apple compared to a non methylated substrate such as polygalacturonic acid (PGA; Fig. 3.25 A). To better quantify the PG activity of both fungi in different substrates, a Milner-Avigad assay on PGA or 70-75% methylesterified apple pectin was performed. This method confirmed that the degree of methylation of the substrate affects PG activity of *B. sorokiniana* and *F. graminearum*. In particular, the reduction of the hydrolytic activity, expressed as number of reducing end-groups released, was 32% and 38% for PGs from *B. sorokiniana* and *F. graminearum*, respectively (Fig. 3.25 B).

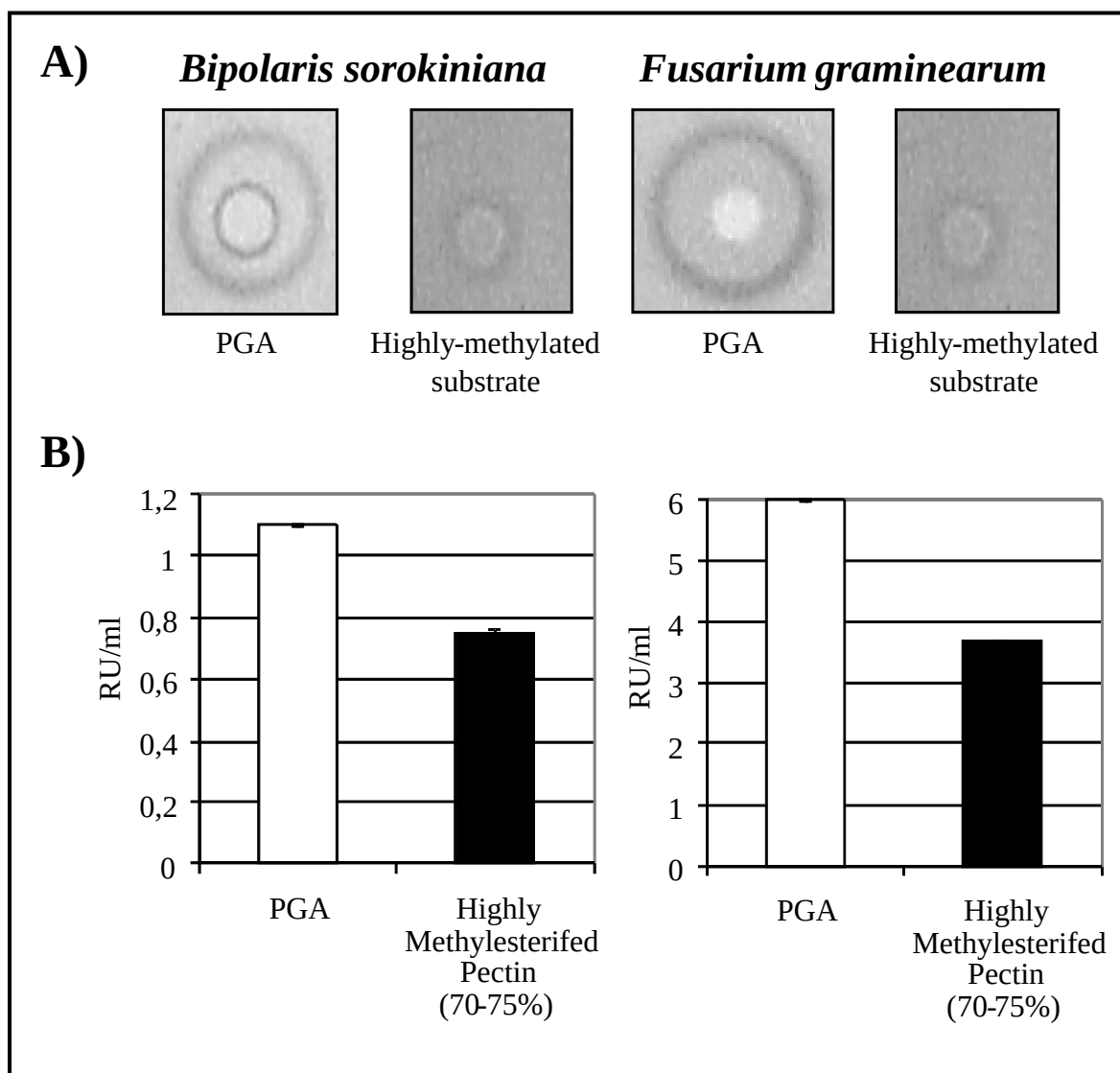


Fig. 3.25 PG activity on pectic substrates with different degree of methylation. (**A**) Agar diffusion assay showing a reduced hydrolytic activity of PGs from *Bipolaris sorokiniana* and *Fusarium graminearum* on a highly methylated substrate compared to a non-methylated substrate (PGA). (**B**) Milner-Avigad assay showing PG activity from *B. sorokiniana* and *F. graminearum* expressed as number of reducing end-groups released on a non-methylated substrate or a highly-methylated substrate. PGA and 70-75% methylesterified pectin from apple were used as pectic substrate. A single test of two different experiments that produced similar results is reported. Bars represent standard deviation between the three replicates of each sample.

3.1.17 Infection of leaf tissue with *Bipolaris sorokiniana*

For the infection experiment on leaf tissue with the fungal pathogen *B. sorokiniana*, the two lines MJ15-151 and MJ15-69 showing a high inhibitory activity of the endogenous PME were

used. The MJ15-57 line was not included because it showed the same degree of pectin methylesterification of the wild-type plants.

Two separate infection experiments were performed: one with 23 plants of the MJ15-151 line and a corresponding number of wild type plants (cv. Svevo), and the other with 15 plants of the MJ15-69 line and a similar number of wild-type plants (cv. Svevo). The first leaf of plants in the third leaf-emerged stage (Zadoks stage 13) was inoculated with 6000 conidia of 62608 strain of *B. sorokiniana*. Disease symptoms were visible 48 hours post infection (hpi) and appeared like reddish-brown spots of variable dimensions on the leaf surface. In order to facilitate the analysis of single lesions, data were collected 72 hpi. Infected leaves were cut, scanned and, using Adobe Photoshop program, the ratio between leaf area showing symptoms and total leaf area was calculated and expressed as percentage. Statistical analysis of the data demonstrated that both lines had a significant reduction in disease symptoms compared to the wild-type plants. In particular, the symptom reduction was of 51% and 79% for MJ15-151 and MJ15-69, respectively (Table 3.7; Fig. 3.26).

Table 3.7 Mean values and standard errors of symptomatic leaf area of transgenic and wild-type plants inoculated with *B. sorokiniana* macroconidia

Line	Symptom severity ^a	Line	Symptom severity ^a
MJ15-151	3.4 ± 0.7 ^{b *}	MJ15-69	2.3 ± 0.5 ^{c **}
WT	7.0 ± 1.1 ^b	WT	10.7 ± 2.7 ^c

^a Symptom severity is expressed as lesion expansion area cm²/ total leaf area cm², x 100.

^b Data represent the means of 23 replicates. ^c Data represent the means of 15 replicates

* and ** = means significantly different at P ≤ 0.05 and 0.01, respectively.

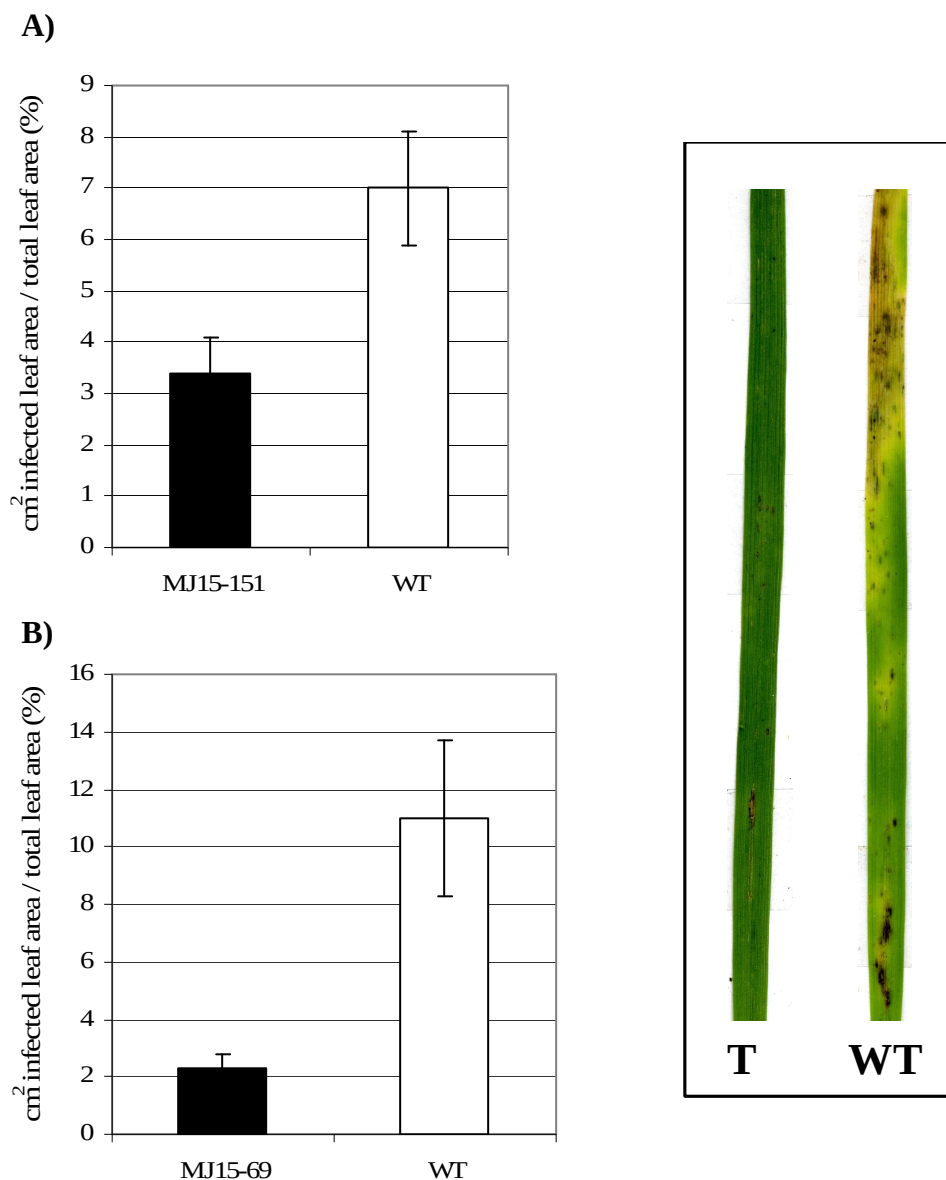


Fig. 3.26 Symptom spread 72h after infection with *B. sorokiniana* for (**A**) MJ15-151 and (**B**) MJ15-69 respect to the wild type (WT) plants. Bars show averages and standard errors of the area of lesions divided by the total leaf area (cm²). Twenty-three plants of MJ15-151 with a corresponding number of WT plants and 15 plants of MJ15-69 with a similar number of WT plants were used in two separate infection experiments.

3.1.18 Infection of wheat floral tissue with *Fusarium graminearum*

For the infection experiments on wheat spike tissue with the fungal pathogen *F. graminearum*, the two lines MJ15-151 and MJ15-69 showing a high inhibitory activity of the endogenous PME were used. The MJ15-57 line was not included because it showed the same degree of pectin methylesterification of the wild-type plants.

The opposite central spikelets in primary spikes (Zadoks stage 68) were point inoculated and visually examined for the spread of disease for a period of 18 days. A single independent infection experiment on at least 25 plants each control and transgenic lines (MJ15-69, MJ15-151, NS and WT) was performed in T₃ generation. *Fusarium* head blight (FHB) disease symptoms, manifested as spike bleaching, usually appeared 3 dpi and reached the maximum expansion 18 dpi.

NS and wild type plants exhibited a similar behaviour and, compared to them, both transgenic lines showed a significant reduction of symptoms starting from 10 dpi to 18 dpi (Fig. 3.27). In particular, at 18 dpi, the reduction of symptoms of the MJ15-69 line was of 31% and 23% as compared to NS and WT respectively while the MJ15-151 line showed a reduction of symptoms of 25% and 17% as compared to the same controls (NS and WT respectively) (Fig. 3.28).

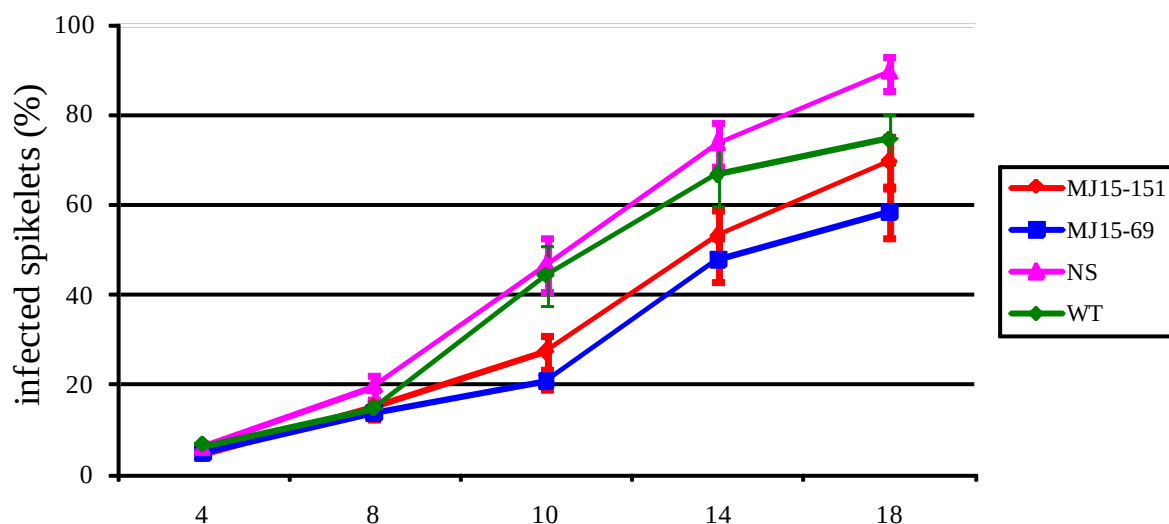


Fig. 3.27 Symptoms spread up to 18 days post infection with *F. graminearum*. Disease symptoms are expressed as percentage of spikelets showing symptoms on the total number of spikelets per spike. Data represent the averages and the standard errors of 1 independent test with at least 25 plants each genotype in T₃ generation.

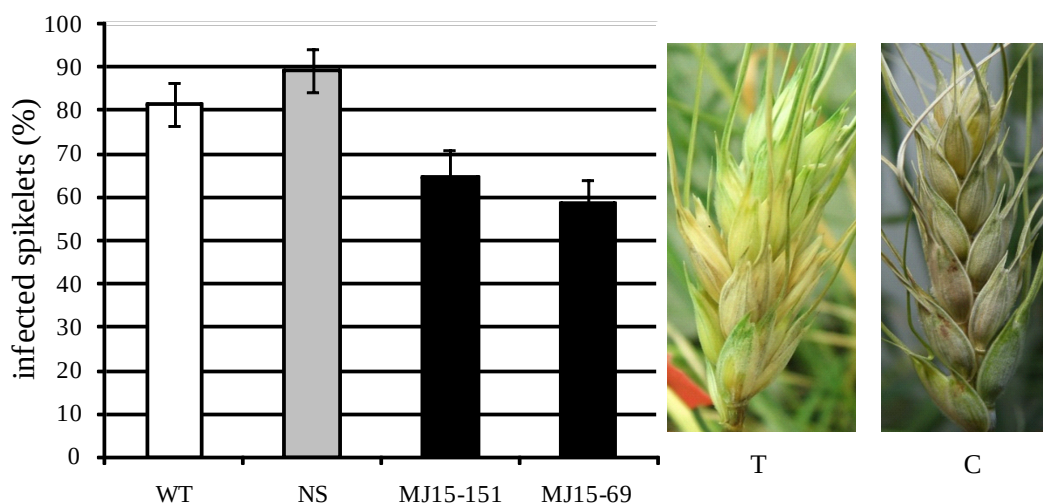


Fig. 3.28 Example of FHB disease symptoms on *Triticum durum*. The figure represents the average of infected spikelets observed at 18 dpi in control and transgenic plants. Bars represent standard error.

3.1.19 *Fusarium graminearum* growth on different pectic substrates

In order to verify if the reduced symptoms observed on *Acpmei* lines after the infection with *F. graminearum* were related to the impaired ability of this fungus to growth on methylesterified pectins, the fungus was grown on a solid medium containing PGA or pectin from apple with 70-75% degree of methylesterification as sole carbon source. After 6 days, fungal growth was determined as linear expansion by measuring the radius of the mycelium (mycelium front distal from the inoculum) from three replicates for each growth medium (Fig. 3.29). The mycelium radius of *F. graminearum* grown on PGA was almost the double of that observed on highly methylated pectin. In particular, the reduction of the fungal growth on medium containing methylated pectin as sole carbon source was 45% compared to its growth on PGA (Fig. 3.30). The same measurements were taken after 8 days of growth when the fungus on growth medium containing PGA reached the edge of the plate (Fig. 3.29). At this stage, the reduction of fungal growth on methylated pectin was even bigger and reached about 73% compared to its growth on PGA (Fig. 3.30). These results highlight that methylesterified pectin can interfere negatively with the growth rate of this fungal pathogen.

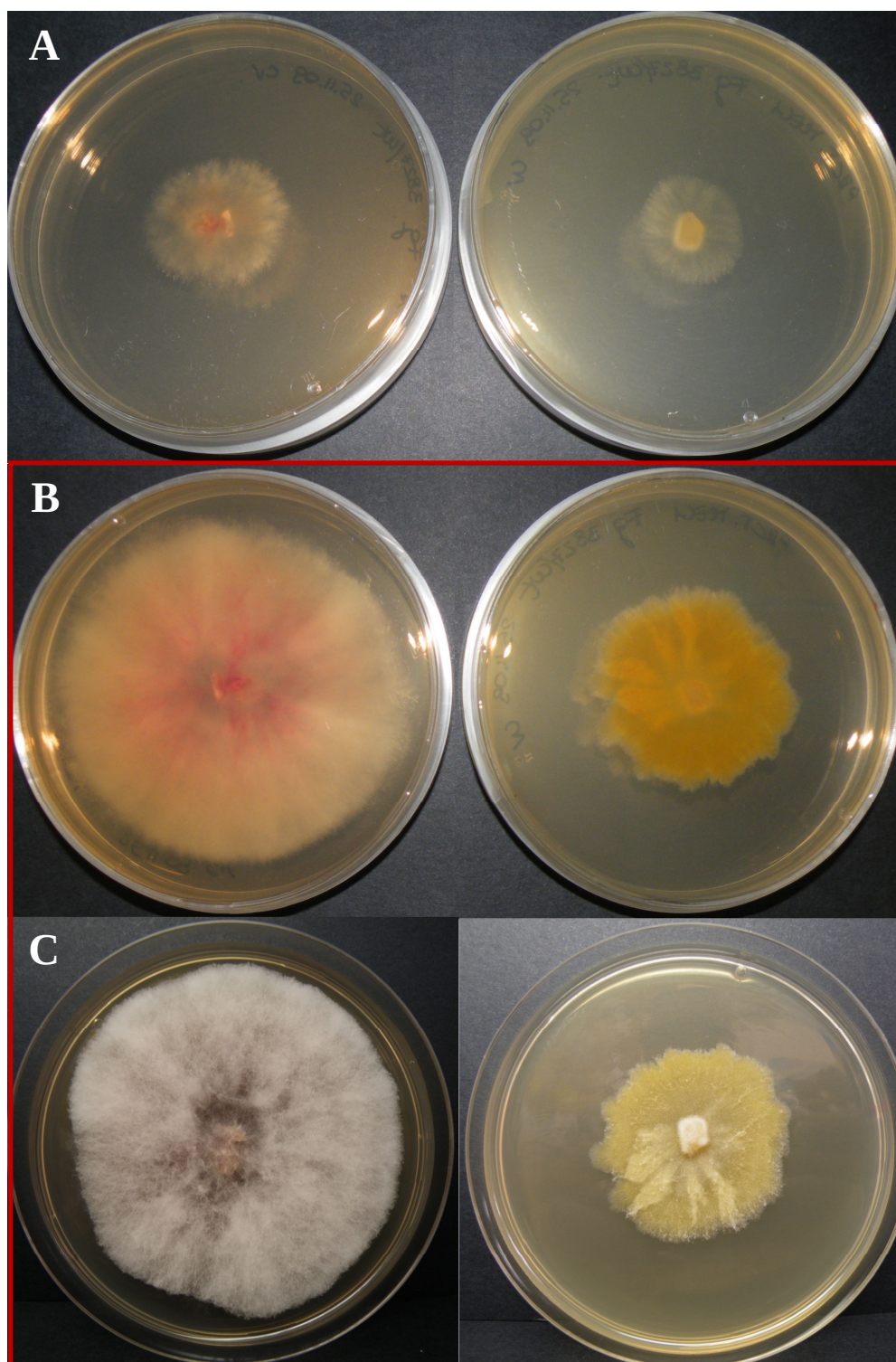


Fig. 3.29 *F. graminearum* grown on solid medium containing PGA (*left*) or pectin from apple with 70-75% degree of methylesterification (*right*) as sole carbon source. **A)** Mycelium after 4 days of growth. **B and C)** Mycelium after 6 days of growth. It's evident a better growth of this fungus on unesterified pectins (PGA) as carbon source compared to highly methylesterified pectins.

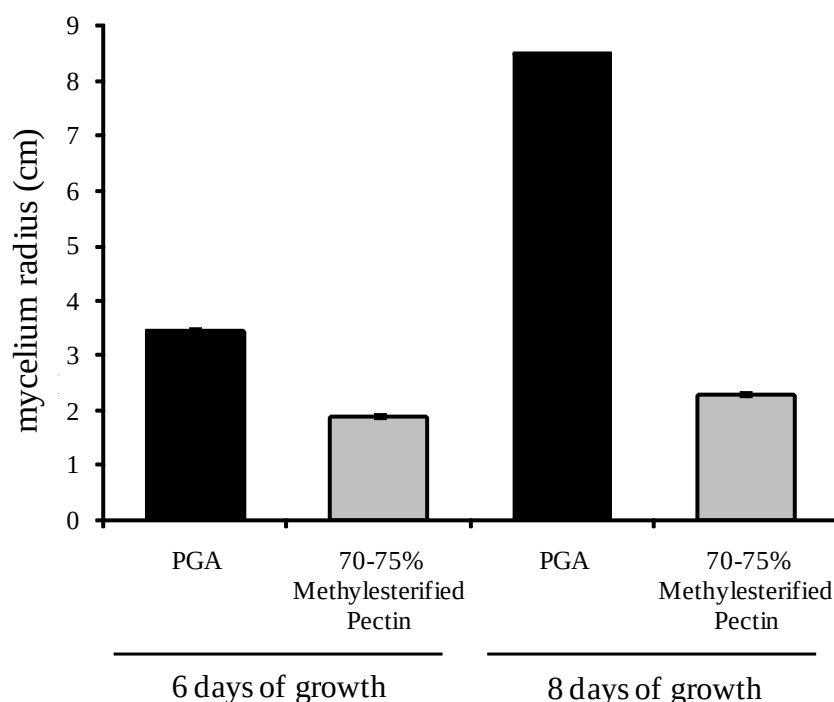


Fig. 3.30 Radius (cm) of *F. graminearum* mycelium after 6 and 8 days of growth on solid medium containing PGA or pectin from apple with 70-75% degree of methylesterification as sole carbon source. Bars represent standard error.

3.2 Production and selection of durum wheat plants expressing PvPGIP2

The objective of this part of the work was the pyramiding of *Acpmei* and *Pvpqip2* transgenes. Transgenic bread wheat (*Triticum aestivum*, hexaploid wheat) plants expressing PvPGIP2 from *Phaseolus vulgaris* (the most effective inhibitor of fungal PGs so far characterized) were already available in our laboratory (Janni *et al.*, 2008), however, the *pmei* lines described above were made in durum wheat (*T. durum*, tetraploid wheat). To speed up the pyramiding of *Acpmei* and *Pvpqip2* transgenes, transgenic durum wheat plants expressing PvPGIP2 were produced.

Immature embryos of *T. durum* cv. Svevo were co-transformed with pUBISR2, containing the *Pvpqip2* under the *Ubi1* promoter (Janni *et al.*, 2008), and *pUBI::bar* as selectable marker, following, as above, the procedure reported by Janni *et al.* 2008. A single transformation experiment, called MJ31, was performed in which 1656 immature embryos were co- bombarded. Fifty-three independent regenerated lines were obtained and analyzed by PCR for the presence of the transgene using the primers 615F/853R which produce an amplicon of 238bp; thirty-three T_0 lines resulted positive and twenty lines negative for a transformation efficiency of 2,3% (Table 3.8).

Table 3.8 Summary of the pUBISR2 bombardment experiment

cv	# bombardment	# bombarded embryos	# regenerated lines in the soil	# T ₀ positive lines by PCR	Transformation efficiency (%)
Svevo	MJ31	1656	53	33	2,3

Some of these T₀ positive lines were also investigated for the presence of the PvPGIP2 protein and for its inhibitory activity. T₀ leaves of this plants were subjected to total protein extraction and western blotting analysis of transgenic and control plants was performed using an antibody raised against the bean PGIP. An example of this analysis is reported in figure 3.31 where a clear immunodecoration signal, corresponding to PvPGIP2, was detected in the transgenic plants, whereas no signal was detected in total protein extracts prepared from the untransformed plants.

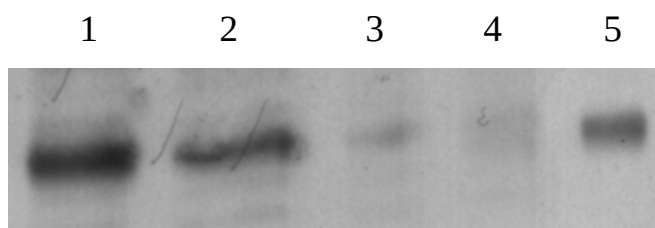


Fig. 3.31 Western blot of total protein extract from transgenic lines transformed with pUBISR2. Analyses were performed using an antibody raised against the bean polygalacturonase-inhibiting protein (PGIP). Ten µg of total protein extract from T₀ wheat leaves were loaded. Samples: 1) MJ31-2, 2) MJ31-11, 3) MJ31-17, 4) Wild type cv. Svevo, 5) Purified PvPGIP2 (50ng).

The activity of the transgenically expressed PvPGIP2 was tested against a *F. phyllophilum* endopolygalacturonase (FpPG) because this enzyme is inhibited by PvPGIP2 (Leckie *et al.*, 1999) but is resistant to the inhibition by the endogenous wheat PGIP. The analysis performed by agarose diffusion assay showed that the transgenic lines exhibited different levels of inhibition of the FpPG, as indicated by a reduced halo or the lack of the halo. In contrast, the crude protein extract from the untransformed plants did not affect the activity of the enzyme (Fig. 3.32). Protein extracts that had been boiled exhibited no inhibitory activity in this assay, excluding the possibility of a non-proteinaceous inhibitor.

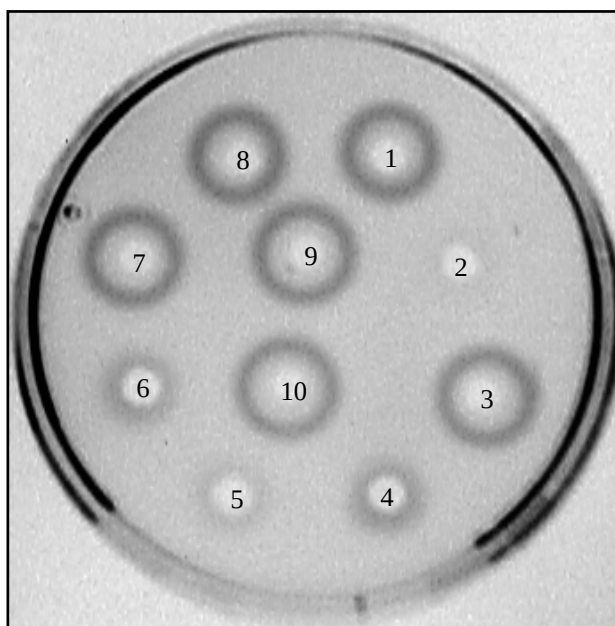


Fig. 3.32 Agarose diffusion assay of crude protein extract of leaves from transgenic and untransformed plants. The assay was performed using 0.0011 reducing units of *Fusarium phyllophilum* endopolygalacturonase (FpPG). Samples: 1) Purified FpPG. Purified FpPG plus crude protein extract from 2) MJ31-2 (5 μ g), 3) boiled MJ31-2, 4) MJ31-2 (1 μ g), 5) MJ31-70 (5 μ g), 6) MJ31-70 (1 μ g), 7) MJ31-17 (5 μ g), 8) MJ31-17 (1 μ g), 9) Untransformed *Triticum durum* cv. Svevo (5 μ g), Untransformed *Triticum durum* cv. Svevo (1 μ g). The lack of the halo indicates the inhibition of FpPG activity.

Two T₀ lines showing a high inhibitory activity, MJ31-2 and MJ31-70 (80% of inhibition of the FpPG loading 1 μ g of crude protein extract) and 1 line with an intermediate inhibitory activity, MJ31-11 (about 60% of inhibition loading 1 μ g of crude protein extract), were multiplied to obtain T₁ plants that were tested again (Zadoks stage 15) for the inhibitory activity against FpPG before crossing them with the two PME1 lines (MJ15-151 and MJ15-69) selected for the high inhibitory activity of the endogenous PME.

In T₁ generation, the MJ31-70 line did not confirmed the inhibitory activity showed in T₀ generation, consequently, this line was not used in the cross with PME1 lines.

3.3 Pyramiding of *Acpmei* and *Pvpgip2* transgenes

In order to combine in one genotype the positive factors, PGIP and PME1, transgenic lines expressing PvPGIP2 (*Pvpgip2* lines hereafter) or AcPME1 were crossed. The two PME1 lines, MJ15-151 and MJ15-69, selected for a high inhibitory activity against the endogenous PME were crossed with the two durum wheat lines, MJ31-2 and MJ31-11, showing a high inhibitory

activity against FpPG. T₃ PMEI lines and T₁ PGIP lines were used as male or female in the cross.

Sixteen F₁ seeds were obtained. DNA from these seeds was extracted and checked by PCR for the presence of both transgenes using the specific primers 615F/853R for *Pvpgip2* and AcpmeiF4/AcpmeiR4 for *Acpmei* (Fig. 3.33). Six F₁ seeds were found to contain both transgenes. These plants had been self-pollinated in order to produce the F₂ generation.

A summary of the PCR results in the PGIP x PMEI cross is shown in Table 3.9.

Table 3.9 Summary of the PCR results in the *Acpmei* x *Pvpgip2* cross

Line	Cross (♀ x ♂)	<i>AcPmei</i>	<i>Pvpgip2</i>
M09-1 (4 seeds)	MJ15-151 x MJ31-11	+	-
		+	+
		+	-
		+	-
M09-2 (1 seed)	MJ15-69 x MJ31-11	-	-
M09-3 (2 seeds)	MJ15-69 x MJ31-2	+	+
		+	+
M09-5 (3 seeds)	MJ15-69 x MJ31-2	+	-
		+	-
		+	-
M09-7 (5 seeds)	MJ15-151 x MJ31-11	+	+
		+	+
		+	-
		+	-
		+	+
M09-8 (1 seed)	MJ31-2 x MJ15-151	-	+

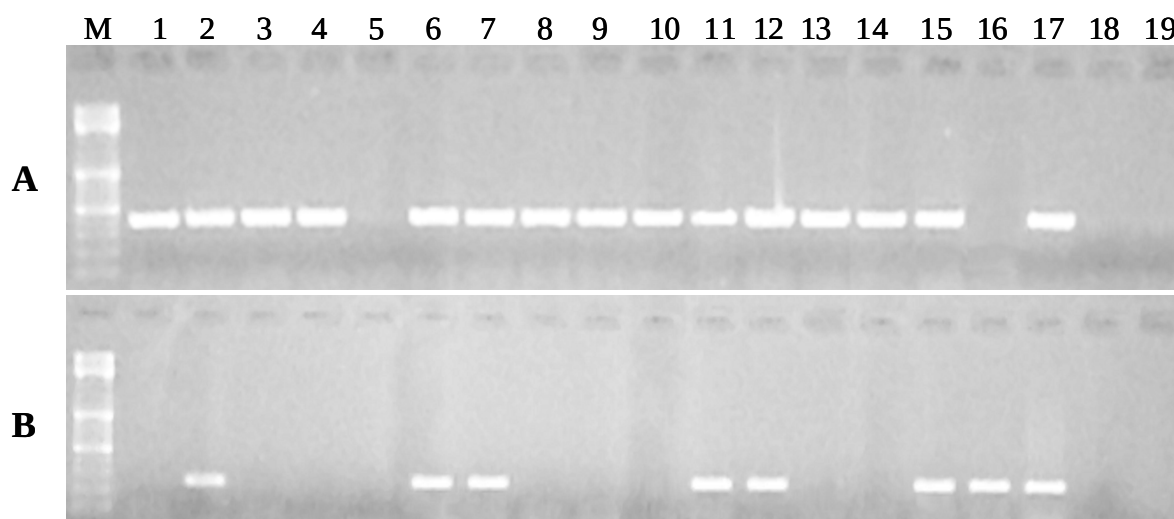


Fig. 3.33 1,5% Agarose gel of the amplification products from F_1 seeds of the cross between *Acpmei* lines (MJ15-151 and MJ15-69) and *Pvpqip2* lines (MJ31-2 and MJ31-11). **A)** PCR products obtained using the specific primers for *Acpmei*, *AcpmeiF4/AcpmeiR4* which produce an amplicon of 407bp. Samples: M) Marker GeneRuler 100 bp DNA Ladder, 1) M09-1-1, 2) M09-1-2, 3) M09-1-3, 4) M09-1-4, 5) M09-2-1, 6) M09-3-1, 7) M09-3-2, 8) M09-5-1, 9) M09-5-2, 10) M09-5-3, 11) M09-7-1, 12) M09-7-2, 13) M09-7-3, 14) M09-7-4, 15) M09-7-5, 16) M09-8-1, 17) MJ15-69 Genomic DNA, 18) *Triticum durum* cv. Svevo, 19) water. **B)** PCR products obtained using the specific primers for *Pvpqip2*, 615F/853R which produce an amplicon of 238bp. Samples: M) Marker GeneRuler 100 bp DNA Ladder, 1) M09-1-1, 2) M09-1-2, 3) M09-1-3, 4) M09-1-4, 5) M09-2-1, 6) M09-3-1, 7) M09-3-2, 8) M09-5-1, 9) M09-5-2, 10) M09-5-3, 11) M09-7-1, 12) M09-7-2, 13) M09-7-3, 14) M09-7-4, 15) M09-7-5, 16) M09-8-1, 17) MJ31-2 Genomic DNA, 18) *Triticum durum* cv. Svevo, 19) water.

3.4 Preliminary test of *Claviceps purpurea* infection on wheat plants expressing PvPGIP2

In order to select additional fungal pathogens to be tested in the cross *Acpmei/Pvpqip2* and in the parental lines, infection experiments with the biotrophic fungal pathogen *Claviceps purpurea*, the causal of the ergot disease of cereals and grasses, were performed. Like *F. graminearum*, *C. purpurea* infects the floral tissue although using a different mode of penetration and colonization of the floral host tissue. In rye, PG activity of this pathogen represents a pathogenicity factor (Oeser *et al.*, 2002). Consequently, a first attempt to verify the possibility to control the development of the ergot disease symptom was performed on wheat plant expressing PGIP, the inhibitor of fungal PG. Moreover, since the *Pvpqip2* durum wheat lines developed during this work were still at the T_1 generation, where part of the progenies can loose the transgene by segregation, the infection experiment was performed on the stable transgenic bread wheat line, J82-23a, that, as specified above, expresses PvPGIP2 and showed a significant reduction of

symptoms following the infection of *Bipolaris sorokiniana* (Janni *et al.*, 2008) and *Fusarium graminearum* (Janni *et al.*, 2007).

3.4.1 Infection of floral tissue with *Claviceps purpurea*

The inhibitory activity of PvPGIP2 in the bread wheat line J82-23a line was confirmed by agarose diffusion assay on total protein extracts from different tissues, including ovary and stigma hairs which are the principal sites of infection of *C. purpurea*.

Conidia suspension of a single isolate (03-20-1) was used to inoculate 20-40 individual florets of 45 ears of each transgenic and control plants (Null Segregant line, the corresponding “null” genotype that lost the transgene by segregation) at the Zadoks stage 59 (Fig. 3.34).

The symptoms of the infection are first recognizable by the presence of the honeydew (a sugary fluid containing millions of conidia produced by the white fungal mass at the sphacelium stage) exuded from inoculated florets and then, by the presence of sclerotia in the spike, which are the over-wintering structures of the fungus which replace the normal grains.

In the infected ears of both transgenic and control plants, honeydew was detectable 8dpi and sclerotia were formed about 20dpi.

Ergot disease symptom was evaluated both by assessing the honeydew production on a scale from 1 to 4 (Fig. 3.35) (as specified in the “Materials and methods”) from 9dpi until 14dpi (Fig. 3.36) and by measuring the sclerotia weight (Fig. 3.37). Both methods produced slightly different mean values between transgenic and control plants, although significantly different.



Fig. 3.34 Wheat spike at Zadoks stage 59 with fluffy ovary.

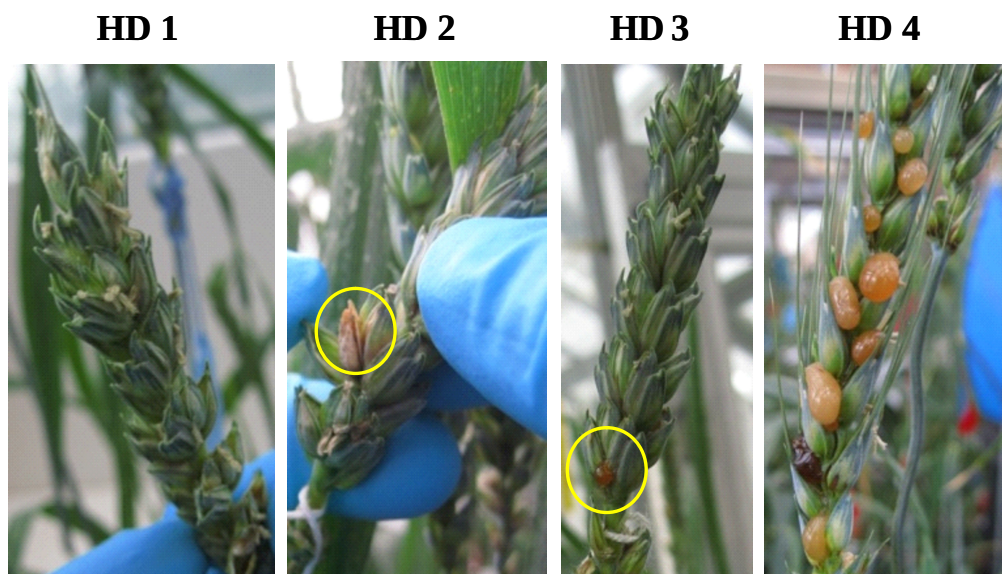


Fig. 3.35 Honeydew score. HD1=none, HD2=honeydew confined within the glumes, HD3=honeydew exuding from the florets in small drops, and HD4=large drops of honeydew running down the spike.

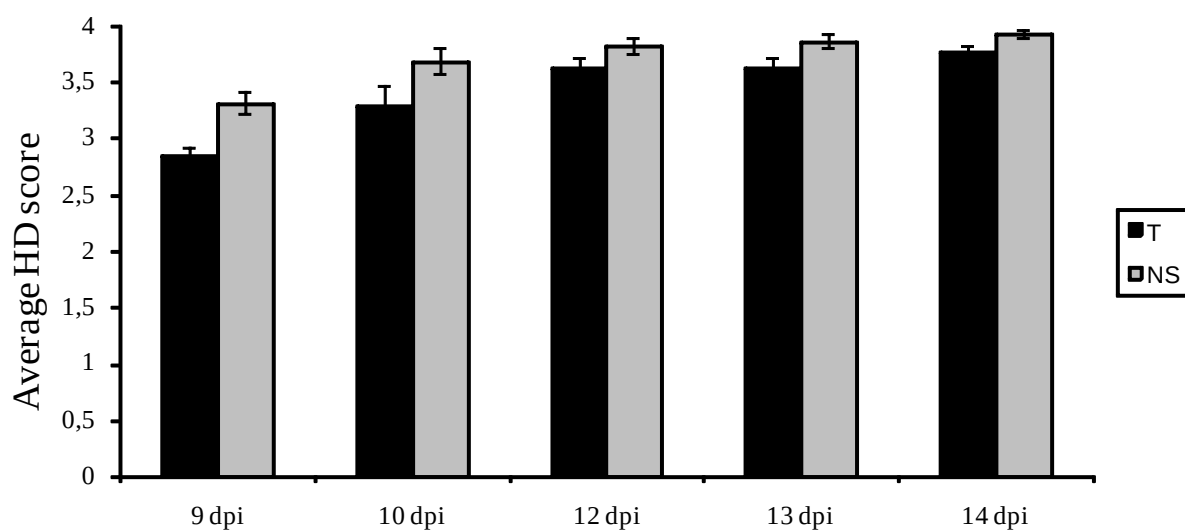


Fig. 3.36 Average honeydew (HD) score of transgenic (J82-23a line) and NS plants from 9dpi until 14dpi. Slightly different, although significant, mean values between transgenic and NS plants were observed. Bars represent standard error.

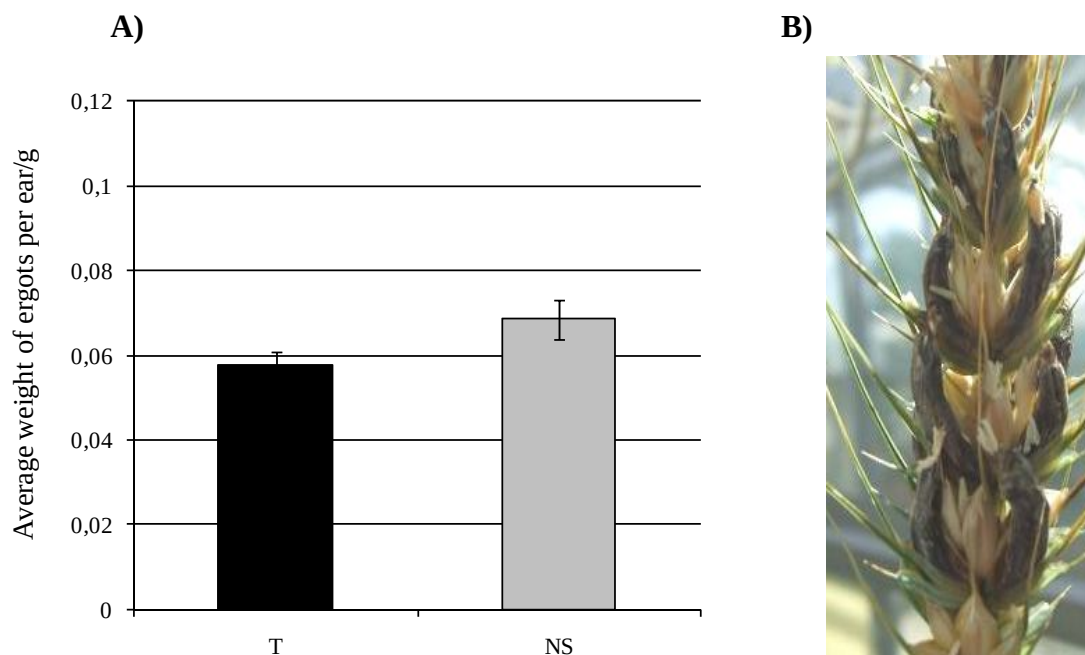


Fig. 3.37 (A) Average weight of ergots per ear/g of transgenic and control (NS) plants. Slightly different, although significant, mean values between transgenic and NS plants were observed. **(B)** sclerotia in the spike. Bars represent standard error.

3.4.2 Microscope analysis of wheat ovaries infected with *C. purpurea*

The progression of the infection on wheat carpel in the first stages of infection was also analyzed by microscope analysis of infected ovaries bisected longitudinally and stained with aniline blue fluorescent dye. The analysis was done on 50 ovaries of each transgenic and control sample excised at 3dpi, 4dpi, 5dpi and 7dpi. The analysis was performed also 1dpi and 2dpi but no infection was observed on any carpels. On the basis of the severity of the infection, it was decided to group in the following 5 classes the symptoms observed in the infected ovaries: Non Infected (NI), Light Infected (LI, when the hyphae do not reach the base of the ovary), Heavy Infected (HI, when the hyphae reach the base of the ovary), Almost Sphacelium (AS, when the hyphae ramify through the host tissue but the host cellular structure and the ovary is still visible) and Sphacelium (S, when the fungus is in the sphacelium stage producing spore and the ovary structure is no longer visible) (Fig. 3.38).

This analysis revealed no relevant differences in the rate of infection between transgenic and control plants from 1dpi until 7dpi when the fungus begins to produce spore in most of the ovaries and the honeydew starts to extrude from the floret (Fig. 3.39).

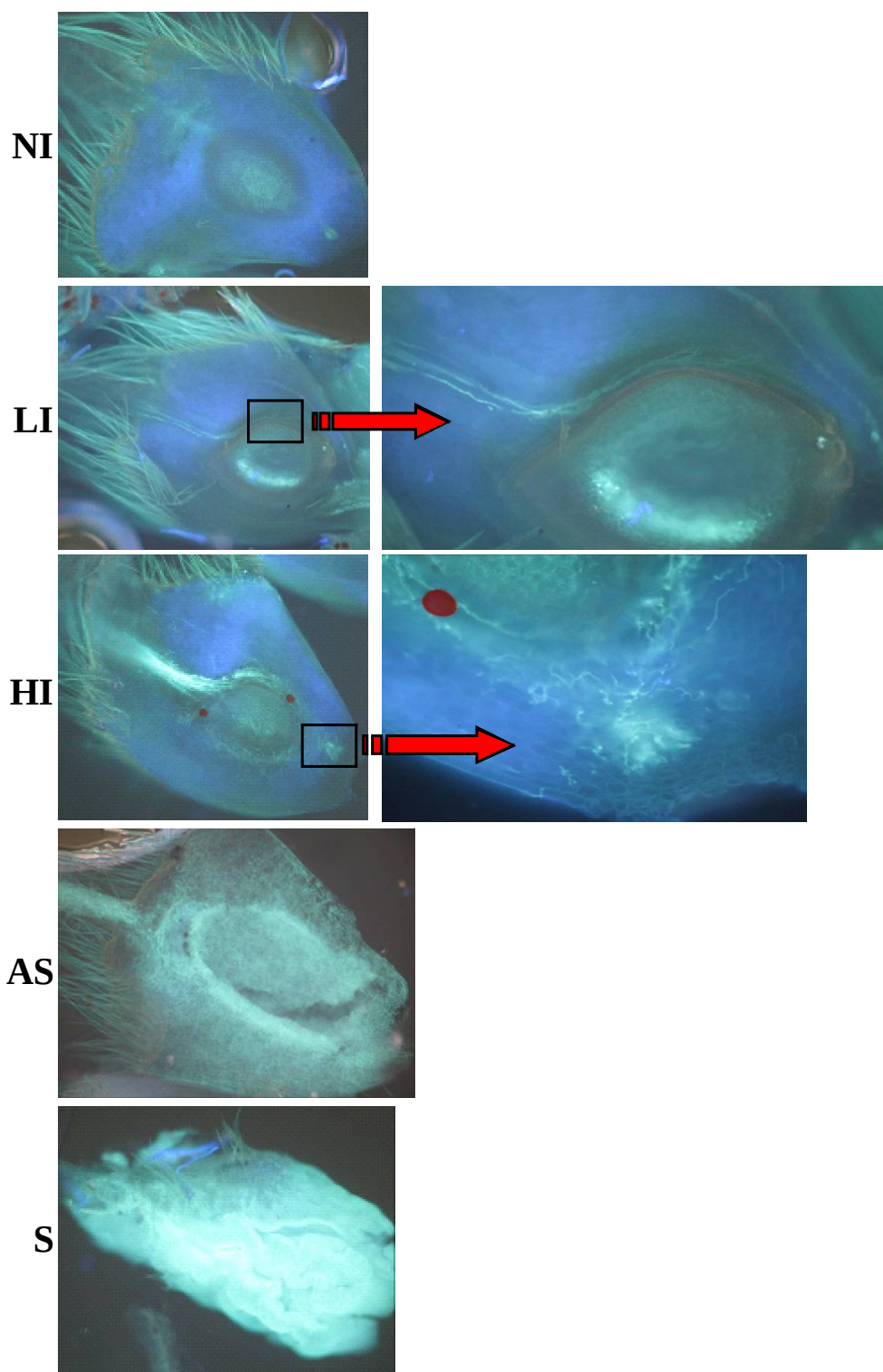


Fig. 3.38 Classes of infected ovaries. NI= Non Infected; LI= Light Infected, when the hyphae do not reach the base of the ovary; HI= Heavy Infected, when the hyphae reach the base of the ovary), AS= Almost Sphaecelium, when the hyphae ramify through the host tissue but the host cellular structure and the ovary is still visible, and S= Sphaecelium, when the fungus is in the sphaecelium stage producing spore and the ovary structure is no longer visible.

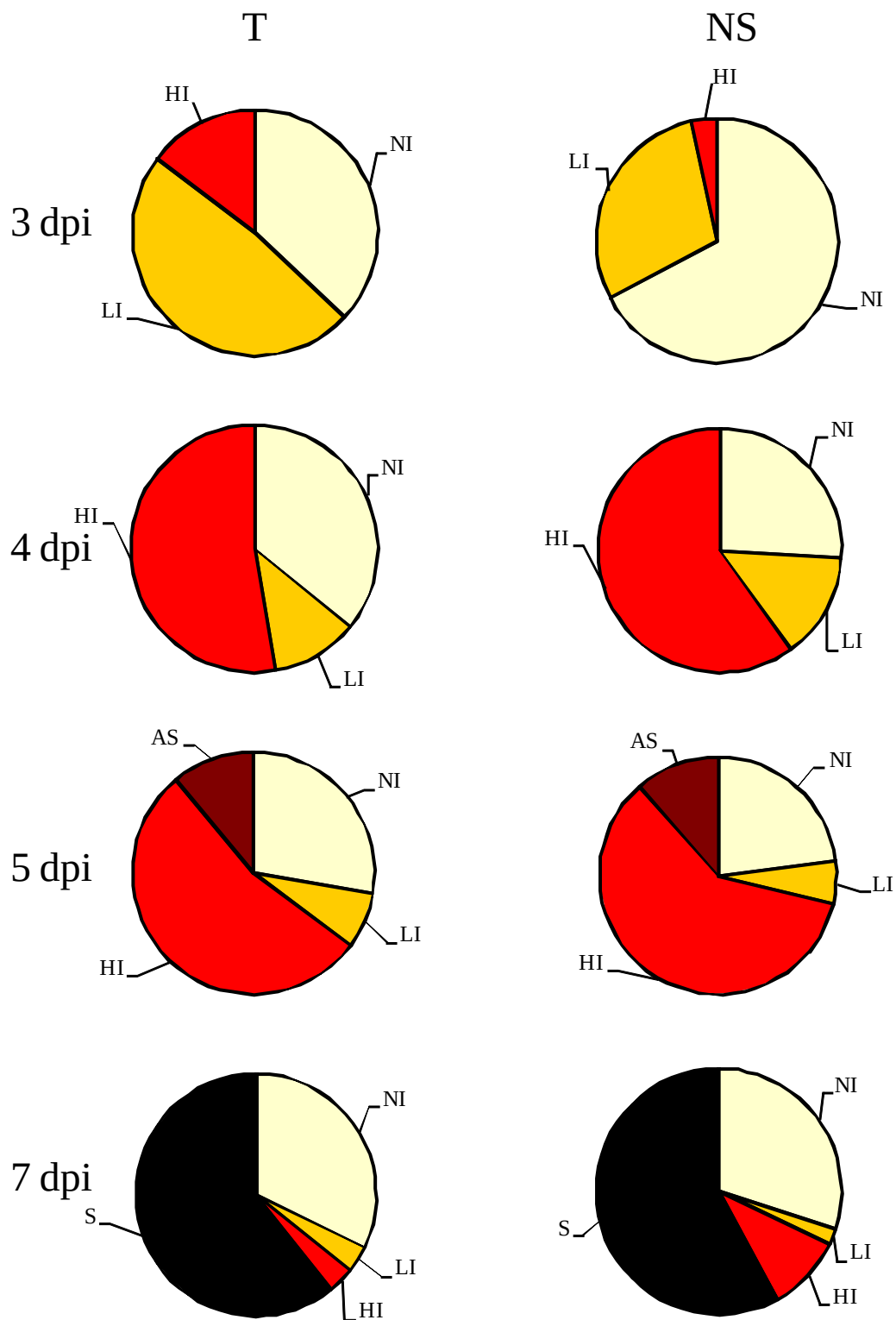


Fig. 3.39 Percentage of infected ovaries of Transgenic and NS plants at 3, 4, 5, and 7 dpi. No relevant differences in the rate of infection between transgenic and NS plants were observed. NI= Non Infected; LI= Light Infected, HI= Heavy Infected, AS= Almost Sphacelium, and S= Sphacelium.

4. Discussion

The development of crop varieties that simultaneously control different pathogens is one of the most attracting strategies in breeding projects aimed at increasing host plant resistance. A way to obtain that, is the improvement of pre-existing plant defence mechanisms against the tools that pathogens commonly use to penetrate and colonize the host tissue. In this view, it is important to consider that plant cell wall represents one of the first obstacles encountered by the microbial pathogens during plant tissue colonization, consequently, the reinforcement of this barrier should increase the capacity of the host tissue to resist against pathogen attacks. Different strategies can be used to reinforce the plant cell wall; among these the reinforcement of the pectic component seems one of the most attracting since several evidences have been reported on the involvement of pectin composition in plant defence. Highly methylesterified pectins are less susceptible to the action of PG and pectate lyase (Willats *et al.*, 2001b). A difference in the degree of pectin methylation was also related to the reaction of tomato cultivars to *Pseudomonas syringae* pv. *tomato* (Venkatesh B., 2002) and in potato the higher percentage of methylated and branched pectins has been reported to correlate with resistance against *E. carotovora* subsp. *atroseptica* (McMillan *et al.*, 1993). A higher degree of methylesterification has been found also in bean cultivars resistant to the fungus *Colletothricum lindemuthianum* as compared to the susceptible ones. Moreover, in this system, when the bean cell walls were digested with the endo-PG of this pathogen, the fragment recovered from resistant cultivars showed higher elicitor activity than the fragment from susceptible cultivars (Boudart *et al.*, 1998). Recently, has been proposed that PME1 might indirectly play a role in plant defence against pathogen attack by inhibiting the endogenous PME activity. In this hypothesis, the reduced PME activity would maintain the pectin in a highly methylated form that would result less sensitive to the action of microbial PGs (Juge, 2006). This hypothesis is supported by the recent demonstration that Arabidopsis transgenic plants over-expressing AtPME1-1 and AtPME1-2 showed an increased degree of pectin methylesterification in their tissues and a reduced disease symptom caused by *B. cinerea*, possibly related to the impaired ability of the fungus to grow on methylesterified pectins (Lionetti *et al.*, 2007).

Additional evidences on the importance of pectin composition in plant resistance have been reported also in monocot plants in spite of a low pectin content in their cell wall. In wheat, differences in methyl ester distribution of homogalacturonan have been found in near-isogenic lines resistant and susceptible to the fungal pathogen *Puccinia graminis* f. sp. *tritici* (Wietholter

et al., 2003). These observations are now extended by our results involving the ectopic expression of *pmei* genes in durum wheat.

Before performing the transformation experiments, the capability of purified AtPMEI-1, AtPMEI-2 (from *Arabidopsis thaliana*) or AcPMEI (from *Actinidia chinensis*) to inhibit wheat PME activity was verified. Both AtPMEIs and AcPMEI recognized and inhibited wheat PME with AcPMEI displaying a stronger inhibitory activity compared to AtPMEIs. Based on this result, AcPMEI and AtPMEI-1 were used for the subsequent transformation experiments. AtPMEI-1 was preferred over AtPMEI-2 because is not glycosylated.

Several wheat transgenic lines were obtained with a reduced PME activity, but only one, the line MJ14-43, contained the *Atpmei-1* transgene. Since this line showed only a slightly reduced endogenous PME activity, about 70% of the control PME activity, was not further characterized. The T₁ generation of the twelve wheat transgenic lines expressing *Acpmei* showed different level of PME activity that was monitored also in T₂ generation. Some transgenic lines confirmed the PME activity observed in T₁ generation (MJ15-57 with 55% reduction of PME activity, MJ15-151 and MJ15-69 with very low PME activity), while other displayed a modified level of PME activity. Moreover, the lines MJ15-93 and MJ15-25, showing reduction of PME activity in T₁ generation, underwent silencing in T₂ generation. Based on these results the three *Acpmei* lines, MJ15-151, MJ15-69 and MJ15-57, showing a stable PME activity over two generations and a different degree of reduction of PME activity were selected for the subsequent analysis.

Southern blotting analysis of the three selected *Acpmei* lines revealed the presence of multiple copies of the transgene in all the lines. Moreover, the presence of additional hybridizing fragments suggests the occurrence of different insertion sites and transgene rearrangement events in all three transgenic lines. In particular, MJ15-151 showed the higher number of additional hybridizing fragments and MJ15-57 possesses more additional hybridizing fragments than MJ15-69. qPCR experiments confirmed the Southern blot results indicating that MJ15-69 possesses about 5-fold and 4-fold less copy numbers of the transgene compared to MJ15-151 and MJ15-57 lines, respectively.

The analysis of *Acpmei* transcript accumulation in MJ15-151, MJ15-69 and MJ15-57 lines revealed a transcripts level in the MJ15-151 and MJ15-69 lines 6-fold and 4-fold higher than in MJ15-57 line, respectively. This was also confirmed at protein level where western blotting analysis of total leaf protein extract of MJ15-57 line showed a lower accumulation of AcPMEI and radial gel diffusion assay demonstrated that the endogenous PME activity of MJ15-57 was higher compared to that of MJ15-151 and MJ15-69. The finding that the line MJ15-57 has a higher transgene copy number compared to the line MJ15-69 but a lower accumulation of the

Acpmei transcript suggests the presence of some non functional transgene *Acpmei* copies or the insertion of some or all of these in a genomic context with a reduced transcription efficiency as compared to that of line MJ15-69. However, the occurrence of silencing events in MJ15-57 can not be excluded.

The same level of PME activity found in leaf tissue of the MJ15-151 and MJ15-69 lines was also detected in the spike tissue, demonstrating, as expected, that the *Ubi1* promoter regulates a constitutive expression of *Acpmei* and none tissue-specific degrading activity towards AcPMEI is activated in these tissues.

The specific hybridizing signals and the high inhibitory activities of the extracellular fluids extracted from vacuum-infiltrated stems indicated that the inhibitor has been correctly targeted to the apoplast as expected since the construct used for the transformation contains the coding region of *Acpmei* with the *Pvpgip1* signal peptide for the apoplastic targeting.

To assess whether the alteration of the endogenous PME activity observed in transgenic plants affects the level of pectin methylation, the degree of pectin methylesterification (DM) was determined in cell walls extracted from leaves of transformed plants indicating that the MJ15-151 and MJ15-69 lines have a significant increase of DM of 20% and 32% compared to the wild type plants, respectively. The MJ15-57 line expressing a lower level of inhibitor and NS line which lost the transgene by segregation, showed no differences compared to WT.

Because the degree of methylesterification in the cell walls of dicot plants can alter plant growth and roots length (Lionetti *et al.*, 2007), a phenotypic analysis of the transgenic plants has been performed. Features like stem size and boot leaf width and length, seed germination, roots length and number of spikelets per spike were considered but any significant differences were found in the transgenic lines compared to the control line. A lower pectin content in the cell wall of monocots such as wheat compared to dicot plants could explain the absence of any clear phenotypic differences.

The infection experiments were performed with the foliar pathogen *B. sorokiniana* and the floral pathogen *F. graminearum* and in both cases a reduced disease symptom development was observed in the lines MJ15-151 and MJ15-69. The infection with *B. sorokiniana* and *F. graminearum* showed a significant reduction of 51% and 17% in the line MJ15-151, respectively, and of 79% and 23% in the line MJ15-69 respectively. The level of leaf symptom reduction observed following the infection with *B. sorokiniana* is similar to that observed in *Arabidopsis* overexpressing AtPMEI-1 or AtPMEI.2 following the infection with *B. cinerea* (Lionetti *et al.*, 2007).

The reduction of symptoms observed in the *Acpmei* lines expressing high level of the transgenic protein could be due to the higher DM of pectins found in these *Acpmei* lines which hampers the activity of endo-PGs produced by these fungi, thus delaying their colonization of the host tissue. Both *B. sorokiniana* and *F. graminearum* produce PGs during their colonization process of the host tissue (Janni *et al.*, 2008; Tomassini *et al.*, 2009) and we demonstrated that the *in vitro* PG activity of these pathogens is affected by the methylation of the pectic substrate. We showed also that *F. graminearum* grows on methylesterified pectin but at a lower rate than on PGA, suggesting that the methyl groups are not efficiently removed by the fungal PME and that this pathogen does prefer unesterified pectins as carbon source. Although we have not tested the inhibitory activity of AcPMEI against the PMEs of *F. graminearum* or *B. sorokiniana*, it has been reported that plant PMEI does not exert any inhibitory activity towards fungal PME (Mattei *et al.*, 2002; Giovane *et al.*, 2004; Di Matteo *et al.*, 2005).

The different methylester distribution in the pectin of transgenic and control plants could be involved in the resistance of transgenic plants. In fact, a different methylester distribution along the pectic polymer could influence the type of oligogalacturonides (OGs) released from the host cell wall. These, in turn, may affect the plant resistance to disease because of their elicitor activity. For example, pectic fragments released by endo-PG treatment from cell walls of bean cultivars resistant to *Colletotrichum lindemuthianum* and having a high DM showed a superior elicitor activity than those released from a cell wall of susceptible cultivars having a low DM (Boudart *et al.*, 1998). On the other hand, the type of OGs released during host tissue infection could act as endogenous suppressors of induced disease resistance mechanisms. In this case, the pectic fragment released from cell wall with a high DM would be less efficient as endogenous suppressors (Moerschbacher *et al.*, 1990; 1999; Wietholter *et al.*, 2003).

In both infection experiments, disease symptoms were more reduced in the MJ15-69 line compared to MJ15-151 line and this could be related to its higher degree of methylesterification of the cell walls compared to MJ15-151 which could be explained by the higher level of accumulation and activity of the transgenic protein in MJ15-69 respect to MJ15-151 line.

To further verify the importance in protecting the pectic component of the cell wall in plant resistance, the pyramiding of two inhibitors AcPMEI and PvPGIP2 was performed but their performance has not been evaluated yet because of the limiting amount of the F₁ progeny.

In order to select additional fungal pathogens to be tested in the cross *Acpmei/Pvpgip2* and in the parental lines, infection experiments with the biotrophic fungal pathogen *Claviceps purpurea*, the causal of the ergot disease of cereals and grasses, were performed.

The infection process of *C. purpurea* on rye (*Secale cereale*) flowers is accompanied by pectin degradation and PG activity represents a pathogenicity factor (Oeser *et al.*, 2002).

Because of the importance of PG in the infection process of rye flower, we tested whether in the interaction system *C. purpurea*/wheat the presence of PvPGIP2 can affect pathogen infection and ergot disease development. Moreover, since the *Pvpgip2* durum wheat lines developed during this work were still at the T₁ generation, where part of the progenies can lose the transgene by segregation, the infection experiment was performed on the stable transgenic bread wheat line, J82-23a, that, as specified above, expresses PvPGIP2 and showed a significant reduction of symptoms following the infection of *Bipolaris sorokiniana* (Janni *et al.*, 2008) and *Fusarium graminearum* (Janni *et al.*, 2007).

Slightly different mean values between transgenic and control plants were found, although significantly different suggesting that PvPGIP2 affects pathogen growth, most probably by reducing *C. purpurea* PG activity, but its contribution does not produce a noticeable phenotypic effect on ergot symptom.

This lack of a remarkable reduction of disease symptom, as obtained with *B. sorokiniana* and *F. graminearum*, could be due to several factors, including a lower inhibitory activity of PvPGIP2 against the PGs secreted by the *C. purpurea* strain used in the infection experiment.

5. CONCLUSIONS

The reported results demonstrated that the ectopic expression of PMEIs can modify the degree and composition of pectin cell wall in wheat. These reported results demonstrated also that the increased degree of methylesterification and/or the modified structure pectin cell wall in the transgenic lines is correlated with a reduced disease symptom development caused by fungal pathogens, including *F. graminearum*, the causal agent of Fusarium Head Blight (FHB), one of the most important wheat disease worldwide. These results led to conclude that the pectin component of the cell wall is an effective target for disease resistance improvement even in a species with a low pectin content of its cell walls such as wheat.

In particular, the specific results obtained were the following:

- Both AcPMEI and AtPMEI-1 endow wheat with a stronger inhibitory activity of endogenous PME; in particular, one *Atpmei-1* line (MJ14-43) displayed a slightly reduced endogenous PME activity (about 70% of the control PME activity) whereas 12 *Acpmei* lines presented different levels of reduced PME activity that varied from about <5% to 80% of the control PME activity;
- Three *Acpmei* lines showing a stable expression and activity of the transgenic product were selected for further analyses. Other *Acpmei* lines were not stable over the generations (MJ21-1, MJ15-61 and MJ15-49) or underwent silencing (MJ15-93 and MJ15-25);
- AcPMEI was correctly targeted to the apoplast as expected by the presence of *Pvpgip1* leader signal for the apoplastic targeting;
- The transgenic *Acpmei* lines did not show any significant phenotypic variation compared to control plants;
- The MJ15-151* and MJ15-69* lines expressing high level of the inhibitor showed a significant increase of DM of about 20% and 32% and a different methylester distribution compared to the wild type plants, respectively;
- The MJ15-151 and MJ15-69 lines showed a significant reduction of symptoms following the infection with the foliar pathogen *B. sorokiniana* of 51% and 79% respectively;
- The MJ15-151 and MJ15-69 lines showed a significant reduction of symptoms following the infection with the floral pathogen *F. graminearum*; in particular, for the MJ15-69 line

the reduction of symptoms was of 31% and 23% as compared to NS and WT respectively and for MJ15-151 line was of 25% and 17% as compared to the same controls;

- *Triticum durum* wheat lines expressing PvPGIP2 had been produced;
- *Triticum durum* wheat lines containing *Acpmei* and *Pvpgip2* had been produced;
- The infection experiment with the biotrophic fungal pathogen *Claviceps purpurea* on the stable transgenic bread wheat line J82-23a that expresses PvPGIP2 showed a slight although significant reduction of symptoms in the transgenic line compared to the control plants.

* These two lines have been also subjected to saccharification experiments that proved an increased efficiency of enzymatic hydrolysis and consequently represent important material for biofuel production [Lionetti V., Francocci F., Ferrari S., **Volpi C.**, Bellincampi D., Galletti R., D'Ovidio R., De Lorenzo G. and Cervone F. (2010). Engineering the cell wall by reducing demethylesterified homogalacturonan improves saccharification of plant tissues for bioconversion. PNAS 107: 616-621].

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