**Dipartimento di Scienze e Tecnologie per l'Agricoltura,
le Foreste, la Natura e l'Energia**

**DOTTORATO DI RICERCA IN
BIOTECOLOGIE VEGETALI
XXIV CICLO**

MULTI-TRANSGENE-STACKING OF GLYCOSIDASE INHIBITOR GENES TO IMPROVE RESISTANCE AGAINST FUNGAL PATHOGENS IN WHEAT

Scientific-disciplinary Area
AGR/07

Tutor: Prof.Renato D'Ovidio **Dottorando:** Raviraj Mahadeo Kalunke

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ABSTRACT

The diseases caused by pathogenic fungi are major problem for grain production. It reduces the productivity and causes contamination of grain with mycotoxins, compounds that are harmful to human health and animals. Disease control can be done by chemical treatments but these are costly and adverse affect on the environment. The most efficient strategy to control crop diseases is the development of resistant genotypes. This goal can be achieved by enhancing specific plant defence mechanisms, among which the reinforcement of plant cell wall compartment can be one of the most efficient because most pathogens have to overcome this barrier to colonize the host tissue. Plant cell walls consist mainly of polysaccharides (i.e. cellulose, hemicelluloses and pectins) and play an important role in defending against pathogens. Many pathogenic fungi can produce a range of cell wall-degrading extracellular enzymes (CWDEs) that are capable of depolymerizing the polysaccharides in the host cell wall. CWDEs are the polysaccharide degrading enzymes, including exo- and endo-polygalacturonases, pectin methylesterases, pectin and pectate lyases, acetyl esterases, xylanases and a variety of endoglucanases that cleave cellulose, xyloglucan and other glucans. During the defence response plant produces protein inhibitors of CWDE. The polygalacturonase inhibitor protein (PGIP) inhibits endopolygalacturonase (PG) secreted by fungal pathogens. The degree and pattern of cell wall pectin methyl-esterification can also influence plant resistance, as highly methyl-esterified pectin is less susceptible to the hydrolysis by fungal PGs. Pectin methyl esterification is controlled by the interaction between pectin methyl esterase (PME) and its protein inhibitor (PMEI). The PGIP and PMEI play an important role in preventing action of PG on pectin. Cereals contain Xylanase inhibitors (XIs) which inhibit microbial Xylanases, from glycoside hydrolase families 10 and 11. Endo β-1,4-xylanases (xylanases; EC 3.2.1.8) are key enzymes in the degradation of arabinoxylans (AXs), the main non-starch polysaccharides from cereal cell walls.

The main objective of this study was to pyramid glycosidase inhibitor genes (PGIP, PMEI and XI), evaluate their co-segregation frequency and their combined effect on wheat resistance against fungal pathogens in wheat. In order to achieve this goal, multi-transgenes-stacking of PGIP, PMEI and XI in wheat was carried out by two approaches, multiple plasmid co-transformation and classical crossing.

In the co-transformation approach, four plasmids containing *Pvpgip, Acpmei, Taxi-III* and *bar* genes in wheat *T. durum* cv. Svevo were co-transformed by particle bombardment. Total sixteen T_0 plants were obtained, among them seven plants showed all four genes which represents 58% co-transformation frequency for four transgenes. Further, seeds were harvested and used for T_1 segregation analysis. All the four transgenes co-segregated at ratio of 3:1, indicating that all four genes are tightly linked in all seven lines. Similar co-transformation experiments were performed in bread wheat (*T. avesticum* cv. Bobwhite) using four plasmids each containing *PvPGIP2, AcPMEI Xip-III* and *bar* genes. Total eighteen T_0 plants were obtained, among them five plants showed all four transgenes which represent 27.77 % co-transformation frequency for four transgenes. Further, seeds were harvested and used for T_1 segregation analysis. All the four transgenes co-segregated at ratio of 3:1, indicating that all four genes tightly linked in all lines.

Segregation analysis in both durum and bread wheat showed about 70% progeny contained all three transgenes of interest together and also tightly linked.

In crossing approach, parental lines of durum wheat cv Svevo each expressing single glycosidase inhibitor were crossed. First, plant containing PvPGIP2/AcPMEI were obtained, and these plants were crossed with a line expressing TAXI-III. In F_1 progeny, a total of nineteen F_1 plants were obtained, with only two plants possessing all three transgenes. Among these two plants, one plant (M011-1-5) exhibited high level of expression for PGIP and TAXI-III along with inhibition activity but, for PMEI no inhibition activity was observed, probably caused by silencing events. The remaining one plant (M011-1-4) exhibited high level expression for all three genes and their corresponding inhibitory activity. The segregation analysis F_2 progeny of this plant (M011-1-4) showed only 10% of the segregating progeny with all three transgenes of interest

Our results indicate that multi-stacking-transgenes using co-transformation can perform better than the classical crossing approach since more than 70% of the progenies were with three transgenes of interest and also tightly linked. On the contrary, in the crossing method only 10% of the segregating progeny contained all three glycosidase inhibitors.

Three transgenic durum wheat lines containing tightly linked PvPGIP2, AcPMEI and TAXI-III (lines MJ56-5, MJ56-16a and MJ56-16b) were analyzed for transgene expression and inhibition activity. All three transgenic lines exhibited high level of expression for PMEI and TAXI-III along with inhibition activity but, for PGIP a very low expression and no inhibition activity was observed, probably becaused of gene silencing events. T_2 generations of these three lines were used to analyze their resistance response to *B. sorokiniana* infection. The results showed about 70 % reduction in Leaf bloch disease symptom. This level of protection is higher than that obtained with the overexpression of only AcPMEI (about 55%) in durum wheat (Volpi et al. 2011), probably because of the co-presence of TAXI-III. However, the possibility that a higher level of PMEI activity in these transgenic plants, compared to those analysed by Volpi et al. (2011) is responsible for the higher protection cannot be ruled out.

RIASSUNTO

La malattie causate da patogeni fungini sono uno dei principali problemi per la produzione cerealicola, queste malattie riducono la produttività e causano la contaminazione del grano con micotossine, composti pericolosi per la salute umana e animale. Il controllo delle malattie può essere effettuato utilizzando trattamenti chimici che però sono svantaggiosi dal punto di vista economico e ambientale. La strategia più efficace per controllare le malattie delle colture di interesse agrario è lo sviluppo di genotipi resistenti. Ciò può essere ottenuto potenziando specifici meccanismi di difesa della pianta, per esempio il rafforzamento della parete cellulare può essere una strategia efficace perchè la maggior parte dei patogeni deve superare questa barriera per colonizzare il tessuto ospite. La parete cellulare vegetale è costituita principalmente da polisaccaridi (cellulosa, emicellulose e pectine) e svolge un importante ruolo nella difesa contro i patogeni. Molti patogeni fungini producono degli enzimi extracellulari che degradano la parete cellulare (CWDE) ;essi sono in grado di depolimerizzare i polisaccaridi presenti nella parete cellulare della pianta ospite. I CWDE sono gli enzimi che degradano la parete cellulare e includono eso e endo-poligalatturonasi, pecti metilesterasi, pectina e pectato liasi, acetil esterasi, xilanasi e una varietà di endoglucanasi che degradano la cellulosa, lo xiloglucano e gli altri glucani. Nella risposta di difesa la pianta produce proteine inibitrici dei CWDE. La proteina inibitrice delle poligalatturonasi (PGIP) inibisce le endopoligalatturonasi (PG) secrete dai funghi patogeni. Il grado e il pattern di metil esterificazione della parete cellulare possono influenzare la resistenza della pianta, infatti la pectina altamente metil esterificata è meno suscettibile all'idrolisi da parte delle PG fungine. La pectin metil esterificazione è controllata attraverso l'interazione tra la pectin metil esterasi (PME) e i suoi inibitori proteici (PMEI). Le proteine PGIP e PMEI svolgono u ruolo importante nel prevenire l'azine delle PG sulle pectine. I cereali contengono inibitori di xilanasi (XI) che inibiscono le xilanasi microbiche appartenenti alle famiglie 10 e 11 delle glicoside idrolasi. Le Endo β-1,4-xilanasi (xilanasi; EC 3.2.1.8) sono enzimi chiave nella degradazione degli arabinoxilani (AXs), il maggior polisaccaride non amidaceo della parete cellulare del frumento.

L'obiettivo principale di questo studio è stato quello di piramidare geni inibitori di glicosidasi (PGIP, PMEI e XI) e valutare il loro effetto combinato sulla resistenza del frumento ai patogeni fungini. Per raggiungere questo obiettivo è stato effettuato un trasferimento multiplo di transgeni (multi-transgenes-stacking) PGIP, PMEI e XI in frumento utilizzando due strategie: la co-trasformazine con plasmidi multipli e l'incrocio tradizionale.

Nell'approccio di co-trasformazione sono stati utilizzati quattro plasmidi contenenti i geni *Pvpgip, Acpmei, Taxi-III* e *bar* per trasformare mediante il metodo biolistico il frumento duro cv Svevo.

Sono state ottenute in tutto sedici piante T_0 di cui sette piante hanno mostrato la presenza di tutti e quatto i transgeni con una frequenza di co-trasformazione del 58%. Quindi sono stati raccolti i semi T_1 e sono stati utilizzati per effettuare l'analisi della segregazione Tutti e quattro i transgeni co-segregano con un rapporto di 3:1, indicando che tutti e quattro i geni sono strettamente associati in tutte e sette le linee. Esperimenti simili di co-trasformazione sono stati effettuati in frumento tenero (*T. avesticum* cv. Bobwhite) utilizzando quattro plasmidi contenenti i geni *PvPGIP2, AcPMEI Xip-III* e *bar.* Sono state ottenute in totale diciotto piante T_0, di queste cinque piante hanno mostrato la presenza di tutti e quattro i transgeni, ciò rappresenta una frequenza di co-trasformazione per i quattro transgeni del 27.77 % .

Quindi sono stati raccolti i semi T_1 ed è stata condotta l'analisi della segregazione. Tutti e quattro i transgeni segregano con una rapporto di 3:1, indicando che tutti e quattro sono strettamente associati in tutte le linee. L'analisi della segregazione in entrambi il grano duro e tenero ha mostrato che circa il 70% della progenie conteneva tutti e tre i transgeni di interesse e che essi erano inoltre strettamente associati.

Nell'approccio che ha previsto l' incrocio tradizionale, sono state incrociate le linee parentali di frumento duro cv Svevo, ognuna esprimente un singolo inibitore di glicoside idrolasi. Per prima cosa sono state ottenute piante contenenti PvPGIP2 e AcPMEI,quindi queste piante sono state incrociate con una linea esprimente TAXI-III. Nella progenie F_1 sono state ottenute in totale diciannove piante di cui due contenenti tutti e tre i transgeni. Di queste due piante, una (M011-1-5) ha mostrato un alto livello di espressione di PGIP e TAXI III con presenza di attività inibitoria ma non è stata rilevata alcuna attività inibitoria da parte di PMEI, probabilmente a causa di un evento di silenziamento. L'altra pianta (M011-1-4) ha mostrato un alto livello di espressione di tutti e tre i transgeni e la presenza della corrispondente attività inibitoria. L'analisi di segregazione della progenie F_2 della pianta M011-1-4 ha mostrato che il 10% della progenie segregante conteneva tutti e tre i transgeni di interesse.

Ciò conferma che nel nostro caso l'approccio della co-trasformazione si è rivelato migliore per l'inserimento di transgeni multipli ,con il 70% della progenie contenente i tre transgeni di interesse e anche strettamente associati.; al contrario nel metodo che ha previsto l'incrocio solo il 10% della progenie segregante mostrava i tre transgeni.

Le linee di frumento duro contenenti i transgeni PvPGIP2, AcPMEI e TAXI-III strettamente associati (linee MJ56-5, MJ56-16a e MJ56-16b) sono state analizzate per l'espressione dei transgeni e per l'attività inibitoria. Tutte e tre le linee hanno mostrato un alto livello di espressione di PMEI e TAXI-III con attività inibitoria, ma per PGIP è stata osservata una espressione molto bassa senza attività inibitoria, probabilmente causata da eventi di silenziamento del transgene. Le generazioni T_2 di queste tre linee sono state usate per analizzare la risposta di resistenza all'infezione di *B. sorokiniana* e hanno mostrato una riduzione dei sintomi della malattia in foglia del 70%. Questo livello di protezione è più alto di quello rilevato in piante di frumento duro sovraesprimenti AcPMEI(55% circa), riportato da Volpi et al. (2011); ciò è probabilmente dovuto alla co-presenza di TAXI-III, comunque non può essere esclusa la possibilità che un più alto livello di attività PMEI in queste piante transgeniche sia responsabile di una maggiore protezione.

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1. Introduction:

1.1 The wheat:

Wheat is a dominant crop in the temperate countries used for human food and livestock feed. It is grown on more land area than any other cash crop. The world wheat production was about 676 million of tonnes (Mt) in 2011 (Table 1.1). The major wheat producing countries are China (143 Mt) followed by India (81.5 Mt), United States of America (56.6 Mt), Russian Federation (55.0 Mt), Canada (25 Mt), Australia (24.0 Mt), Pakistan (24.0 Mt), Ukraine (21.0 Mt), Turkey (19.8), Kazakhstan (15.6), Iran (13.2), Argentina (13.2 Mt), Egypt (8.6), and Uzbekistan (6.6 Mt). It is a major diet component in most of the countries because of its agronomic adaptability, high protein content , starch content facilitating digestion, easier grain storage and c grain to flour conversion for making it edible, palatable. Its high content of starch (60–70% of the whole grain and 65–75% of white flour) with 8-15% of protein, minerals and vitamins made it an important source of nutrition. When wheat based food is accomplished with other foods that supply amino acids like lysine and methionine (legume or meat protein), it becomes a highly nutritive diet. In addition, wheat is used as source of animal feed, adhesives, paper additives and several other products making it economically important. In recent years wheat is contributing for biofuel production also.

Table: 1.1 Top wheat producing countries in the world (FAO., 2011. www.fao.org)

	Average 2008-10	2009	2010 estimate	2011 forecast
EU	141.8	138.5	136.5	142.0
China (Mainland)	114.2	115.1	115.1	113.0
India	80.0	80.7	80.8	81.5
United States	62.8	60.4	60.1	56.6
Russian Federation	55.7	61.7	41.5	55.0
Canada	26.2	26.8	23.2	25.0
Australia	23.2	21.9	26.3	24.0
Pakistan	22.8	24.0	23.3	24.0
Ukraine	20.7	20.8	17.2	21.0
Turkey	19.3	20.6	19.5	19.8
Kazakhstan	14.3	17.0	10.0	15.6
Iran Islamic Rep. of	12.4	13.0	14.5	13.2
Argentina	10.4	8.8	14.0	13.5
Egypt	8.3	8.5	8.5	8.6
Uzbekistan	6.5	6.6	6.7	6.6
World	**674.4**	**684.5**	**653.7**	**676.0**

1.2 Origin and phylogeny of wheat:

The first cultivation of wheat occurred at about 10,000 years ago. The genetic base of wheat can be classified as diploid (AA), tetraploid (AABB) and hexaploid (AABBDD) with a combination of three different types of genome A, B and D.

The diploid wheat is composed of two sets of seven chromosomes from genome A (2n=14). Example: Einkorn wheat (*Triticum monococcum* AA, 2n=14).

The tetraploid wheat (e.g. emmer and durum wheat) is derived from wild emmer, *T. dicoccoides*. The wild emmer is the result of hybridization between two diploid wild grasses, *T. urartu* and an unknown wild grass. The unknown grass had never been identified among the wild grasses that are surviving now. But the closest relative is *Aegilops speltoides* (Liu et al. 2003). The hybridization with wild emmer (AABB, 2n=28) occurred long before domestication and was driven by natural selection.

Hexaploid wheat (AABBDD) originated from the hybridization between domesticated emmer or durum wheat (AABB) and wild diploid grass *Triticum tauschii* (DD). Example: spelt wheat and bread wheat, they have three sets of paired chromosomes (AABBDD, 2n=42).

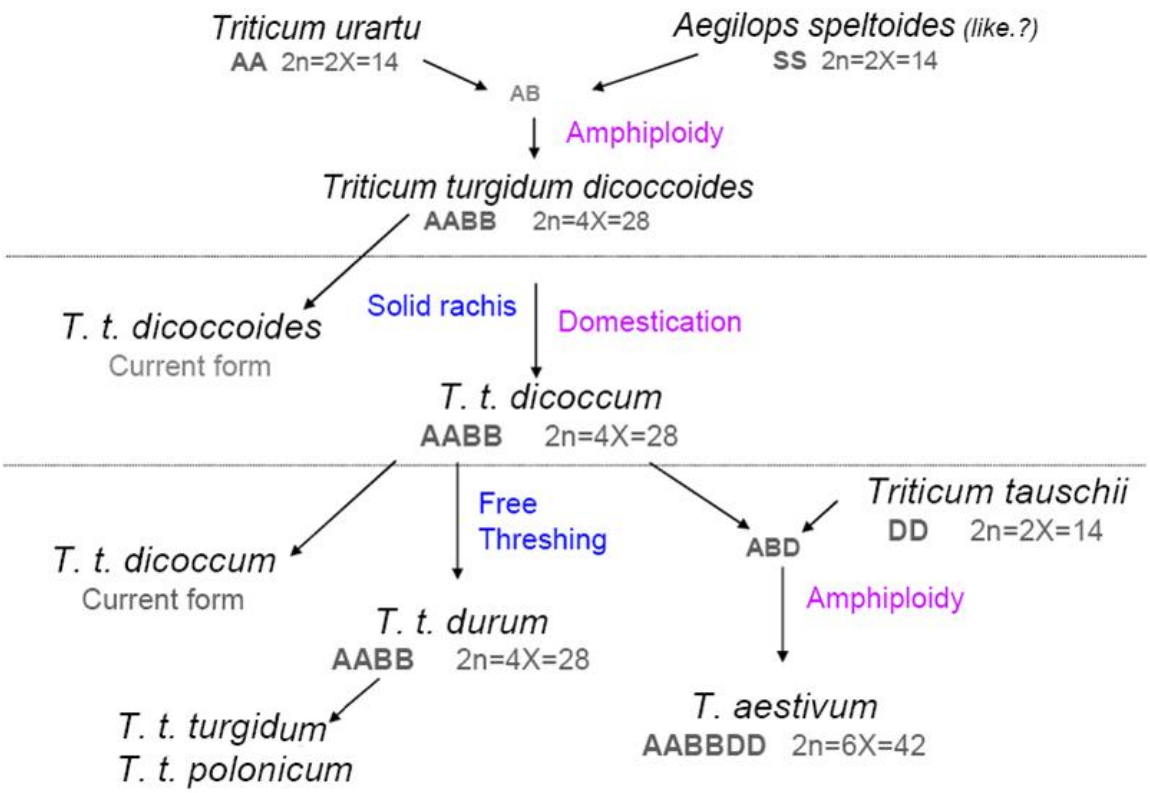

Fig. 1.1 Genetic base of wheat

1.3 Plant immune system for against pathogens:

Plant disease is defined as any physiological abnormality or significant disruption in the "normal" health of a plant. It can be caused by biotic agents including fungi and bacteria or by abiotic factors such as nutrient deficiency, drought, temperature, ultraviolet radiation or pollution. Bacteria, fungi, protists, insects and vertebrates attack plants for their nutritional requirements. Although there is lack of an immune system as in animals, plants have developed a stunning array of structural, chemical and protein-based to prevent invading organisms from causing extensive damage. Plants have developed a wide variety of constitutive (basal) and inducible defence mechanisms.

Among constitutive defence, plants possesses barriers such as cell walls, waxy epidermal cuticles, and bark. These substances not only protect the plants from invasion but also provide strength and rigidity and to become fortified against attack (Ferreira et al., 2007). Basal resistance can be triggered when plant cells recognize microbe-associated molecular patterns (MAMPs) including specific proteins, lipopolysaccharides and cell wall components commonly found in microbes. In inducible defence mechanism, plant cells detect invading pathogens and counteract through the production of toxic chemicals, pathogen-degrading enzymes, inhibitors of hydrolytic enzymes (Lawrence et al., 2000) and deliberate cell suicide (Coll et al., 2011). The activation of inducible defence mechanism responses consists of three phases: recognition, signal transmission and induction. After the recognition event, active defence mechanisms mainly involve Hypersensitive Response (HR). The HR is characterized by deliberate plant cell suicide at the site of infection. In HR, plant limits the access of water and nutrients to the pathogen by sacrificing a few cells in order to save the rest of the plant. HR is typically more pathogen-specific than basal resistance and is often triggered when the gene products in the plant cell recognizes the presence of specific disease-causing effector molecules introduced by the pathogens. Bacteria, fungi, viruses, and microscopic worms called nematodes are capable of inducing HR in plants. Once the hypersensitive response has been triggered, plant tissues may become highly resistant to a broad range of pathogens for an extended period of time. This phenomenon is called systemic acquired resistance (SAR) and represents a heightened state of readiness in which plant resources are mobilized in case of further attack. (Jones and Dang 2006).

1.4 Plant cell wall structure and composition:

Cell walls, waxy epidermal cuticles, and bark participate in basal defence since they form barriers to pathogen entry. Plant cell wall is the key determinant in providing strength, flexibility, texture and overall shape of higher plants (Carpita and Gibeaut, 1993). It is a complex, diverse and

dynamic structure which is involved in changing the overall processes of cell division, growth and differentiation.

The composition of cell wall varies on the basis of growth phase, cell type and cell position. (Carpita et al., 2001). There are two general types of primary plant cell wall based on the relative amounts of pectic polysaccharides and the structure and amount of hemicellulosic polysaccharides (Carpita and Gibeaut, 1993). Type I cell wall, which are found in all dicotyledons and the non graminaceous monocotyledons, typically contain 20–25% xyloglucan and/or 5–10% glucomannan and 20–35% pectin (Carpita and Gibeaut, 1993; Vogel, 2008). Type II cell walls, which is present in graminaceous monocotyledons are rich in (glucurono-) arabinoxylans [(G)AXs, 20–40%] and mixed linkage glucans (10–30%) but contain about 5% pectin (Carpita and Gibeaut, 1993; Carpita, 1996; Vogel, 2008). The cell wall is organized in a primary and secondary cell wall structure.

The primary cell wall structure is organized into a network with the cellulose microfibrils, the cross-linking glycans increasing the tensile strength of the cellulose, and the co-extensive network of pectins that provides the cell wall with the ability to resist compression. Some proteins in the cell wall are thought to increase the mechanical strength and part of them consists of enzymes, which initiate reactions that form, remodel, or breakdown the structural networks of the wall. Such changes in the cell wall directed by enzymes are particularly important for fruit to ripen and leaves to fall in autumn. The secondary cell wall is deposited inside the primary cell wall as a cell matures, sometimes has a composition nearly identical to that of the earlier-developed primary wall. In addition, lignin provides strength to the structure of the secondary wall. Lignin also makes plant cell walls less vulnerable to attack by fungi or bacteria.

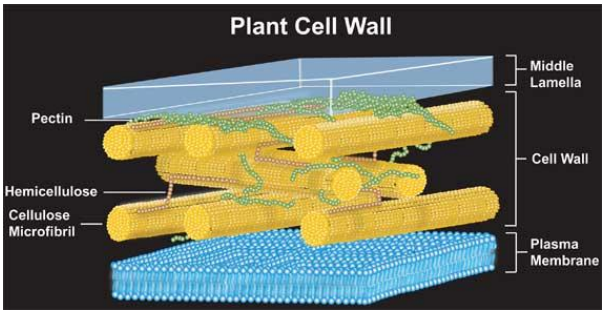

Fig. 1.2 Struture of plant cell wall (Carpita et al., 2000)

1.5 Pectins:

Pectin is one of the major components of primary cell wall present in the middle lamellae between primary cell walls where it functions in regulating intercellular adhesion. 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). Pectin of higher plants forms the structurally most complex family of polysaccharides in nature. Pectinaceous polysaccharides can be distinguished as five classes: homogalacturonan (HG), xylogalacturonan (XGA), apiogalacturonan (AP), and rhamnogalacturonan I (RG-I) and II (RGII).

All of these polysaccharides are built on a HG backbone except RG-I which is built by 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 subunits (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. The distribution of methyl group on HG is not uniform and the different patterns can be detected by specific antibodies (Willats et al., 2001). A stretch of a minimum of nine un-methyl-esterified galacturonic acid residues should be present to form Ca^{2+} linkages promoting the formation of the 'egg-box' or "junction zones" (Liners et al., 1989).

In these structures, carboxyl group of the two GalA residues belonging to different HG chains form a negatively charged pocket that can accommodate a Ca^{2+} cation. The egg-box can induce gel formation and strengthens 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 XGA (Le Goff et al., 2001). 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 α-1→4 linked and is substituted by 4 different side chains consisting of a variety of different sugars residues 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 dimerized by means of borate ester links through apiosyl residues in 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 various domains can vary considerably and the sugar composition of RG-I is highly heterogeneous, RG-II appears to have a highly conserved structure.

The structure of pectin is because HG as backbone or side chains will not contribute in the same way as the 3-D structure of the pectin matrix and will consequently affect cell wall structure and properties differently (Wolf et al., 2009). Pectins are synthesized in the *cis* Golgi, methylesterified in the medial Golgi and substituted with side chains in the *trans* Golgi cisternae before being secreted into the wall as highly methylesterified (70-80%) forms (Goldberg et al., 1996). Subsequently, pectins can be modified by pectinases such as pectin methylesterases (PMEs) which catalyse the demethylesterification of homogalacturonans in a developmental regulated manner and release of acidic pectins and methanol.

1.6 Arabinoxylans:

Arabinoxylans (AX) consists of a main backbone of β-(1, 4)-linked D-xylopyranosyl units, mainly substituted with α-L-arabinofuranosyl residues at O-2 and/or O-3 position. The degree of substitution of an AX molecule is expressed as its arabinose to xylose ratio (A/X-ratio). Phenolic acids, such as ferulic or p-coumaric acids, can be ester-linked to these arabinose units at O-5 position (Fig. 1.3).

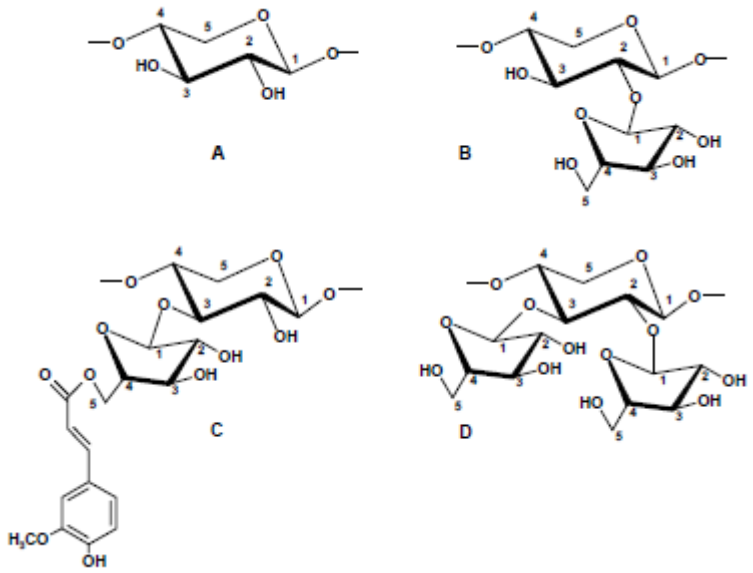

Figure 1.3: Structural elements of arabinoxylan. (A) unsubstituted D-xylopyranosyl residue; (B) D-xylopyranosyl residue substituted at O-2 with an L-arabinofuranosyl residue; (C) D-xylopyranosyl residue substituted at O-3 with an L-arabinofuranosyl residue and showing the linkage pattern of ferulic acid to O-5 of an L-arabinofuranosyl residue; (D) D-xylopyranosyl residue substituted at O-2 and O-3 with L-arabinofuranosyl residues (Courtin and Delcour, 2002).

1.7 Cell wall degrading enzymes and their inhibitors:

Plant-saprophytic and plant-pathogenic microorganisms use a large arsenal of cell wall degrading enzymes (CWDEs) to break the barrier in order to invade plants or to feed on the released nutrients

(Walton, 1994; Williamson et al., 1998; ten Have et al., 2002). Efficient cell wall degradation requires a concerted action of several CWDEs.

1.7.1 Pectic enzymes and Polygalacturonases (PGs):

Pectin degrading enzymes are among the first CWDE produced by pathogens during plant infection, accounting for the rapid and extensive degradation of cell wall (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 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 infection of *Colletotrichum gloeosporioides* (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 (PG) and pectate lyase (Fries et al., 2007). PGs are the most extensively studied pectinases. They are distinguished into 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. PGs 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 further degradation of the cell wall by other degrading enzymes (De Lorenzo et al., 2001). The importance of PG in pathogenesis had been demonstrated for several fungi such as *Botrytis cinerea, Aspergillus flavus, Alternaria citri, Claviceps purpurea* and *Sclerotinia sclerotiorum* (Shieh et al., 1997; Li et al., 2004). The degradation of pectin by PGs not only loosens the primary cell wall allowing the cellulose-hemicellulose network to become more accessible for digestion by others CWDEs but also leads to the release of cell wall pectin-derived oligosaccharides, oligogalacturonides (OGs), which can act as elicitors of a variety of plant defences (Cervone et al., 1989; D'Ovidio et al., 2004a).

1.7.2 Polygalacturonase inhibiting protein (PGIP):

Many plants possess a cell wall glycoprotein, the polygalacturonase inhibiting protein (PGIP), which is able to inhibit fungal endo-PGs. The interaction between fungal PGs and plant PGIP

favors the accumulation of oligogalacturonides (OGs), which elicit a wide range of responses (Cervone et al., 1997; Ridley et al., 2001). PGIP belong to the subclass of proteins containing leucine-rich repeats (LRRs) of the extracytoplasmic type (Jones and Jones 1997). They typically contain 10 imperfect LRRs of 24 residues each, which are organized to form two α-sheets, one of which (sheet β1) occupies the concave inner side of the molecule and contains residues crucial for endo-PG recognition (Di Matteo et al., 2003; D'Ovidio et al., 2004; Leckie et al., 1999).

Biochemical and molecular analyses both revealed the existence of a small PGIP-encoding gene family in several plant species and that members of the same PGIP family can possess distinct PG-inhibiting activities (De Lorenzo et al. 2001). These analyses also indicate that the bean PvPGIP2 is one of the most effective inhibitors of fungal PGs (D'Ovidio et al. 2004). The effectiveness of PGIP in limiting host tissue colonization has been shown in tomato, *Arabidopsis*, tobacco, and grape. In these dicot plant species, the overexpression of PGIP reduces the symptoms caused by the infection of *B. cinerea* (Aguero et al. 2005; Ferrari et al. 2003; Joubert et al. 2006; Manfredini et al. 2006; Powell et al. 2000). The demonstration that PG is a pathogenicity factor for *Claviceps purpurea* during the infection of rye (Oeser et al.2002) has reinforced interest in understanding the role of PGIP in monocots. Kumar and associates (2002) demonstrated the involvement in leaf penetration of the endo-PG produced in vitro by the cereal pathogen *Bipolaris sorokiniana* (Sacc.) and similar work was performed by Clay et al. (1997) in *Cochliobolus sativus*, the causal agent of foliar spot blotch, root rot and black point of grains. The effectiveness of PGIP in limiting host tissue colonization has been demonstrated not only in dicot plants but also in monocot plants, transgenic wheat plants expressing PvPGIP2 showed a significant reduction of symptoms following the infection of *Bipolaris sorokiniana* (Janni et al., 2008) or *Fusarium graminearum* (Farrari et al. 2012) 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.

1.7.3 Pectin methyl esterase (PME):

Homogalacturonan is highly methyl-esterified when exported into cell walls and is subsequently de-esterified by the action of Pectin methyl esterase (PME's). PME catalyzes the hydrolysis of methylester groups of cell wall pectins. This yields the substrates for depolymerising enzymes and methanol. PME is a ubiquitous cell-wall-associated enzyme that is presents in several isoforms and facilitates plant cell wall modification and subsequent breakdown. It is found in bacteria and fungi as well as in all higher plants. In addition to the role of microbial PMEs in pathogenesis as CWDEs, also plant PME activity can be is involved in pathogen infection. PME activity is necessary for complete degradation of pectin by PGs and PLs since these enzymes are unable to cleave highly

methyl-esterified pectin. The functional PME from the necrotrophic *Botrytis cinerea* had been shown to be an important determinant of its pathogenicity (Valette-Collet et al., 2003). It has been also provided that the disruption of *pmea* gene in the bacterium *Erwinia chrysantemi* reduces its virulence on *Saintpaulia ionanta* (Boccara 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). AtPME3 is also induced in Arabidopsis following infection with *Pectobacterium carotovorum* and *Botritis cinerea* and its silencing improve resistance to these pathogens (Raiola et al., 2011). This phenomenon has been also observed after the herbivore attack. Some reports highlighted the up-regulation of Plant PME mRNA and protein levels (Divol et al., 2005; von Dahl et al., 2006), suggesting that PME contribute indirectly by affecting the cell wall properties and induces anti-herbivore responses (Korner et al., 2009).

Plant PME acts also as a host receptor for the tobacco mosaic virus (TMV) in binding the movement proteins (MPs) of some viruses required for their dispersal to new cells via plasmodesmata (Lewis et al., 2009). Indeed, Arabidopsis microarray database revealed that the expression levels of 75% 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).

1.7.4 Role of pectin methyl esterase inhibitor:

The activity of PME is regulated at the transcriptional and post-transcriptional level. In this latter case, PME is tightly regulated by its endogenous inhibitor protein called pectin methyl esterase inhibitor (PMEI) (Giovane et al., 2004; Peaucelle et al., 2008). PMEI is active against plant PME by forming a stoichiometric 1:1 complex (Fig 1.4) in which the inhibitor binds the putative active site of the enzyme (Di Matteo et al., 2005). The effectiveness of PMEI in controlling PME activity had been demonstrated by the over expression of AtPMEI-1 or AtPMEI-2 in transgenic *Arabidopsis* plants. Both proteins interacted with endogenous PME isoforms and transgenic tissues showed a significant reduction of PME activity (Lionetti et al., 2007). These transgenic plants also showed an increased level of cell wall pectin methyl esterification and a significant reduction of symptoms caused by *Botrytis cinerea* (Lionetti et al., 2007). Similarly, Volpi et al., (2011) demonstrated that the ectopic expression of a AcPMEI from kiwi can modify the degree of methylesterification (DM) and the transgenic plants showed a significant reduction of disease symptom caused by *B. sorokiniana* and *F. graminearum*. Grass species contain a low level of pectin in their cell wall (Vogel 2008), however, 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). In particular, there is a high random distribution of methyl ester had been detected in near-isogenic resistant lines compared to a more block wise distribution found in the susceptible lines (Wietholter et al., 2003).

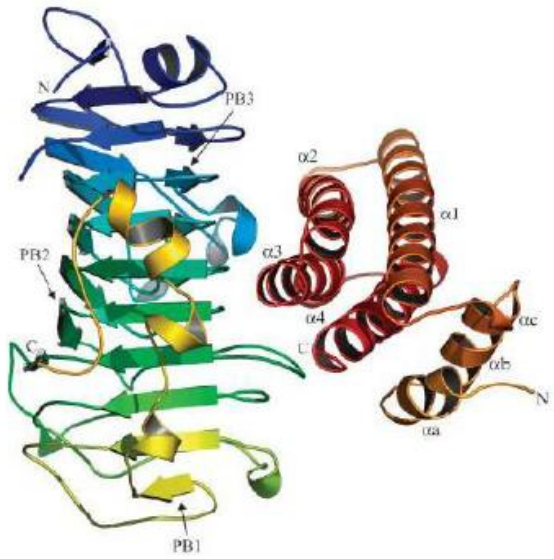

Fig. 1.4 Ribbon representation of the PME-PMEI complex. The enzyme (PME-1 from tomato) is shown in green-blue on the left side. The inhibitor (AcPMEI) is represented in yellow-red on the right side; the α-helices of the four-helix bundle are indicated as α1 to α4, whereas helices of the N-terminal region are named αa, αb, and αc (Di Matteo et al., 2005).

1.7.5 Xylanase:

Endo-*β*-(1, 4)-xylanases (EC 3.2.1.8. xylanases) cleaving internal *β*-(1, 4)-linkages between xylose units in AXs are believed to be an important part of the offensive arsenal of microorganisms necessary to penetrate and colonize the plant tissues. *Fusarium graminearum* expresses a number of differentially regulated xylanase genes during *Fusarium* head blight disease progression in wheat (Champeil et al., 2004, Hatsch et al., 2006). *Claviceps purpurea* also expresses two xylanase genes during infection. While deletion of *CpXyl1* had no significant effect on virulence (Giesbert et al., 1998), deletion of*CpXyl2* significantly retarded plant infection (ten Have et al., 2002). Disruption of xylanase gene *BcXynA* had a pronounced effect on virulence of *Botrytis cinerea* on tomato leaves and grape berries (Brito et al., 2006). However, targeted disruption of xylanase genes had been carried out without any significant conclusion on their pathogenic importance from *Cochliobolus carbonum* (Apel-Birkhold and Walton, 1996), *Fusarium oxysporum* (Gomez-Gomez et al., 2002) and *Magnaporthe grisea* (Wu et al., 2006).

1.7.6 Role of Xylanase inhibitors (XIs):

The cell wall contains Xylanase inhibitors (XIs) that are protein components which inhibit microbial xylanases of glycoside hydrolase families 10 and 11. The role of the xylanase inhibitors (XI) in plant defence has been studied less intensely than that of PGIP, however, several observations suggest that these inhibitors are important plant defence components. In particular, XIs are effective against microbial xylanases and not against the endogenous ones, share a significant sequence similarity with pathogenesis–related proteins (PR). They are localized in the apoplastic region and induce following pathogen infection (Dornez et al., 2010). In wheat, there are three classes of XIs: *Triticum aestivum* xylanase inhibitor (TAXI), xylanase inhibiting protein (XIP) and thaumatin-like xylanase inhibitor (TLXI).

Triticum aestivum xylanase inhibitor (TAXI):

TAXI-type XIs are widely represented in cereals. In general, they are characterized by molecular masses (MMs) of about 40 kDa and alkaline pI values (> 8.0) (Goesaert et al., 2004). TAXI-type XIs exist as two molecular forms. The first consists of a single polypeptide chain. The second is derived from the former and is made up of two polypeptides of approximately 30 and 10 kDa, held together by a disulfide bridge (Debyser and Delcour, 1998; Debyser et al., 1999). TAXI-type proteins are only slightly glycosylated (Sansen et al., 2004b; Croes et al., 2008). Genomic and transcriptomic information identified different TAXI-type XI gene sequences in common wheat and other cereals. The sequences of TAXI-III and TAXI-IV almost perfectly correspond to those of TAXI-Ib and TAXI-IIb respectively and were referred to as TAXI-Ib/III and TAXI-IIb/IV. Indeed, it can be stated that TAXI-III is an isoform of TAXI-IB while TAXI-IV is an isoform of TAXI-IIB (Raedschelders et al., 2005). TAXI-type XIs most likely also occur as multigene families in other cereals (Goesaert et al., 2003a; Raedschelders et al., 2004). TAXI-type XIs specifically inhibit bacterial and fungal xylanases belonging to glycoside hydrolase family (GH) 11. As they do not inhibit GH10 xylanases, all plant endogenous xylanases identified so far, all belonging to this family (Simpson et al., 2003), are not inhibited by TAXI-type XIs. TAXI-type XIs cover the xylanase cleft (Fig 1.5A) in which the active site is located, resulting in competitive inhibition (Pollet et al., 2009) Mutations on the surface of xylanases in the vicinity of the active site cleft have a large effect on the interaction with TAXI (Tahir et al., 2004). Weng et al. (2010) has been demonstrated that Xylanase inhibitor *TAXI-I* gene expression was drastically induced by methyl jasmonate (MeJa) treatment. It suggested that TAXI-I might be involved in plant against fungal and bacteria xylanases.

Xylanase inhibiting protein (XIP):

XIP-type XIs are present in seeds of different monocotyledons, although in variable levels and occurs as monomeric proteins with MMs of approximately 30 kDa and pI values varying between 5.5 (rice) and > 9.3 (maize) (Goesaert, et al., 2002; Goesaert et al., 2003b; Goesaert et al., 2005). XIP-type XIs possess two N-glycosylation sites and are approximately 2% (w/w) glycosylated (Payan et al., 2003). In common wheat, several *Xip* gene sequences have been identified. The presence of XIP-I, XIP-III and XIP-R2 in wheat has been demonstrated (Croes et al., 2009a, Igawa et al., 2005). So far, XI activity has been demonstrated only for XIP-I, XipII (Elliot et al 2009) and XIP-R1. The specificity of XIP-R1 to some extent differs from the specificity of XIP-I as XIP-R1 inhibits GH11 xylanases of *Thermomyces lanuginosus*, *Hypocrea jecorina*, *Trichoderma viride* and *Aureobasidium pullulans* but does not affect the activity of the GH11 *A. niger* xylanase (Takahashi-Ando et al., 2007). XIP-type XIs possess a $(\beta / \alpha)_8$ barrel fold, the top of which is decorated by loops arranged to form a long depression running along one side of the molecule (Payan et al., 2003). They have structural features and N-terminal amino acid sequences typical for GH18 chitinases. However, detailed structural analysis of XIP-I revealed some important differences, explaining the lack of activity towards chitin (McLauchlan et al., 1999; Payan et al., 2003). They inhibit fungal but not bacterial GH10 and GH11 xylanases (Flatman et al., 2002), although several fungal xylanases insensitive to XIP have been reported. Sequence alignments and structure comparisons of several GH10 and GH11 xylanases indicated that XIP-type XIs indeed do not display an absolute specificity for fungal xylanases, but rather target GH10 and GH11 xylanases that lack extended insertions, which cause steric clashes with the inhibitor (Payan et al., 2004). The molecular structures of XIP-I in complex with xylanases provide the structural basis for xylanase recognition by XIP-I (Payan et al., 2004). Remarkably, XIP-I binds GH10 and GH11 xylanases at distinct locations, possibly allowing simultaneous binding of the inhibitor to both target enzymes (Fig. 1.5 B).

Thaumatin-like xylanase inhibitor:

TLXI-type XIs are present in common wheat (Fierens et al., 2007). TLXI-type XIs have a MW of approximately 18 kDa and a pI value of at least 9.3 (Fierens et al., 2007). TLXI contains five disulfide bridges, resulting in a very stable protein that withstands extreme pH and temperature conditions (Fierens et al., 2009). TLXI shows that it consists of two domains. The first domain, domain I, consists of a β-sandwich built up of two β-sheets comprising five (A1 to A5) and six (B1 to B6) β-strands. Apart from the N-terminal (A1) and the C-terminal (A5) strand, all these β-strands run antiparallel within each β-sheet. The second domain is formed by a β-hairpin turn followed by

an extended loop and corresponds to domain III of thaumatin (Vandermarliere et al., 2010). The α-helices of domain II, present in thaumatin, are absent in TLXI (Vandermarliere et al., 2010). Alignment of the amino acid sequence of TLXI with that of thaumatin and with those of other short-chain of Thaumatin-Like Protein (TLPs) indeed shows that TLXI, like other short-chain TLPs with a MMs between 15 and 19 kDa, lacks about 50 amino acids, corresponding to domain II of thaumatin (Fierens et al., 2009). TLXI specifically inhibit bacterial and fungal xylanases belonging to GH11 and not GH10 xylanases. TLXI-type XIs inhibit xylanases in a non-competitive manner, *i.e.* by binding outside the active site (Fig. 1.5C). His22TLXI, located at a flexible loop, is a key residue for inhibition activity (Rombouts et al., 2009).

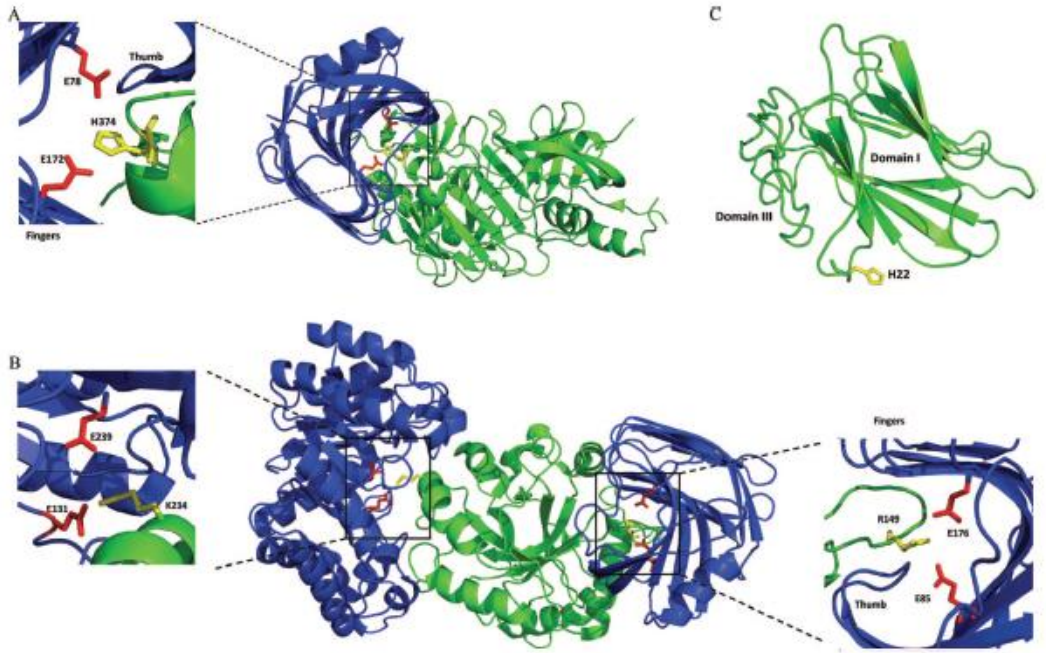

Fig. 1.5. Overall structure of (**A**) TAXI-IA in complex with GH11 *Bacillus subtilis* XynA (PDB 2B42), (**B**) putative three protein complex of XIP with GH10 *Aspergillus nidulans* XlnC (PDB 1TA3) and GH11 *Penicillium funiculosum* XynC (PDB 1TE1) and (**C**) TLXI (PDB 3G7M). Xylanases are represented in blue and their catalytic residues are shown in red. XIs are represented in green and key residues for inhibition are shown in yellow. All structures were generated with the PyMOL (DeLano, 2002) program (Dornez et al., 2010)

1.8 Multi-transgene-stacking in plants:

The experiments reported to date on genetic engineering of plants to enhance their resistance to pathogens mostly involve the manipulation of a single gene. However, many important traits like complex metabolic pathways depend on interactions between a number of genes and so genetic engineering must be now advanced to manipulation of polygenic traits, multiple traits or multiple gene products. A number of approaches had been used to introduce multiple genes into plant genomes and then coordinate them into transgene expression (Halpin et al., 2001). These approaches include sexual crossing between plants carrying separate transgenes (Ma et al., 1995; Bizily et al., 2000), sequential re-transformation (Lapierre et al., 1999), co-transformation with

multiple plasmids (Chen et al., 1998; Ye et al., 2000) or with single plasmids on which several transgenes are linked. The various strategies that had been employed for the transfer of two or more genes to target plants are briefly reviewed as follows.

1.8.1 Crossing between plants with different transgenes:

Two or more transgenes can be sequentially introduced into a plant by conventional breeding procedures, e.g. a plant containing one transgene is crossed with individuals harbouring other transgenes. This approach has been followed at the research level to combine existing transgenic traits. For example, crossing plants expressing different *Bacillus thuringiensis* (Bt) toxins can provide an effective way of delaying the emergence of Bt-resistant pests as recently illustrated in broccoli. Pyramided *cry1Ac* and *cry1C* Bt genes controlled the diamond back moths resistant to either single protein (Cao et al., 2002; Zhao et al., 2003). Similarly, disease- and pest-resistant rice had been developed by crossing plants expressing the *Xa21* gene (resistance to bacterial blight) with plants expressing both Bt fusion gene and chitinase gene (Datta et al., 2002). The crossing had also been used to introduce new biochemical pathways in plants. Two genes for a bacterial organic mercury detoxification pathway (mercuric reductase, *merA* and organomercurial lyase, *merB)* were combined by crossing in *Arabidopsis* and plants expressing both genes were able to grow on 50-fold higher methylmercury concentrations than wild-type plants (Bizily et al., 2000). Biodegradable polymer Polyhyroxybutyrate (PHB) had been produced in plants by incorporating three genes coding for a biosynthetic pathway from the bacterium *Alcaligenes eutrophus* in *Arabidopsis* by serial crossing (James et al., 2003). Transgenic tobacco plants, individually harbouring rice chitinase or alfalfa glucanase genes were crossed and the hybrids with both the genes showed 75% reduction in the number of lesions produced by *Cercospora nicotianae* (Zhu, 1994). Expression of *chitinase* and *β-1, 3-glucanase* genes in carrot reduced the disease severity caused by *Alternaria dauci, A. radicina, C. carotae* and *Erysiphe heraclei* (Melchers and Stuiver, 2000). A successful combination of chitinase and glucanase transgenes in pea line via crossing was achieved (Selatsa et al., 2008). Limitation of crossing is segregation in the subsequent generations and larger progeny population need to be maintained and screened. There is only one report in bread wheat by León and associates (2010). They performed crossing between the seprate plants containing three transgenes genes of HMW-GS and showed improved mixing properties and dough functionality.

1.8.2 Sequential transformation:

Re-transformation is also proven to be one of the viable strategies. Flower colour had been modified in *Forsythia* by inducing anthocyanin synthesis through sequential transformation with the genes for dihydroflavonol 4-reductase from *Antirrhinum majus* (*AmDFR*) and anthocyanidin synthase from *Matthiola incana* (*MiANS*). The double transformants displayed a novel bronze-orange petal colour caused by the *de novo* accumulation of cyanidin-derived anthocyanins over the carotenoid yellow background of the wild-type (Rosati et al., 2003). Similarly, the introduction of a two-gene glyoxalase pathway into tobacco led to enhanced salinity tolerance with the double transgenics responding better under salinity stress than plants harbouring either of the single transgene (Singla-Pareek et al., 2003). In potato, antisense inhibition of three starch synthase genes was achieved by retransforming plants that were already altered in amylopectin synthesis with the suppression of SSII and SSII by an antisense gene for granule-bound starch synthase (Jobling et al., 2002). This resulted in the production of an extremely freeze–thaw stable starch. This result could have significant environmental and consumer benefits in since it can replace the current technology where freeze– thaw-stable starch is produced by chemical modification. Recently, three genes involved in the synthesis of long-chain polyunsaturated fatty acids were sequentially introduced into *Arabidopsis* in order to illustrate the potential of transgenic plants for producing omega-3 and omega-6 fatty acids normally obtained from fish oils (Qi et al., 2004). The first and foremost limitation of the above mentioned techniques is that the transgenes introduced are not linked. They got segregated apart in the subsequent generations and larger progeny population need to be maintained and screened. The incorporation of a three-gene trait such as PHB synthesis potentially requires an eightfold increase in plant numbers (Hitz, 1999). Another problem associated with the re-transformation strategy is that it requires a variety of selectable marker genes so that a different one can be used with each sequential transformation. Although the range of potentially useful selectable markers is constantly increasing, GM crops with an accumulation of such genes are encountering significant hurdles in regulatory approval and public acceptance. Several systems have been developed that enable the removal of selectable marker genes which may help to overcome this limitation. Selectable marker gene removal systems is a slow multistep procedure that involves sexual crossing and are therefore not applicable to vegetatively propagated plant species (Hohn et al., 2001 or Hare and Chua, 2003).

1.8.3 Co-transformation:

Co-transformation is defined as the simultaneous introduction of multiple genes followed by the integration of the genes in the cell genome. The genes are either present on the same plasmid used in transformation ('single-plasmid co-transformation') or on separate plasmids ('multiple-plasmid co-transformation'). For single-plasmid co-transformation, the limitation is the difficulty to assemble complex plasmids with multiple gene cassettes. Standard transformation vectors are not optimal for such a task. The major problem is that their multiple cloning sites consists of hexa-nucleotide restriction sites which are often present within one or more of the sequences that are to be inserted in the vector. A combination of single plasmid and multiple-plasmid *A. tumefaciens*-mediated co-transformation was exploited by Ye et al., (2000) to introduce the entire β-carotene biosynthetic pathway into carotenoid-free rice endosperm. Co-transformation using Multiple-plasmid is relatively a simple method. Two or more plasmid containing genes were mixed and used in co-transformation. The success of this multi-transgene-stacking technique depends on the frequency with which the two (or more) independent transgenes are transferred and integrated into the cell genome. In a co-transformation experiment with two separate plasmids used for biolistic transformation of rice embryogenic tissues, co-transformation efficiency was correlated with the ratio at which the plasmids were mixed with respect to the selectable marker gene. In a co-transformation experiment using 14 different plasmids (one of which containing a selectable marker gene), it was found that 85% of the obtained transgenic plants contained more than two genes, 17% of the transgenic plants contained even more than nine transgenes. As many as 13 genes could be inserted into rice genome by this method (Chen et al., 1998). An average co-transformation frequency of 39% was obtained with a 1:0.7 molar ratio of each of the individual transgenes to the selectable marker gene. It is postulated that higher frequencies can be obtained by increasing the amount of target gene in relation to the selectable marker gene. All target genes integrated in the genome indicated that all genes had an equal chance of integration. Hence the nature of the coding region had no effect on the efficiency of integration. In most cases, gene copy number ranged from one to four which leads to the estimation that segments of DNA between 78 and 300 kb were integrated (Chen et al., 1998). In a biolistic co-transformation experiment on soybean embryogenic suspension cultures using to 12 different plasmids, all the 26 tested regenerated plants contained at least eight different plasmids while 73.0% of the clones contained 12 plasmids. Other workers also reported the insertion of multiple genes or fragments into a single locus (Kumpatla et al., 1997; Kohli et al. 1998, and Agrawal et al. 2005), maize (Register et al. 1994), bean (Aragao et al., 1996), and wheat (Campbell et al., 2000). Campbell et al. (2000) performed the co-transfromation by

particle bombardment in bread wheat cv. Bobwhite. They were used three types of different plasmids each containing RNase L, 2-5A synthetase gene and neomycin phosphotransferase II (NPT II) as selectable marker for transformation. On the basis of the inheritance of the two genes of interest they demonstrated that the genes were linked. Similarly, Anand et al., (2003) performed co-transformation in bread wheat cv Bobwhite using genes: *chitinase* and *β-1,3-glucanase*. They showed significant delay in spread of Fusarium head blight (FBH) disease caused by *Fusarium graminearum* under the greenhouse conditions.

1.8.4 Internal ribosome entry site for multi-transgenes- stacking:

Multi-transgenes- stacking (multiple transgenes under single promoter) is one way of overcoming the difficulties of co-ordinating the expression of different transgenes without duplicating regulatory sequences. There are currently several alternative single-transgene systems although none had been yet widely applied in plants. Chimeric polycistronic constructs that incorporate internal ribosome entry sites (IRESs) from different viruses have been tested and shown to function in plant systems (Urwin et al., 2000; Urwin et al., 2002; Dorokhov et al., 2002; Jaag et al., 2003). IRESs are specific sequences of several hundred nucleotides that can directly recruit ribosomes to internal positions within mRNAs and initiate translation in a cap-independent manner. IRES-mediated expression had also been shown to be significantly lower than normal cap-dependent expression in certain plant tissues (Urwin et al., 2002). Using this strategy, a short, two-step, biochemical pathway for mannityl opines production was introduced into tobacco cells (von Bodman et al., 1995).

1.9 Aim of work:

The aim of this study was to perform multi-transgene-stacking of three different glycosidase inhibitor genes in addition to the bar selectable marker gene in wheat to verify its feasibility, inheritance pattern and the more suitable between two different approaches. We compared co-transformation with multiple plasmids using particle bombardment and crossing between transgenic plants carrying different genes. In co-transformation, four constructs containing transgenes *Pvpgip2*, *Acpmei*, *TaxiIII*/*XipIII* and *bar* were co-bombarded into the immature embryos of durum wheat cv Svevo or bread wheat cv. Bobwhite. It was then analyzed for co-transformation frequency and their inheritance pattern. Further, these multi-transgene-stacked glycosidase inhibitor genes (*Acpmei* and *Taxi-III*) in wheat were characterized and tested against the fungal pathogen *B.sorokiniana*, a causal agent of Leaf bloch symptom in wheat.

In crossing approach, the separate plant lines carrying *Acpmei*, *Pvpgip2* and *Taxi-III* transgenes have been also crossed and analyzed for their segregation.

2. Materials and Methods:

2.1 Vectors:co-transformation by particle bombardment:

The following plasmids were used in co-transformation by particle bombardment in durum and bread wheat.

(A) pAHC17-SR2:

Polygalacturonase inhibiting protein2 (*Pvpgip2*) gene with signal peptide for apoplastic targeting (Lekie et al. 1999) was inserted into the *Bam*HI site of pAHC17. The expression cassette in pAHC17 contains maize ubiquitin *Ubi1* promoter and the NOS terminator element (Christensen et al 1992; Fig 2.1 A).

(B) pAHC17-Acpmei:

The complete coding region of pectin methylesterase inhibitors gene *Acpmei* from *Actinidia chinensis* (GenBank accession number P83326) with the signal peptide of the polygalacturonase inhibiting protein 1 (*Pvpgip*1) for the apoplastic targeting was inserted into the *Bam*HI site of pAHC17, under control of the constitutive *Ubi*1 promoter and NOS terminator (Fig 2.1 B).

(The coding region of Acpmei was kindly provided by Prof. Bellincampi, University of Rome La Sapienza, Italy)

(C) pAHC17-Taxi-III:

The complete coding region of *Taxi-III* gene was isolated from *Triticum aestivum* cv Chinese Spring and was inserted into the *Bam*HI site of pAHC17. The expression cassette is under control of the constitutive *Ubi*1 promoter and NOS terminator (Fig 2.1 C)

(D) pAHC17-XipIII:

The complete coding region of *Xip-III* gene was isolated from *T. durum* cv. Longdon and along with Flag-tag sequence was inserted into the *Bam*HI site of pAHC17. The Flag-tag sequence encodes a FLAG octapeptide that allows the identification of the XIP-III tagged protein. This tag can be also useful for expression analysis and purification by affinity chromatography of XIP-III produced in the transgenic wheat lines. The *Xip-III* gene was under control of the constitutive *Ubi*1 promoter and NOS terminator (Fig 2.1 D)

(E) pUBI:BAR:

This construct carry the *bar* gene, which confers resistance to the bialaphos herbicide. The pUBI:BAR contruct allows the selection of herbicide resistant transgenic calli and shoots during the regeneration and growth of co-tansformed embryos. The *bar* gene is under control of the constitutive *Ubi*1 promoter and NOS terminator (Fig 2.1 E).

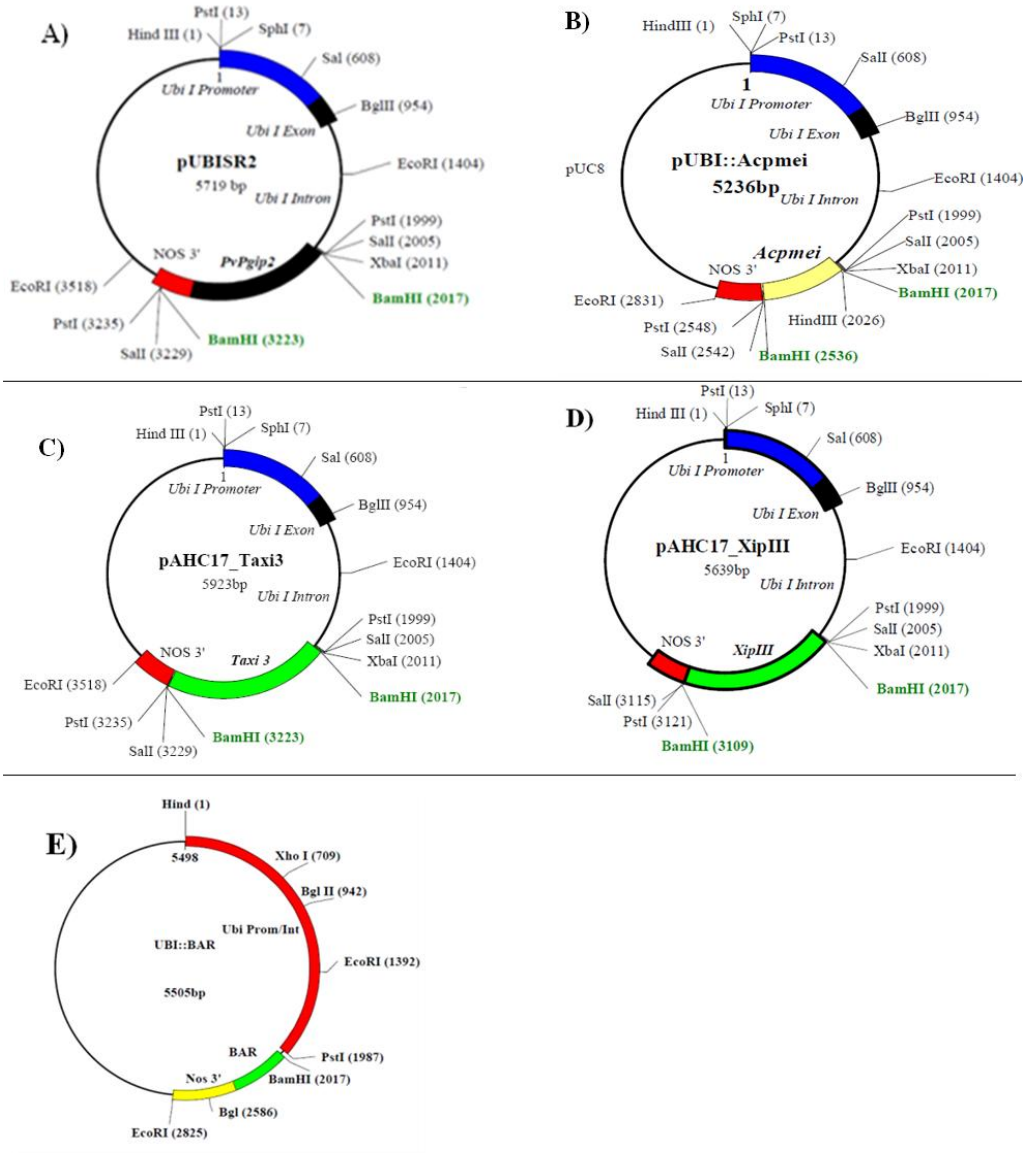

Fig. 2.1 The construct maps of plasmid containing gene (A) *Pvpgip2* (pAHC17-SR2), (B) A*cpmei* (pUBI:Acpmei), (C) *Taxi-III* (pAHC17-Taxi-III), (D) *Xip-III* (pAHC17-XipIII) and (E) *bar* (pUBI:BAR) used as selectable marker in wheat transformation. All genes are under control of *Ubi*1 promoter and NOS terminator.

2.2 Production of transgenic wheat plants:

2.2.1 Media:

Murashige and Skoog (MS) medium, at pH 5.85 (Murashige and Skoog, 1962) supplemented with Maltose, 2,4-dichlorophenoxyacetic acid (2,4-D), Thiamine-HCl, L-asparagine, was used for wheat cultures. All the culture media were solidified using Phytagel as gelling agent. After autoclaving of the medium, 2, 4-D was added and poured in 100mm x 15mm size Petri dishes except for the bombardment medium for which 60mm x 15mm size Petri dishes were used. Different media composition is described in Table 2.1 (Weeks et al. 1993).

Table 2.1 Culture media compositions.

Dissecting medium	Murashige & Skoog Salt mixture 4.3g/lt; Maltose 40g/l; 10ml/l Thiamine-HCl (25mg/500ml); 0.15g/l L-asparagine; 0.25% w/v phytagel and 2ml/500ml 2.4 D (0.5mg/ml).
Recovery medium	Murashige & Skoog Salt mixture 4.3g/lt; Maltose 40g/l; 10ml/l Thiamine-HCl (25mg/500ml); 0.15g/l L-asparagine; 0.25% w/v phytagel and 2ml/500ml 2.4 D (0.5mg/ml).
Bombardment medium	Murashige & Skoog Salt mixture 4.3g/lt; Maltose 40g/l; 10ml/l Thiamine-HCl (25mg/500ml); 0.15g/l L-asparagine; 0.25% w/v phytagel; Sucrose 171.5 g/l and 2ml/500ml 2.4 D (0.5mg/ml).
Regeneration medium	Murashige & Skoog Salt mixture 4.3g/lt; Maltose 40g/l; 10ml/l Thiamine-HCl (25mg/500ml); 0.15g/l L-asparagine; 0.25% w/v phytagel; 2ml/500ml 2.4 D (0.5mg/ml) and 1.5ml/500 ml Bialaphos (1 mg/ml).
Rooting medium	Murashige & Skoog Salt mixture 2.15g/l; Maltose 20g/l; 5ml/l Thiamine-HCl (25mg/500ml); L-asparagine 0.075g/l and 1.5 ml/500 ml Bialaphos (1mg/ml)
Early germination medium	Murashige & Skoog Salt mixture 4.3g/l; Maltose 40g/l; 10ml/l Thiamine-HCl (25mg/500ml) L-asparagine 0.15g/l and 1.5 ml/500ml Bialaphos (1 mg/ml)

2.2.2 Embryos isolation:

Triticum durum cv. Svevo and *Triticum aestivum* cv. Bobwhite plants were grown in the field. Spikes were collected of Caryopses at 10 to 18 days after post anthesis stage (Zadoks stage 72) and surface-sterilized using 70% ethanol containing 50µl/100ml of Tween 20 followed by three times washing with distilled water. Caryopses were collected and sterilized in 70% ethanol for 5 min and 20% sodium hypochlorite for 15 min followed by washing thrice with sterile distilled water.

Immature embryos of 0.5 to 1 mm long were aseptically removed under the stereo microscope (Fig. 2.1A). They were kept in the dissecting medium inside the scutella side up (Fig. 2.1B) and incubated in dark at 23°C for 5 days for callus induction (Fig. 2.1C).

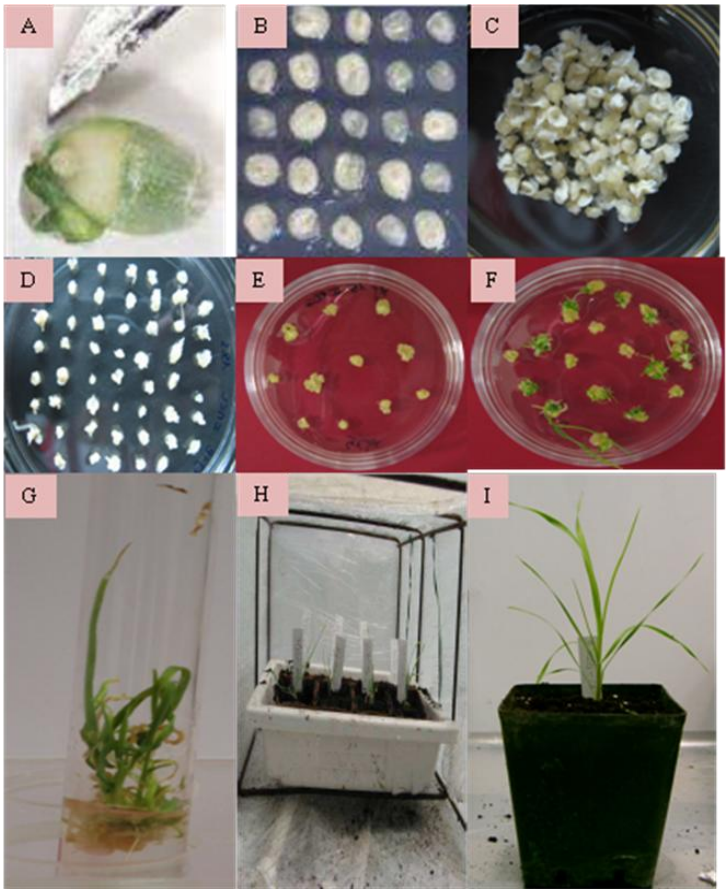

Fig. 2.2 (A) Isolation of immature embryo from wheat caryopsis. (B) Immature embryos with scutella side up and plated for bombardment. (C) Formation of embryogenic callus. (D) Calli in the bombardment medium, (E) Bombarded calli in the recovery medium. (F) Regeneration of plantlet on selective medium, (G) Plantlets resistant to the herbicide and in rooting stage (H) plants in the acclimation stage (I) Putative T_0 transgenic plants.

2.2.3 Coating of gold particles with DNA, co-transformation and plant regeneration:

Plasmid DNA was coated with gold particles and used for bombardment. 30mg of gold particles (0.6µm) was re-suspended in 500µl of 100% ethanol. The suspension was aliquoted (35µl) into 1.5ml tubes and after a brief centrifugation the ethanol was removed. For co-transformation of durum wheat, 10µg of each plasmid containing *Pvpgip2* (pAHC17-SR2), *Acpmei* (pUBI:Acpmei), *Taxi-III* (pAHC17-Taxi-III) and *bar* (pUBI:BAR) were used. For co-transformation of bread wheat, plasmids containing *Pvpgip2* (pAHC17-SR2), *Acpmei* (pUBI:Acpmei), *Xip-III* (pAHC17-Xip-III;) and *bar* (pUBI:BAR) in 1:1:1:1 molar ratio were used. The microprojectiles' pellet was re-suspended in a solution containing 2.5M $CaCl_2$ (250µl), spermidine (50µl), plasmid DNA and water. The content were mixed by vortexing 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 pellet was re-suspended in 36µl of 100% ethanol and stored in ice. About 30-40 embryo derived calli were transferred onto the bombardment medium 4 hours before bombardment. For each bombardment, 10µl of the DNA-gold suspension was placed in the centre of the macroprojectile and bombarded using biolistic particle delivery system- Model PDS-1000/He (Bio-Rad, Hercules, CA, U.S.A.) as described by Weeks et al. (1993). The distance from the stopping plate to the target was 13cm and the rupture disc strength was 1100psi (Fig. 2.3). Immediately after bombardment, the callus were kept in the bombardment medium (Fig. 2.2D) and incubated in dark at 23°C for 24 hours. They were then maintained in the recovery medium for 4 weeks and transferred into a fresh medium at 2-week intervals (Fig. 2.2E). For regeneration the calli were transferred to the regeneration medium containing bialaphos herbicide and incubated for 6 weeks at 27°C with 16hr light/8hr dark period. They were transferred to a fresh medium at an interval of 2-weeks. From the third week onwards, the calli resistance to the herbicide gave rise to new shoots (Fig. 2.2f). The shoots were transferred into Pyrex tubes containing rooting medium for 2-3 weeks to maintain herbicide selection. Plants capable of forming long, highly branched roots in the bialaphos-containing medium were defined as resistant (Fig. 2.2G). Sensitive plantlets initiated root formation but the primary root soon stopped growing and gave out only a few short lateral roots). Within one week, the sensitive plantlets exhibited yellow necrosis and reduced vigor whereas the resistant plantlets thrived in the rooting medium. Once the plants had sufficient leaves and roots, they were transferred into pots and kept in the growth chamber at 23°C, 16-h day light for 5-10 days. They were completely covered with plastic bags to maintain high humidity and allow them to acclimatize in greenhouse conditions (Fig. 2.2H).

The plants were then transferred into bigger pots and the primary regenerant plants were called as T_0 plants (Fig.2.2I). The total process starting with the excision of embryos up to the production of T_0 plants took about 168 days. The presence of transgene in the regenerated plants was checked by PCR.

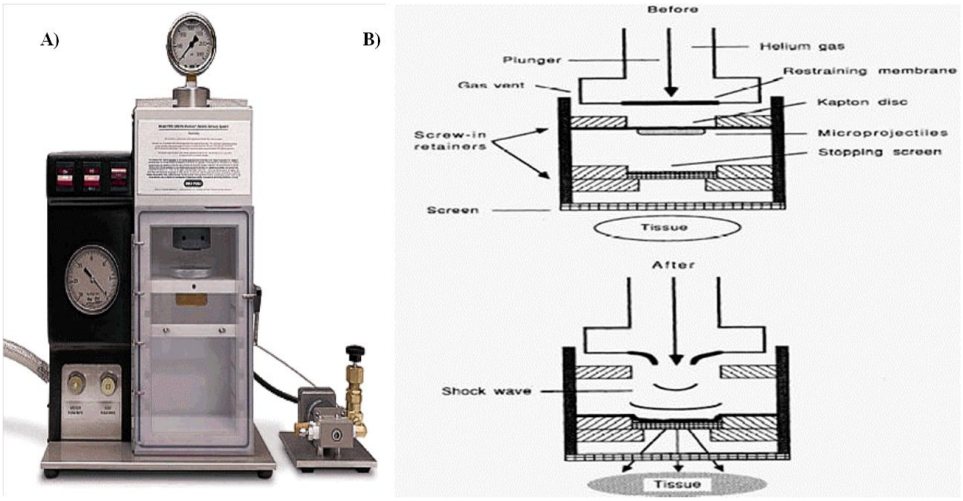

Fig. 2.3 A) Biolistic PDS-1000/He ™ System (BioRad/Dupont) B) Overview of biolistic bombardment process.

2.3 Screening of transgenic plants:

Before subjecting the seeds to germination, they were analyzed for the presence of transgenes by PCR using the total DNA extracted from half-seed (D'Ovidio and Porceddu, 1996). Half-seed was homogenized with 80µl of extraction buffer (100mM Tris-HCl pH 8.0, 50mM EDTA pH 8.0, 500mM NaCl) and 32µl of 10% SDS and incubated at 65°C for 10mins. 26.7µl of 5M potassium acetate was added to the samples and mixed and kept in ice for 20mins.

After centrifugation at 14000 rpm for 20mins, the supernatant containing the DNA was recovered. DNA precipitation was carried out by isopropanol precipitation. Then it was again centrifuged at 14000 rpm for 5mins. DNA pellet was washed with 70% ethanol, then vacuum dried and re-suspended in 20µl of sterile water. 1µl of DNA was used for PCR.

The PCR was performed according to the procedures specified for GoTag PCR Master Mix (Promega, Italia). The annealing temperature was 60°C and specific oligonucleotides designed for each transgenes (Table 2.3) were used.

2.4. Specific primer design:

The gene specific primers were designed using DNAMAN software (Lynnon Biosoft, Quebec, Canada). Following is the list of oligonucleotides used in the screening and characterization of transgenic wheat lines by PCR (Table 2.2 and 2.3)

Table 2.2 List of oligonucleotides used in the screening and characterization of transgenic lines.

Oligonucleotide name	Sequence-5'-3'
F753	ATGACTCCATTCAATATC CC
Pvpgip2 615F	CCTCACCGGGAAGATTCCG
Pvpgip2 853R	TTAGCTGCGTCAGTCCCTGC
AcpmeiF4	CTTGTATCTTTGAGAACTGCAC
AcpmeiR4	TGAGTTGGAATATTTGGTGGA C
TaxiIII-3R	TGACCGGGTTGGATGGGTA
TaxiIII-1F	ATGGCACGGGTCCTCCTCCTG
TaxiIII-1R	CTAGCTGCCGCAACCCGTAAAG
Xip-III 527R	TGCTTCGCCAGCTCCAGC
BAR1	CATCGAGACAAGCACGGTCA
BAR2	GAAACCCACGTCATGCCAGT
77F (Actin)	TCCTGTGTTGCTGACTGAGG
UBI-49F	TCGATGCTCACCCTGTTGTTT
312R (Actin)	GGT CCA AAC GAA GGA TAG CA

Table 2.3 List of oligonucleotides and their annealing temperature used in the screening and characterization of transgenic lines.

Genes	Utilization	Forward primer	Reverse primer	Annealing temperature
Pvpgip2	Transgenic screening	UBI-49F	Pvpgip2 853R	60ºC
	RT-PCR and probe amplification	F753	Pvpgip2 853R	60ºC
Acpmei	Transgenic screening	UBI-49F	Pvpgip2 853R	60ºC
	RT-PCR and probe amplification	AcpmeiF4	AcpmeiR4	60ºC
Taxi-III	Transgenic screening	UBI-49F	TaxiIII-3R	60ºC
	RT-PCR and probe amplification	TaxiIII-1F	TaxiIII-1R	65ºC
Xip-III	Transgenic screening	UBI-49F	XipIII-527R	60ºC
Bar	Transgenic screening	UBI-49F	BAR2	60ºC
Actin	Reference gene in RT-PCR	Actin-77F	Actin-312R	60ºC

2.5 Nucleic acid extraction:

2.5.1 Midi and Maxi preparation of plasmid DNA:

The Midi-and Maxi preparation of plasmid DNA was performed according to "PureLinkTM HiPure Plasmid Filter Purification" kit from Invitrogen. Midi-and Maxi preparation of plasmids pAHC17-SR2, pUBI:Acpmei, pAHC17-Taxi-III, pAHC17-Xip-III and pUBI:BAR were used in co-transformation by particle bombardment.

2.5.2 Total DNA extraction:

Total DNA was extracted from leaves using Tai and Tanksley (1990) method. The fresh or frozen (-80°C) leaves (up to 0.15g) of transgenic and control plants were crushed and homogenized in a mortar and pestle. 700µl of extraction buffer (100mM Tris-HCl pH 8.0, 50mM EDTA pH 8.0, 500mM NaCl, 1.25% SDS, 8.3mM NaOH, 0.38% Sodium bisulphite) preheated at 65°C was added and incubated at 65°C in water bath for 10 min. Then 220µl of 5M potassium acetate was added and the samples were kept in ice for 40 min in ice and centrifuged for 3 min at 4°C. The recovered supernatant was filtered and allowed to precipitate the DNA using 600µl of isopropanol. After centrifugation for 3mins at 4°C, the supernatant was discarded and the pellet was rinsed with 800µl of 70% ethanol twice. The pellet was re-suspended in 300µl of T5E buffer (50mM Tris-HCl pH 8.0, 10mM EDTA pH 8.0) and kept at 65°C for 5 min. 150µl of 7.4M ammonium acetate was added and again centrifuged for 3 min. The supernatant was taken for isopropanol precipitation and centrifuged for 3 min. The pellet was rinsed twice using 70% ethanol. Then the pellet was re-suspended in 100µl of T5E buffer and incubated at 65°C for 5 min. 10µl of 3M sodium acetate was added and the DNA was precipitated with 75µl of isopropanol. It was again centrifuged at 14,000 rpm for 5min. DNA pellet was washed with 70% ethanol and vacuum dried. Then it was re-suspended in 20µl of sterile water at 4°C for overnight. The DNA was heated at 65°C for 5 min and stored at 4°C (-20°C for longer periods). When the initial leaf material was more than 0.15g, the same procedure was used but with increased volumes of extraction buffers.

2.5.3 Total RNA extraction:

Total RNA extraction was performed according to the procedures specified by"RNeasy Plant mini" Kit (Qiagen) using 100mg of frozen leaf tissue. Extracted RNA was stored at -80°C. DNA contamination during RNA extraction process was removed using DNase treatment according to the procedures specified in "DNAfreeTM" kit (Ambion).

2.5.4 Agarose gel electrophoresis:

The DNA was run on 1.2-1.5% agarose gels prepared in 1x TBE buffer (Tris, Boric acid and EDTA) with 0.5µg/ml ethidium bromide. DNA samples were prepared by adding 6x loading dye (Fermentas, Italy). Molecular weight markers GeneRuler 100bp DNA Ladder or GeneRuler 1kb DNA Ladder (Fermentas, Italy) were used.

2.5.5 Gel extraction and purification of DNA fragments:

DNA fragment was extracted from agarose gels using "Wizard® SV Gel and PCR Clean-Up System" kit (Promega, Italy).

2.5.6 Restriction digestion:

All the restriction enzymes were purchased and used according to the manufacturer's instructions (Promega, Italy). Different quantities (500ng - 5µg) of plasmid or genomic DNA were used for analytical or preparative digestion. Incubation was performed for 1-2 hrs at 37°C with specific restriction enzymes in appropriate reaction conditions. 1U of enzyme/µg of DNA was added. For southern blotting, 5-10µg of genomic DNA was used in a 40µl of final volume.

2.6 Transcripts analysis:

Total RNA extracted from transgenic and *Triticum durum* cv. Svevo plants was used to generate first strand cDNA by following the manufacturer's instructions given in "QuantiTect Reverse Transcription" kit (Qiagen). Transcription accumulation was determined using quantitative PCR in a total reaction volume of 20µl using 1µl of cDNA and using primers for *Pvpgip2, Acpmei* and *Taxi-III* (Table 2.3). Reaction conditions were as follows: one cycle at 94°C for 5min followed by 35 cycles at 94°C for 1min, 60°C for 1min and 72°C for 1 min (except for *TaxiIII* whose annealing temperature is 65°C).

2.7 Southern blotting:

Total DNA was extracted from transgenic and control wheat plants following the Tai and Tanksley (1990) method and used for southern blotting. Genomic DNA 5µg was cleaved with *Bam*HI restriction enzyme, fractionated on 1.2% (w/v) agarose gel and transferred to a positively charged nylon membrane (Roche Diagnostics) as described by Sambrook et al. (1989). The gel was incubated for 45min in denaturation buffer (0.5M NaOH and 1.5M NaCl) and 1 hour in neutralization buffer (1.5M NaCl; 1M Tris-HCl pH 8.0). The DNA fragments were transferred onto the nylon membrane by capillarity using 10x SSC (150mM NaCl; 15mM sodium citrate) transfer

buffer. After the transfer (about 16 hours), the membrane was air dried for 30 mins and fixed with UV light at 150Joules. Before hybridization with the digoxigenin-labelled probe, the membrane was rinsed with 2X SSC.

2.7.1 Molecular hybridization:

This experiment was done in 4 phases: pre-hybridization, hybridization, washing and immunological detection.

Pre-hybridization:

The membranes containing the transferred DNA fragments were saturated in order to avoid the background on the filter generated by non specific binding of the probe. The buffer used for the pre-hybridization contained 5x SSC, 0.1% N-Laurilsarcosine, 0.2% SDS, 0.5% blocking reagent (Boehringer Mannheim). The membrane was incubated for 3 hrs at 65°C.

Hybridization:

Before mixing the probe for hybridization, the DNA fragments were denaturated by keeping it at 95°C for 5 mins and suddenly kept in ice for 1 min. The membrane was kept in the buffer for at least 16 hours at 65°C for hybridization at high stringent conditions. The recovered probe was stored at 4°C for reuse.

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.

Immunnological detection:

The immunological detection was conducted using a chemiluminescent assay in which a specific anti-digoxigenin-antibody, conjugated to alkaline phosphatase reacts with the CSPD® (Disodium 3-(4-methoxyspiro {l,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.13,7]decan}-4-yl) phenyl phosphate; Roche Diagnostics) substrate that is dephosphorylated leading to a distinct luminescent signal recorded on a film. The buffers and the washing conditions used were: 5 min washing with buffer 1 (0.1M Maleic acid, 0.15M NaCl, pH 7.5 containing 0.3% (V/V) Tween 20) and 30 min washing 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 by washing

twice with buffer 1 for 15 mins and once with buffer 3 (100mM Tris-HCl pH 9.5; 100mM NaCl; 50mM $MgCl_2$).The filter was put into a plastic bag and 1ml of CSPD® 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.

2.7.2 Digoxigenin labelling:

The coding region of *Pvpgip2*, *Acpmei*, and *Taxi-III* was labelled with digoxigenin (digoxigenin-11-uridine-5-triphosphate; Roche Diagnostics) by PCR by following the procedure reported by D'Ovidio and Anderson (1994). 1µl of plasmid DNA (15ng) was added to the reaction mixture containing 20µl of 5x buffer, 10mM dATP, dCTP, dGTP, 1mM dTTP, 0.1mM DIG-11dUTP- alkali labile (Roche Diagnostics), 100mM primers (Table 2.2) , 5units of Go Tag polymerase (Promega Italy) and the final volume was adjusted to 100µl with sterile water.

The DNA amplification conditions were: 94°C-1 min, 60°C-1 min, 72°C-1 min, for 35 cycles (except for *Taxi-III* whose annealing temperature was 65°C). The amplified DNA was then purified using the "Wizard® SV Gel and PCR Clean-Up System" kit (Promega, Italy). The efficiency of the labelling was verified by DIG Quantification Teststrip (Roche Diagnostics) by following the manufacturer's procedure and stored at 4°C.

2.8 Protein extraction and analysis:

2.8.1 Total protein extraction and Bradford assay:

The fresh or frozen (-80°C) leaves of transgenic and control plants were crushed in a mortar and pestle using liquid nitrogen. Then they were homogenized in 20mM sodium acetate and 1M NaCl, pH 4.6 (2mL/g) and kept for 1hr vortexing at 4°C. It was centrifuged 10,000 rpm for 20 min at 4°C and the supernatant was recovered. In order to obtain extracts free of debris, it was centrifuged again for 5min. Protein concentration of the crude protein extract was determined with the "Bio-Rad Protein assay" kit (Bio-Rad; Bradford 1976).

2.8.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE):

SDS-PAGE was performed as described by Laemmli (1970) using separating gel and stacking gel with 15% and 3.75% polyacrylamide, respectively. The composition of the running buffer was: 0.2M Glycine, 0.02M Tris pH 8.8 and 0.1% SDS. For each sample, 10µg of protein extract was loaded into the gel and fractionated at a constant voltage of 200V until the run off of the bromophenol blue dye front.

2.9 Western blotting:

The SDS-PAGE gel was incubated in transfer buffer (25mM Tris- HCl, pH8.0, 192mM glycine and 0.04% SDS) for 15 minutes. Blotting was performed in the 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 8.0, 150mM NaCl, 0.1% Tween20 and 5% dry milk rid of fat) at room temperature in the orbital shaker for 2hrs. The membrane was then washed twice with washing buffer (10mM Tris-HCl pH 8.0, 150mM NaCl and 0.2% Tween20) and incubated overnight with specific primary antibodies. A polyclonal antibody that recognizes the N-terminal peptide of mature bean PGIP (Bergmann et al., 1994) was used. After removing 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 "Western blotting Luminol reagent" kit (Santa Cruz Technologies).

2.10 Inhibition assays:

2.10.1 PGIP inhibition assay:

Enzymatic activity of PGs was evaluated using modified agarose diffusion assay (Taylor and Secor 1988) also called "Cup-plate" and also 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 in 0.5cm wells on plates containing 100mM sodium acetate pH 4.6, 0.5% polygalacturonic acid and 0.8% agarose. The plates were incubated for 19 hrs 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 (one agarose diffusion unit defined as the amount of enzyme that produced a halo of 0.5cm radius external to the inoculation well) after 19 hrs at 30°C. Inhibitory activity was expressed as inhibitory units (one inhibitory unit defined as the amount of PGIP that reduced by 50% 1 agarose diffusion unit of PG). In the second method, the incubation mixture contains 200µl of 0.5% polygalacturonic acid from orange or 0.5% pectin from apple 70-75% methylesterified (Sigma-Aldrich) in 100mM sodium acetate pH 4.7 and 0.1 mg/ml bovine serum albumin. After enzyme addition, the mixture was incubated at 30°C for 1 hr. PG activity was expressed as reducing units (One reducing units was defined as the amount of enzyme required to release reducing groups at 1 µmol/min using D-galacturonic acid as standard).

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

2.10.2 PMEI inhibition assay:

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 casted 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. Plates were incubated at 30°C for 16 hrs. The gel was stained with 0.02% (w/v) ruthenium red (Sigma) for 45 min. Then it was 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 the protein extracts from leaves which were carefully chosen at the same stage of development.

2.10.3 Xylanase inhibition assay:

Xylanase activity was quantified by the radial gel diffusion assay as described by Emami and Hack (2001) with some modifications. *A. niger* xylanase M4 (Megazyme) was placed into 0.5 cm diameter wells in 1% agarose (w/v) plates containing 1.0% Birchwood xylan (Sigma) in McIlvaine's buffer pH 5.0 (0.2M Na2HPO4, 0.1M Citric acid). The plates were incubated at 30°C for 16 hrs. The halo caused by enzyme activity was visualized after 30 mins of treatment with 95% ethanol. For inhibition with TAXI-III, varying amount of total protein extract from transgenic plant along with *A. niger* xylanase (0.005U) as loaded in well and incubated and developed as above specified In the second method, Xylanase activity was measured using the dinitrosalicylic acid (DNS) assay as described by Brutus et al. (2004) with some modifications. Aliquot of the xylanase was mixed with 480µl of 1.0% Birchwood xylan prepared in McIlvaine's buffer, pH 5.5. Final volume of reaction up to 500µl was by adding McIlvaine's buffer, pH 5.5 and then incubated at 40°C for 5 min. The reaction was terminated with the addition of 500µl DNS reagent and boiled for 5 min. The reactions were cooled and centrifuged for 5 min at 12 000×g and optical density measured at 545 nm. One unit of xylanase activity was defined as the amount of protein that released 1 µmol of xylose $min^{-1.}$ For inhibition assay, *A. niger* xylanase was mixed with total plant extract from transgenic plant and incubated for at Room Tempreture (RT) for 2 min. Final volume of reaction up to 500µl was adjusted by adding McIlvaine's buffer, pH 5.5 and then analyzed for

xylanase activity. Xylanase activity was performed in boiled sample of same protein extract at same time. The inhibition calculated by the decrease of xylanase activity in total plant extract.

2.11 Infection of transgenic plants:

2.11.1 Fungal cultures:

Fusarium graminearum strain 3827 was cultured in SNA medium (Urban et al., 2002) containing 0.1% KH_2PO_4, 0.1% KNO_3, 0,1% $MgSO_4.7H_2O$, 0.05% KCl, 0.02% glucose, 0.02% sucrose, 2% bactoagar (Sigma) with 200ppm biotin and 200ppm thiamine. *Bipolaris sorokiniana* (strain 62608) was cultured on potato dextrose agar (PDA; Fluka)

Macroconidia was harvested by gently scraping the culture surface with 3ml of sterile water. Macroconidia concentration was estimated by Thoma chamber. For plant inoculation, final condia concentration was adjusted to 500conidia/20µl for *F. graminearum* and 6,000conidia/20µl for *B. sorokiniana*. Tween 20 was added to a final concentration of 0.05% into the conidia solution.

2.11.2 Plants growth:

Wheat seeds were surface sterilized by 0.5% v/v sodium hypochlorite for 15 min and rinsed thoroughly in sterile water. Then it was kept on a filter paper in dark at 4°C for 4 days to ensure synchronous germination. The Plants were grown in a climatic chamber with 16-h light period at 18 to 23°C and fertilized monthly with 21:7:14 fertilizers until maturation. Once the Zadoks stage 92 was reached, dried seeds were trashed by hand and stored at 4°C.

2.11.3 Leaf infection with *Bipolaris sorokiniana*:

The upper surface of first leaf from the T_2 transgenic lines and control wheat plant at the third leaf emerged stage (Zadoks stage 13) was inoculated with 20µl conidia solution (300conidia/µl) of *B. sorokiniana* strain 62608. During experiment, the plots were covered with plastic film and sprayed with water in order to maintain high relative humidity. Leaf blotch symptoms (Fig. 3.8) appeared as reddish-brown spots of variable dimensions on the leaf surface after 72 hrs of post infection (hpi) and the leaf surface was used for scanning. The lesion area (cm^2) was measured using Adobe Photoshop program (Microsoft, Segrate, Italy). The arithmetic mean was calculated using the average lesion size in transgenic and wild type plants and used to calculate the disease severity. In each experiment, three lines of transgenic durum wheat plants: T_2MJ56-10 (seventeen plants), T_2MJ56-16a-12 (nine plants) and T_2 MJ56-16b-1 (eight plants) along with their corresponding wild type-*T. durum* cv. Svevo plants (15 plants) were used. Further, these plants were allowed to grow

and used to extract the total protein. Plants that showed combined inhibitory activity to endogenous PME and Xylanase M4 from *A. niger* were used for analysis of disease severity. Similarly, T_1 generation of bread wheat plants from T_0 MJ65-28 (20 plants) along with their corresponding wild type-*T. aestivum* L. cv. Bobwhite (20 plants) were used for infection experiment.

2.12 Crossing:

In classical crossing experiments, spikes of transgenic durum wheat line expressing separately *Pvpgip2 or Acpmei* were emasculated and crossed (Table 3.15 and Table 3.16). The F_1 seeds were collected and then used in PCR screening to verify the presence of transgenes. Those plants containing both transgenes were crossed again with transgenic plants expressing *Taxi-III* gene. Seed progenies were collected and then used in PCR screening to verify the presence of transgenes.

2.13 Statistical analysis of data:

T_1 and T_2 segregation data based on PCR analysis was subjected to goodness of fit test (chi-square) for independent segregation and homogenous segregation between the lines. Yates correction was applied for the low number of samples. Data from *B. sorokiniana* and *F. graminearum* infection experiments were analyzed by applying the student's t-test with 2 tails hypothesizing different variances between the two groups.

3. Results:

3.1 Multi-stacking transgenic production in durum wheat by particle bombardment:

3.1.1 Transformation frequency:

The constructs containing genes *Pvpgip2* (pAHC17-SR2), *Acpmei* (pUBI:Acpmei), *Taxi-III* (pAHC17-Taxi-III) and *bar* (pUBI:BAR) as selectable marker gene were used to co-bombard immature embryos of *Triticum durum* cv. Svevo. Particle gun bombardment of embryos, selection, and regeneration were carried out as described by Janni et al, 2008. Total 2253 immature wheat embryos were co-transformed in two batches MJ56 and MJ58 (Table 3.1). Twelve T_0 plants were produced from batch MJ56 at a transformation frequency of 1.16% whereas four T_0 plants were produced from the MJ58 batch with a transformation frequency of 0.33%.

Table 3.1. Bombardment experiments in durum wheat using pAHC17-SR2, pUBI:Acpmei, pAHC17-Taxi-III and UBI:BAR plasmids, as determined by PCR.

Name	Cv	# No embryos bambarded	# No plants regenerated	Transformation frequency %
MJ56	*Triticum durum* cv. Svevo	1053	12	1.16%
MJ58	*Triticum durum* cv. Svevo	1200	4	0.33%

3.1.2 Co-transformation frequency:

Total DNA was extracted (Tom and Tanksley 1991) from leaf of T_0 plants and was used for PCR screening using gene specific primers (Fig. 3.2). In batch MJ56, a total of twelve 12 T_0 plants were produced. Among them seven plants (58.33%) contained all four transgenes (Table 3.2), one plant with *Acpmei & bar* genes (8.33%) and the remaining four plants contained *bar* genes (33.33%). In batch MJ58 only four T_0 plants were produced. Among them one plant contained *Acpmei & bar* (25%), one plant with *Taxi-III & bar* (25%) and remaining two plants contained only the *bar* gene (50%) (Table 3.2).

Table 3.2. Co-transformation frequency of *Pvpgip2*, *Acpmei*, *TaxiIII* and *bar* transgenes in durum wheat produced by particle bombardment, as determined by PCR.

	Total T_0 plants	**Gene(s)**			
		Pvpgip2, Acpmei, Tax-III & bar	***Acpmei & bar***	***Taxi-III & bar***	***Bar***
T_0 MJ56 Plants	12	7	1	0	4
Co- transformation frequency (%)		58.33	8.33	0	33.33
T_0 MJ58 Plants	4	0	1	1	2
Co-transformation frequency (%)		0	25	25	50

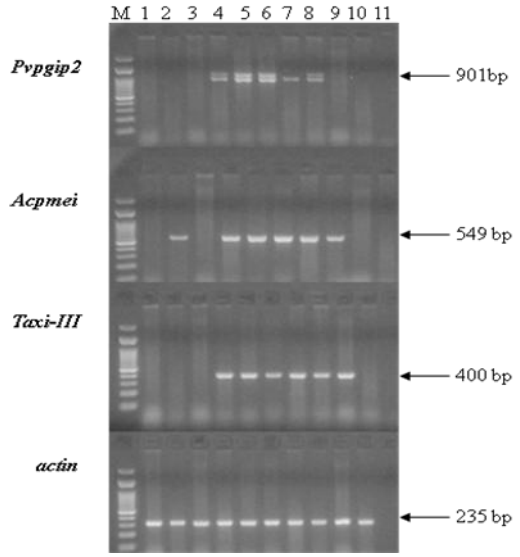

Fig. 3.1: Example of the PCR amplification performed on total DNA of T_0 plants of durum wheat. Amplicons were separeted on 1.5% Agarose gel. PCR amplification for *Pvpgip2, Acpmei, Taxi-III* and *actin* using the gene specific primers. Marker GeneRuler 100 bp DNA Ladder: 1 to 9) T_0 transgenic plants; 10) non-transformed durum wheat cv. Svevo used as negative control; 11) water.

3.1.3 T_1 segregation analysis of transgenic durum wheat:

The T_1 generation seeds from T_0 plants containing all four transgenes (MJ56-5, MJ56-9, MJ56-12, MJ56-15a, MJ56-15b, MJ56-16a and MJ56-16b) were used for segregation analysis. Total DNA was extracted from half-seed (D'Ovidio and Porceddu, 1996) and used for screening by PCR with primers for *Pvpgip2, Acpmei, Taxi-III* and *bar* transgenes (Table 2.3). Total of 198 T_1 progeny seeds were analyzed, amongst them 139 seeds contained all four genes, that is about 70% of T_1

progeny integrated all four transgenes and three plants with only gene of interest lack of *bar* gene(Table 3.3).

The segregation data, based on PCR analysis, was subjected to a goodness of fit testing (chi-square) for independent segregation. All transgenic lines showed significant associations between the four transgenes during segregation and fit 3:1 ratio (Table 3.4), indicating that all four transgenes are tightly linked.

In addition, data were analyzed for homogenous segregation between the lines showing tightly linked transgenes. This relationship was based on the expected frequency calculated considering all lines. Only three lines, MJ56-9, MJ56-12 and MJ56-15b, were significantly different from the expected segregation ratio. In constrast, the remaining four lines, MJ56-5, MJ56-15a, MJ56-16a and MJ65-56-16b, were not significantly different in the expected mode of segregation.

Table 3.3 T_1 progeny of transgenic durum wheat, as determined by PCR.

T_0 lines	**Total T_1 seeds analyzed**	**Gene(s) present**											
		pgip2	*Taxi-III*	*bar*	*pgip2 & pmei*	*pmei & TaxiIII*	*Taxi-III & bar*	*pgip2 & bar*	*pgip2, Taxi-III & bar*	*pmei, Taxi-III & bar*	*pgip2, pmei & Taxi-III*	*pgip2, pmei, Taxi-III & bar*	*Null-lines*
T_0 MJ56-5	20				1							19	0
T_0 MJ56-9	39							2		2		27	8
T_0 MJ56-12	40		2	2								27	9
T_0 MJ56-15a	11	1		1		1			1	1		5	1
T_0 MJ56-15b	30	2		3	1		4	1			2	15	2
T_0 MJ56-16a	22			2	1							17	2
T_0 MJ56-16b	36								1	2	1	29	3

Table 3.4 Chi-square test with independent segregation and homogenous segregation between lines analyzed in T_1 progeny of durum wheat, as determined by PCR.

T_0 lines	**X^2 for independent segregation based on T_1 progeny**	***p* value***	**X^2 for homogeneous segregation based on T_1 progeny**	***p* value***
T_0 MJ56-5	269.18	0.0	3.102	0.530
T_0 MJ56-9	286.04	0.0	19.70	0.000
T_0 MJ56-12	297.11	0.0	25.86	0.000
T_0 MJ56-15a	029.60	0.0	4.197	0.381
T_0 MJ56-15b	99.688	0.0	9.547	0.048
T_0 MJ56-16a	192.84	0.0	1.436	0.830
T_0 MJ56-16b	341.60	0.0	7.375	0.120

*$X^2 \geq 9.49, P \leq 0.05$, 4 d.f and $X^2 \geq 13.28, P \leq 0.01$, 4 d.f

3.1.4 T_2 segregation analysis of transgenic durum wheat:

T_2 seeds from three lines: T_1 MJ56-5-10, T_1 MJ56-16a-12 and T_1 MJ56-16b-1 were used for segregation analysis. Total DNA was extracted from half-seed (D'Ovidio and Porceddu, 1996) and used for PCR screening using specific primer for *Pvpgip2, Acpmei, Taxi-III* and *bar* genes. Total of 70 T_2 progeny seeds were analyzed, amongst them 51 seeds contained all four genes, which is 72% of T_2 progeny integrated all four transgenes. The segregation data, based on PCR analysis, was subjected to a goodness of fit testing (chi-square) for independent segregation. All transgenic lines showed significant associations between the four transgenes during segregation and fit 3:1 ratio (Table 3.5), indicating that all four transgenes are tightly linked. Further, segregation data was analyzed for homogenous segregation between the lines showing tightly linked transgenes. This relationship was based on the expected frequency calculated considering all lines. In T_1 MJ56-5-10 line was significantly different from the expected segregation ratio but remaining two lines: T_1 MJ56-16a-12 and T_1 MJ56-16b-1 were not significantly different in the expected mode of segregation.

Table 3.5 Chi-square test for the independent segregation and homogenous segregation between lines analyzed of T_2 progeny of durum wheat, as determined by PCR.

T_1 lines	**Total T_2 seeds Analyzed**	**Gene(s) presents**						**X^2 independent segregation for T_1 progeny**	***p* value***	**X^2 homogeneous segregation for T_1 progeny**	***p* value***
		Taxi -III	***bar***	***pgip2, pmei & bar***	***pmei , Taxi- III & bar***	***pgip2 , pmei , Taxi- III & bar***	**Null - lines**				
T_1 MJ56-5-10	25	1	1		1	17	5	175.78	0.0	19.48	0.006
T_1 MJ56-16a-12	22			1		17	4	193.57	0.0	0.412	0.891
T_1 MJ56-16b-1	23					17	6	200	0.0	0.3111	0.981

*$X^2 \geq 9.49$, $P \leq 0.05$, 4 d.f

3.1.5 Co-expression analysis in T_1 generation:

T_1 seeds from three lines, MJ56-5, MJ56-16a and MJ56-16b, PCR positive for all four transgenes were grown and analyzed for transgene expressions.

Transcripts and protein expression of PvPGIP2:

T_1 leaves from MJ56-5 line (3 plants) were subjected to *Pvpgip2* transcript accumulation analysis by qRT-PCR. The transcripts level of *Pvpgip2* was found to be very low (Fig. 3.2). Leaves of these plants were subjected to total protein extraction and used in inhibition activity assays against the polygalacturonase (PG) of *Fusarium phyllophilum* (FpPG) because PvPGIP2 is very effective against this PG. No inhibition activity was observed against FpPG (Fig. 3.3). These results were also confirmed by western blot analysis on the same total protein extracts using an antibody raised against the bean PGIP protein. No immunodecoration signal corresponding to PvPGIP2 was observed in all the transgenic plants analyzed (Fig. 3.4). Similarly, total protein extracts from T_1 plants of MJ56-16a and MJ56-16b lines did not show any inhibition activity against FpPG, nor Pvpgip2 trascript accumulation or western blotting immunodecoration signal, indicating that gene silencing have occurred for *Pvpgip2* in all transgenic durum wheat lines.

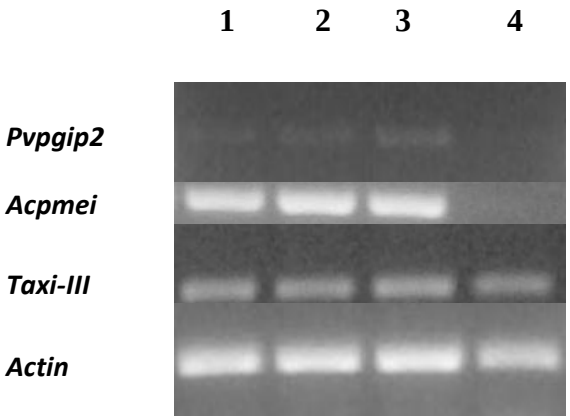

Fig. 3.2 Transcript accumulation of *Pvpgip2*, *Acpmei* and *Taxi-III* genes in transgenic durum wheat lines. Transcript analysis was performed by RT-PCR and amplicons analyzed on 1.5% agarose gel. 1) MJ56-5-1 T_1 line; 2) MJ56-5-6 T_1 line; 3) MJ56-5-8 T_1 line; 4) *Triticum durum* cv. Svevo.

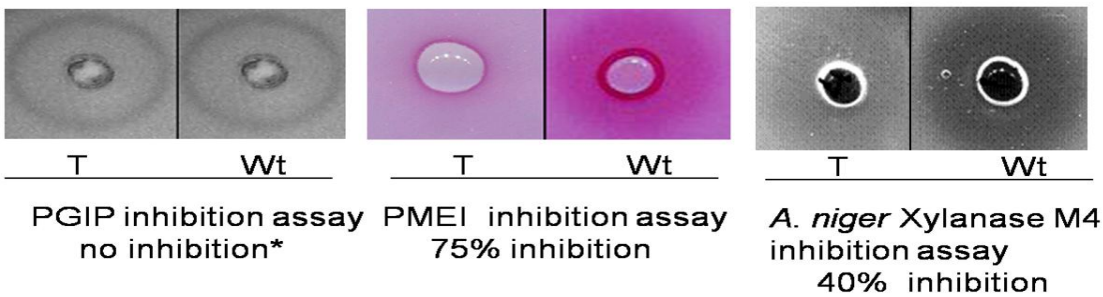

Fig. 3.3 Examples of agarose diffusion assay with total protein extract from T_1 generation plants of transgenic (T) wheat expressing PvPGIP2, AcPMEI and TAXI-III and with wild type plants (Wt). FpPG and *A. niger* xylanase M4 were used for the inhibition assays with PvPGIP2 and TAXI III, respectively. Endogenous PME activity was analyzed to verify the level of PMEI inhibition.

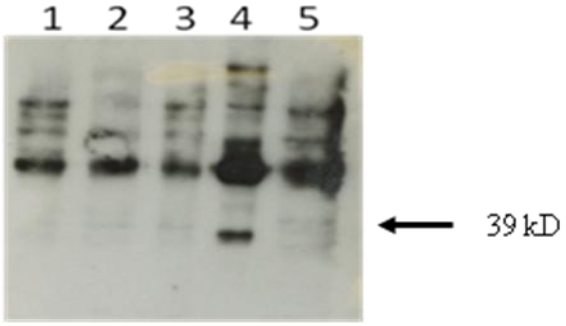

Fig 3.4 Western blotting with total protein extracts (10µg) from the transgenic durum wheat lines and a polyclonal *Ab* anti-PvPGIP was used. 1) MJ56-5-1; 2) MJ56-5-6; 3) MJ56-5-8; 4) Bread wheat transgenic line 23a expressing PvPGIP2 used as positive control (Janni et al., 2008); 5) Durum wheat cv. Svevo.

Transcripts and protein expression of AcPMEI and TAXI III:

In qRT-PCR analysis in T_1 leaves from MJ56-5 line (3 plants), showed transcript accumulation of Acpmei and Taxi-III genes (Fig. 3.2). Then, the expression of AcPMEI and TAXI-III at protein level were analyzed on basis of their inhibition activity against endogenous PMEs and Xylanase M4 from *A. niger* respectively (Fig. 3.3). Total fifteen T_1 plants were analyzed of MJ56-5 line, among them all plants gives PMEI inhibition except four plants, which exhibited Xylanase inhibition, representing 73.30 % of silencing for *Taxi-III* gene as there was no inhibition with *A. niger* xylanase M4 (Table 3.5).

In T_1 plants of MJ56-16a, all ten plants showed PMEI inhibition and only four plants with TAXI III inhibition. Total thirteen plants T_1 plants were analyzed of MJ56-16b line, among them eleven plants showed PMEI inhibition and three plants with TAXI-III inhibition.

T_1 plants contained both inhibitory activity, AcPMEI and TAXI III, were grown and the corresponding T_2 generations were used in infection experiments with the fungal pathogen *B. sorokiniana*.

Table 3.5 Expression of AcPMEI and TAXI-III in T_1 generation of transgenic durum wheat lines as determined by AcPMEI and TAXI-III inhibition assays. AcPMEI inhibition was determined by analyzing the endogenous PME activity, whereas TAXI III inhibition was evaluated against the xylanase M4 of *A. niger.*

T_0 line	**No of T_1 plants analyzed**	**Plant presents**		
		AcPMEI inhibition +/-	**TAXI-III *A. niger* xylanase M4 inhibition assay +/-**	**AcPMEI and Xylanase inhibition**
T_0 MJ56-5	15	15/0	4/11	4
T_0 MJ56-16a	10	10/0	4/06	4
T_0 MJ56-16b	13	11/2	3/10	3

3.1.6 Co-expression analysis in T_2 generation:

T_2 generation plants from the T_1 lines-MJ56-5-10, MJ56-16a-12 and MJ56-16b-1 were used for co-expression analysis of *Acpmei* and *Taxi-III* transgenes. The inhibition activity of AcPMEI and Taxi III was based on the endogenous PME activity and against the Xylanase M4 of *A. niger*, respectively.

Total eighteen T_2 plants of MJ56-5-10 were analyzed, amongst them fifteen plants showed both AcPMEI and TAXI-III inhibition and the remaining three plants had no inhibition activity for both inhibitors (Table 3.6). Total nineteen T_2 plants from MJ56-16a-12 were analyzed, among them eighteen and eight plants showed PMEI and TAXI-III, respectively. For MJ56-16b-1 line, a total seventeen T_2 plants were analyzed, among which fourteen and eight plants showed PMEI and TAXI-III inhibition, respectively.

Table 3.6 Expression of AcPMEI and TAXI-III in T_2 generation of transgenic durum wheat lines as determined by AcPMEI and TAXI-III inhibition assays.

T_1 lines	**No of T_2 plants analyzed**	**Plant presents**		
		AcPMEI inhibition +/-	**Taxi-III inhibition +/-**	**AcPMEI & Taxi-III inhibition**
T_1 MJ56-5-10	18	15/3	15/03	15
T_1 MJ56-16a-12	19	18/1	09/10	9
T_1 MJ56-16b-1	17	14/3	08/09	8

3.1.7 Southern blot analysis of MJ56-5, MJ56-16a and MJ56-16b:

Southern hybridization was performed with the transgenic lines: MJ56-5, MJ56-16a and MJ56-16b. Genomic DNA (5µg) was digested with *Bam*HI and hybridized separately with DIG-labeled probe for *Pvpgip2, Acpmei* and *Taxi-III* genes. Restriction digestion with *Bam*HI enzyme causes the excision of the complete coding region and hybridization signal demonstrated the presence of the expected fragment in all transgenic plants. As expected, no hybridization signals were present in genomic DNA of non transformed durum wheat cv Svevo (Fig. 3.5). Results from southern blotting confirmed the accuracy of our PCR detection system in a subset of progeny and their integration in genome

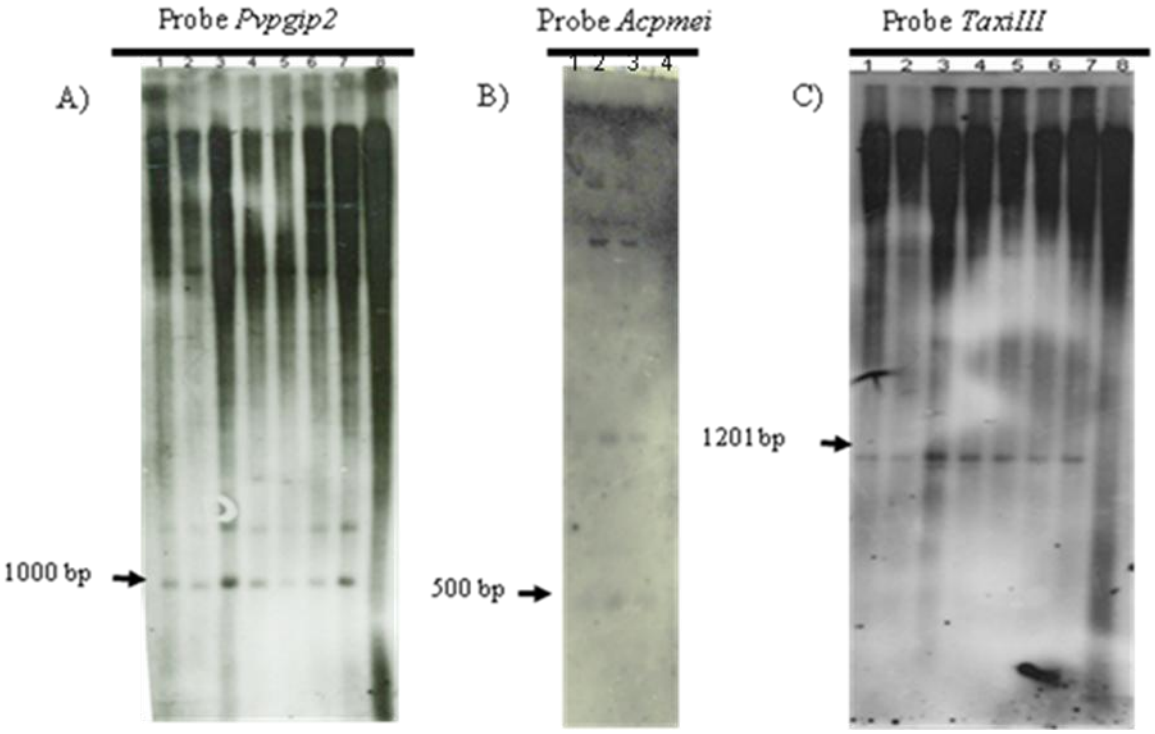

Fig. 3.5 Southern blot on durum wheat genomic DNA (5µg) digested by *Bam*HI and hybridized using DIG-labeled probe. .A) Hybridization pattern with Pvpgip2 probe. 1) T_1MJ56-16a-1; 2) T_1MJ56-16a-2; 3) T_1MJ56-16a-12; 4) T_1MJ56-16a-14; 5) T_1MJ56-16b-1; 6) T_1MJ56-16b-3; 7) T_1MJ56-16b-7; 8) *Triticum durum* cv. Svevo. B) Hybridization pattern with *Acpmei* probe. 1) T_2MJ56-5-10-26; 2) T_2MJ56-16a-12-18; 3) T_2MJ56-16b-1-11; 4) *Triticum durum* cv. Svevo. C) Hybridization pattern with *Taxi-III* probe. 1) T_1MJ56-16a-1; 2) T_1MJ56-16a-2; 3) T_1MJ56-16a-12; 4) T_1MJ56-16a-14; 5) T_1MJ56-16b-1; 6) T_1MJ56-16b-3; 7) T_1MJ56-16b-7; 8) *Triticum durum* cv. Svevo.

3.1.8 *Bipolaris sorokiniana* infection on leaf tissue:

Those T_2 generation plants from T_1MJ56-5, T_1MJ56-16a, and T_1MJ56-16b lines showing presence of all three transgenes were used for infection experiment on leaf tissue with the fungal pathogen *B. sorokiniana*. In each separate experiments, transgenic plants from the three lines, T_2MJ56-10, T_2MJ56-16a-12, T_2 MJ56-16b-1 and the control *T. durum* cv. Svevo were used. The upper surface

of first leaf, at the third leaf-emerged stage (Zadoks stage 13) was inoculated with 6000 conidia of *B. sorokiniana* strain 62608. Leaf Bloch symptoms were visible 48 hours post infection (hpi) and appeared like reddish-brown spots of variable size on the leaf surface (Fig. 3.6A). In order to facilitate the analysis of single lesions, data was collected at 72 hpi. Disease symptoms were evaluated as the ratio between leaf area showing symptoms and the total leaf area, expressed as percentage. After symptom evaluation plants were subjected to total protein extraction. Those plants showing combined inhibitory activity of both AcPMEI and TAXI III were analyzed for disease severity. Statistical analysis of the data showed that all three lines had a significant reduction in disease symptoms compared to the wild-type plants (Table 3.7). All infection experiments were performed separately with the same procedure. Two T_2 lines, MJ56-5-10 and MJ56-16a-12, showed 73.0 % of symptom reduction and T_2 line MJ56-16b-1 showed 68.0% of reduction in symptoms (Fig. 3.6B).

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

Infection expt.	**Lines**	**Symptoms severity[a]**	**No of plants**	**% of reduction in symptoms**
1	T_2 MJ56-5-10	2.65 ± 1.3*	15**	73.12
	Svevo	9.6 ± 1.9	24	
2	T_2 MJ56-16a-12	1 ± 0.5*	9**	73.33
	Svevo	3.75 ± 0.72	19	
3	T_2 MJ56-16b-1	1.3 ± 0.74*	8**	70.66
	Svevo	3.75 ±0.72*	19	

[a] - Symptom severity is expressed as lesion expansion area cm2/ total leaf area cm2, x 100.
* - means significantly different at $P \leq 0.05$
** - number of plants with AcPMEI and TAXI-III inhibition.

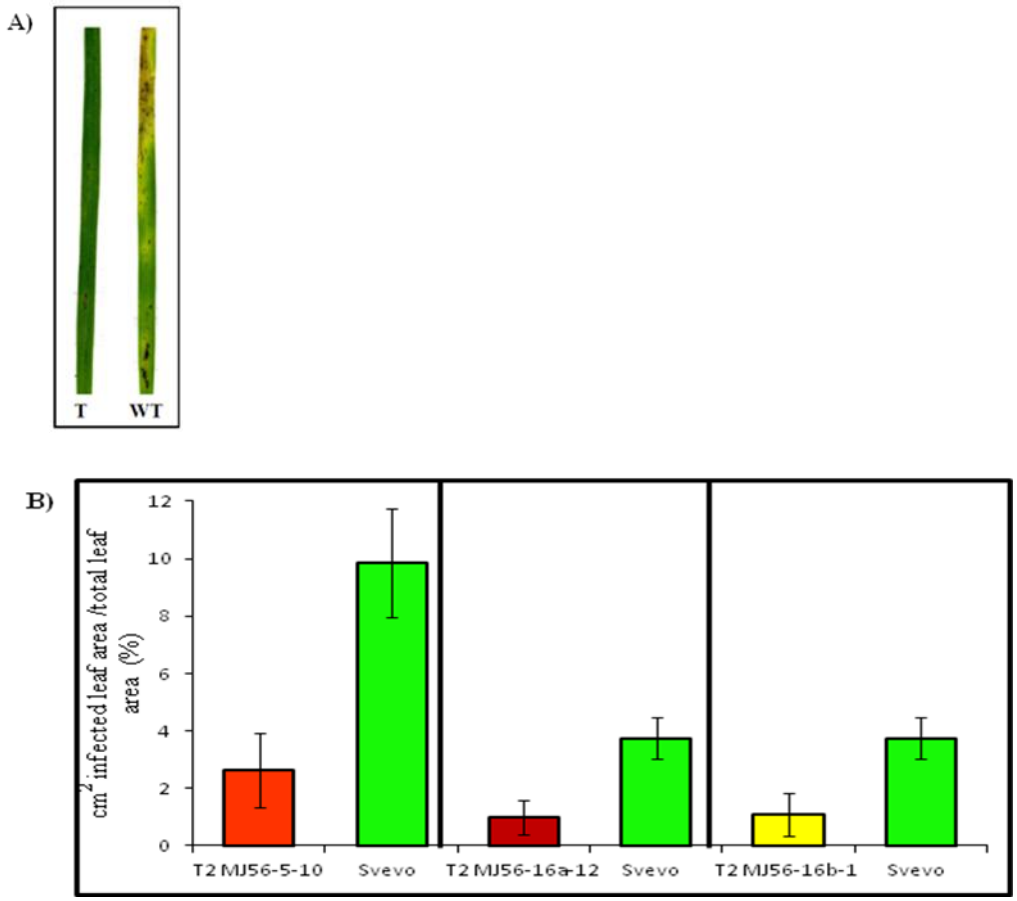

Fig 3.6 Infection of leaf tissue with *B. sorokiniana.*(A) Example of lesions caused by *B. sorokiniana* on wheat leave 72h after infection. T) transgenic leave; WT, wild type (cv Svevo) plants. (B) T_2 generation of transgenic plants were used for infection with *B sorokiniana* along with control wild type (Wt) plants. A bar represents averages and standard errors of the area of lesions divided by the total leaf area (cm2)

3.2 Multi-stacking transgenic production in bread wheat by particle bombardment:

3.2.1 Transformation frequency:

The construct containing genes *Pvpgip2* (pAHC17-SR2), A*cpmei* (pUBI:Acpmei), *Xip-III* (pAHC17-XipIII) and *bar* as selectable marker in plasmid (pUBI:BAR) were used to co-bombard on *T. avesticum* cv. Bobwhite immature embryos. Particle gun bombardment of embryos, selection, and regeneration were carried out as described by Janni et al. 2008 (Table 3.8)

In MJ63 batch, total 932 immature embryos were co-transformed and and only two T_0 plants were produced at a transformation frequency of 0.32%. Similarly, 862 immature embryos co-transformed in batch MJ65 and eighteen T_0 plants were produced at 2.08% transformation frequency.

Table 3.8. Bombardment experiments in bread wheat using pAHC17-SR2, pUBI:Acpmei, pAHC17-XIP-III and UBI::BAR plasmids.

Name	Cv	# No of embryos bombarded	# No of regen. plants	% of regen.	# No of plants in soil*	Total T_0 plants	Positive T_0 plants**	% of trans.
MJ63	Bobwhite	932	21	2.25	4	2	0	0.32
MJ65	Bobwhite	862	41	4.76	20	18	10	2.08

*some plants died during process of acclimatization/due to contamination in soil.
**as determined by PCR.

3.2.2 Co-transformation frequency:

Total DNA was extracted (Tom and Tanksley 1991) from leaf of T_0 plants and was used for PCR screening using gene specific primers

In batch MJ65, total eighteen T_0 plants were produced. Among them five plants (27.77%) contained all four genes transgenes (Table 3.9), three plants with *Acpmei, XipIII* & *bar* genes (16.66%), two plants with *Acpmei & bar* genes (11.11%) and remaining eight plants contained *bar* gene (44.33%).

Table 3.9 Co-transformation frequency of *Pvpgip2, Acpmei, Xip-III* and *bar* transgenes in bread wheat produced by particle bombardment, as determined by PCR

	Total T_0 plants	Gene(s) presents			
		Pvpgip2, Acpmei, Taxi-III & bar	***Acpmei, XipIII & bar***	***Acpmei & bar***	***bar***
T_0 MJ63 Plants	2	0	0	0	2
Co-transformation frequency (%)		0	0	0	100
T_0 MJ65 Plants	18	5	3	2	8
Co-transformation frequency (%)		27.77	16.66	11.11	44.44

3.2.3 T_1 segregation analysis of transgenic bread wheat:

T_1 generation seeds from T_0 plants containing all four transgenes (T_0 MJ65-2, T_0 MJ65-11, T_0 MJ65-18b, and T_0 MJ65-28) which were used for segregation analysis. Total DNA was extracted from half-seed (D'Ovidio and Porceddu, 1996) and used for screening by PCR with primers for *Pvpgip2, Acpmei, Xip-III* and *bar* transgens (Table 3.12). Total 114 T_1 progeny was analyzed amongst them, 72 seeds contained all four genes, that is about 70% of T_1 progeny integrated all four transgenes (Table 3.10). Among the analyzed seeds, five seeds from MJ65-2 showed presence of three transgenes of interest (*Pvpgip2, Acpmei, Xip-III*).

The segregation data, based on PCR analysis, was subjected to a goodness of fit testing (chi-square) for independent segregation. All transgenic lines showed significant associations between the four transgenes during segregation and fit 3:1 ratio (Table 10), indicating that all four transgenes are tightly linked. Then, data was analyzed for homogenous segregation between the lines showing tightly linked transgenes. This relationship was based on the expected frequency calculated considering all lines. Only MJ65-2 was significantly different from the expected segregation ratio, the remaining three lines: MJ65-11, MJ65-18b and MJ65-28 were not significantly different in the expected mode of segregation.

Table 3.10 Chi-square test for the independent segregation and the homogenous segregation between lines of T_1 progeny of bread wheat, as determined by PCR.

T_0 lines	**Total T_1 seeds**	**Gene presents**						**X^2 independent segregation based on T_1 progeny**	***p* value***	**X^2 homogeneous segregation based on T_1 progeny**	***p* value***
		bar	***pgip2* + *pmei* + *bar***	***pmei* + *xip-III* + *bar***	***pgip2* + *pmei* + *Xip-III***	***pgip2* + *pmei* + *Xip-III* + *bar***	**Null-lines**				
T_0 MJ65-2	43	2	2	0	5	30	4	301.78	0.0	11.24	0.023
T_0 MJ65-11	19	0	0	0	0	10	9	130.56	0.0	3.92	0.416
T_0 MJ65-18b	17	0	0	0	0	9	8	116.62	0.0	5.43	0.245
T_0 MJ65-28	35	0	0	2	0	23	10	251.04	0.0	0.29	0.994

*$X^2 \geq 9.49$, $P \leq 0.05$, 4 d.f and $X^2 \geq 13.28$, $P \leq 0.01$, 4 d.f

3.2.4 Co-expression analysis in T_1 generation:

T_1 seeds from MJ65-28 lines, MJ56-5, MJ56-16a and MJ56-16b, PCR positive for all four transgenes were grown and analyzed for their expressions.

Expression of PvPGIP and AcPMEI:

The expression of PvPGIP2 and AcPMEI protein in transgenic lines were analyzed on basis of their inhibition activity against FpPG and endogenous PMEs respectively (Fig. 3.7). The leaf from T_1 plants MJ65-28 was used extract total protein. Total twenty T_1 plants were analyzed, among them sixteen plants showed PGIP inhibition and twelve plants with AcPMIEI inhibition (Table 3.11).

Expression of XipIII:

The inhibitory activity of XIPIII in these plants has not been determined because of limiting wheat material and the lack of information about the inhibiting activity of XIPIII.

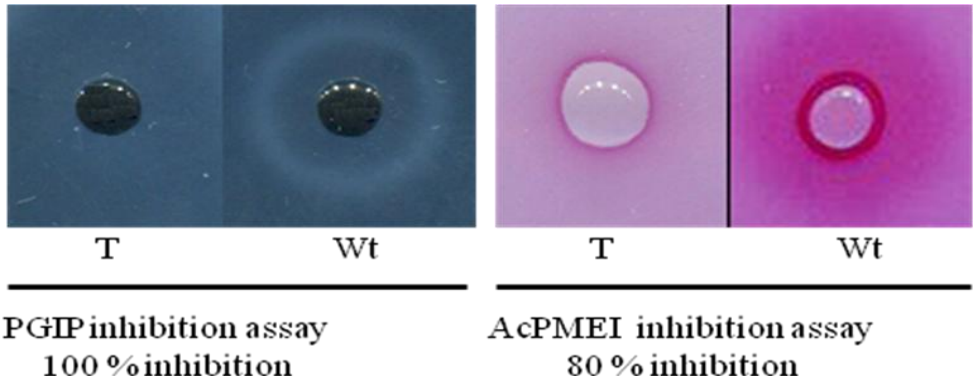

Fig. 3.7 Examples of agarose diffusion assay with total protein extract of transgenic (T) bread wheat line T_1MJ65-28 and with wild type plants (Wt). FpPG was used for the inhibition assays with PvPGIP2 and Endogenous PME activity was analyzed to verify the level of PMEI inhibition.

Table 3.11 Expression of PGIP and AcPMEI in T_1 generation of transgenic bread line MJ65-28. As determined by PGIP and AcPMEI inhibition assays.

T_0 line	**No of T_1 plants analyzed**	**Plants presents**		
		PGIP inhibition +/-	**Acpmei Inhibition +/-**	**PvPGIP and AcPMEI inhibition**
T_0 MJ65-28	20	16/4	12/8	12

3.3.5 *Bipolaris sorokiniana* infection on leaf tissue:

Those T_1 generation plants from T_0 MJ65-28 showing presence of all transgenes were used for infection experiment on leaf tissue with the fungal pathogen *B. sorokiniana*. The upper surface of first leaf, at the third leaf-emerged stage (Zadoks stage 13) was inoculated with 6000 conidia of *B. sorokiniana* strain 62608. Leaf Bloch symptoms were visible 48 hpi and appeared like reddish-brown spots of variable size on the leaf surface. In order to facilitate the analysis of single lesions, data was collected at 72 hpi. Disease symptoms were evaluated as the ratio between leaf area showing symptoms and the total leaf area, expressed as percentage (Fig 3.8). After symptom evaluation plants were subjected to total protein extraction. Those plants showing combined inhibitory activity of both PGIP and AcPMEI were analyzed for disease severity. Statistical analysis of the data showed that transgenic plants had 54.71 % significantly reduction in disease symptoms compared to the wild-type plants (Table 3.12).

Table 3.12 Mean values and standard errors of symptomatic leaf area of transgenic bread wheat plants and wild-type plants lines inoculated with *B. sorokiniana* macroconidiaa

Infection expt.	Lines	Symptoms severity[a]	No of plants	% of reduction in symptoms
1	T_1 MJ65-28	5.9 ± 1.8*	12**	54.71%
	Bobwhite	12.65 ± 1.94	22	

[a] Symptom severity is expressed as lesion expansion area cm2/ total leaf area cm2, x 100.
* means significantly different at P ≤0.01
** number of plants with PvPGIP and AcPMEI inhibition.

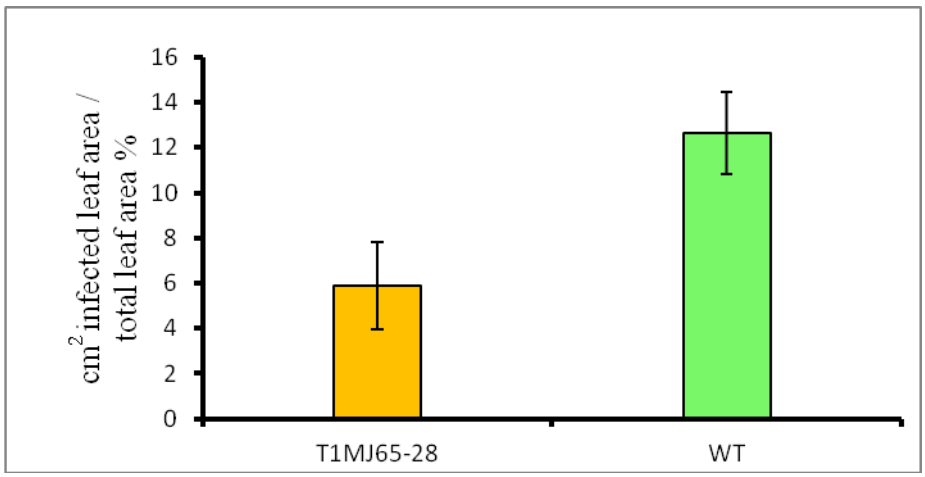

Fig 3.8 *B. sorokiniana* infection on leaf tissue of transgenic wheat leaves 72h after infection. A bar represents averages and standard errors of the area of lesions divided by the total leaf area (cm2) in transgenic T_1MJ65-28 and with control wild type plants (WT).

3.3 Pyramiding of *Pvpgip*2, *Acpmei* and *Taxi-III* transgenes through crossing in durum wheat:

In case of pyramiding of *Pvpgip*2, *Acpmei* and *Taxi-III* transgenes through crossing, we performed two crossing experiments. (1) First cross was produced between transgenic lines over expressing PGIP and PMEI. (2) Second cross was performed between lines over expressing PGIP and PMEI and transgenic lines over expressing TAXI-III.

3.3.1 Pyramiding of *Pvpgip*2 with *Acpmei* transgenes:

The PMEI line MJ31-11-4-19, exhibited high inhibitory activity against the endogenous PME and were crossed with the PGIP lines MJ15-69-3-7-33-10, showing a high inhibitory activity against FpPG. Five F_1 seeds were obtained from the cross and the DNA from these seeds was used for PCR screening using the specific primers to verify the presence of both transgenes (Table 3.13). All five seeds contained both transgenes and exhibited high expression at protein level for *Pvpgip2* and *Acpmei* showing high inhibition activity for those transgenes (Fig. 3.12). Further, two F_2 plants from F_1 S10-05-1 were crossed with transgenic lines over expressing *Taxi-III*.

Table 3.13 Characterization F_1 lines from cross between *Pvpgip* and *Acpmei* lines in durum wheat

Name of line	Parental lines (♀× ♂)	F_1 lines	PCR		Inhibition assay (% of inhibition)	
			Pvpgip2	***Acpmei***	**PGIP**	**AcPMEI**
S010-05	MJ15-69-3-7-33-10 × MJ31-11-4-19	F_1 S10-05-1	+	+	83.5	100
		F_1 S10-05-2	+	+	83.5	100
		F_1 S10-05-3	+	+	83.5	100
		F_1 S10-05-4	+	+	83.5	100
		F_1 S10-05-5	+	+	58.4	100

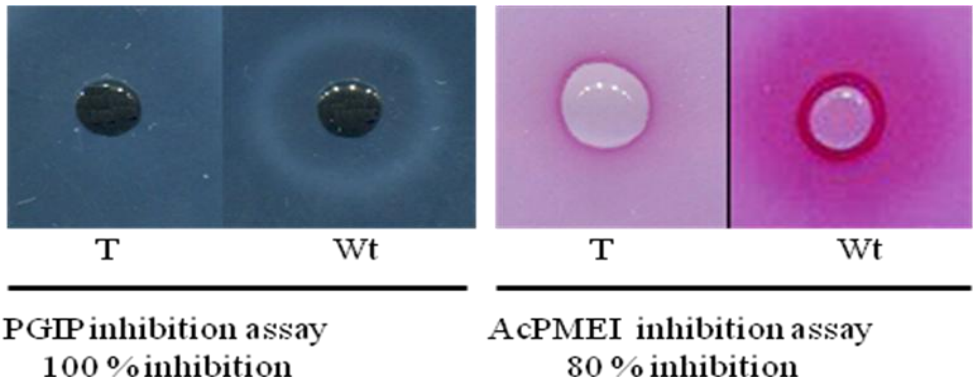

Fig. 3.12 Examples of agarose diffusion assay for PGIP and AcPMEI inhibition assay with total protein extract from F_1 S010-05 plants (T) and with wild type plants (Wt). FpPG was used for the inhibition assays with PvPGIP2 and .Endogenous PME activity was analyzed to verify the level of PMEI inhibition.

3.3.2 Pyramiding of *Pvpgip*2, *Acpmei* with *Taxi-III* transgenes:

The F_2 lines S010-05-1-2, S010-05-1-21 exhibited high expression for *Pvpgip2* and *Acpmei* were used in crossing with T_1 MJ30-23-1 (line over expressing Taxi-III). Total sixteen F_1 seeds were obtained and the DNA from these seeds was used in PCR screening for the presence of transgenes using specific primers.

Two F_1 seeds (M011-1-4 & M011-1-5) containing all three transgenes were analyzed for their inhibition activity. F_1 M011-1-4 gives high expression for all three transgenes, as determined by inhibition assays (Table 3.16). Differently, F_1 M011-1-5 showed silencing for *Acpmei* gene as no inhibition for endogenous PMEs was observed, although the presence of *Acpmei* in this plant was confirmed by PCR. The harvested F_2 seeds from F_1M011-1-4 plant were used for PCR screening

analysis. Total 32 seeds were analyzed. Among them only three seeds (10%) with of all three transgenes, *Pvpgip2 Acpmei*, and *Taxi-III* were identified (Table 3.14).

Table 3.13 Characterization of F_1 lines from cross between *Pvpgip2*, *Acpmei* lines with *Taxi-III* line in durum wheat

Line	Parental lines (♀× ♂)	F_1 lines	PCR			Inhibition assay (% of inhibition)		
			Pvpgip2	*Acpmei*	*Taxi-III*	PGIP2	PMEI	*A. niger* xylanase M4
M011-1 (9 seeds)	MJ30-23-1 × S010-05-1-2	MO11-1-1	-	-	+			88
		MO11-1-2	-	-	+			88
		MO11-1-3	-	-	+			88
		MO11-1-4	+	+	+	100	88.00	50
		MO11-1-5	+	+	+	100	No	30
		MO11-1-6	-	-	+			88
		MO11-1-7	-	-	+			88
		MO11-1-8	-	-	+			88
		MO11-1-9	-	-	+			88
M011-2 (3 seeds)	S010-05-1-21 × MJ30-23-1	MO11-2-1	+	+	-	100	No	
		MO11-2-2	+	+	-	100	No	
		MO11-2-3	+	-	+	100		100
M011-3 (2 seeds)	S010-05-1-36 × MJ30-10-4-16	MO11-3-1	-	+	-		No	
		MO11-3-2	-	+	+		100	100

Table 3.14 Characterization of F_2 seeds from F_1 M011-1-4 for presence of *Pvpgip2*, *Acpmei* and *Taxi-III* transgenes, as determined by PCR.

F_1 line	Total F_2 seeds	Gene(s) presents							
		pgip2	***pmei***	***Taxi-III***	***pgip2 + pmei***	***pgip2 + Taxi-III***	***pmei + Taxi-III***	***pgip2 + pmei + Taxi-III***	**Null-lines**
F_1 M011-1-4	32	1	4	11	5	4	2	3	2

4. Discussion:

The plant cell wall is one of the obstacles encountered by the microbial pathogens during plant tissue colonization. This compartment contains a number of components involved in host defence. Among the protein component of this compartment is the Polygalacturonase inhibiting protein (PGIP) that inhibits the endopolygalacturonase (PG) secreted by pathogens during the initial phase of infection (Cervone et al., 1986; Ridley et al. 2001). The over expression of *Pvpgip2* gene from *Phaseolus vulgaris* in wheat showed a significant reduction in the disease symptoms following the infection by *B. sorokiniana* (Janni et al., 2008) and *F. graminearum* (Ferrari et al., 2012). The cell wall contains also Xylanase inhibitors (XIs) that are protein components which inhibit microbial xylanases of glycoside hydrolase families 10 and 11. Endo-β-1, 4-xylanases are the key enzymes in degradation of arabinoxylans (AXs), the main non-starch polysaccharides from cereal cell walls (Juge, 2006). The direct involvement of these protein inhibitors in host defence has not been demonstrated yet, but several observations indicate that they can play an important role (Igawa et al., 2004;Igawa et al., 2005; Weng et al. 2010). Among the polysaccharide components of the cell wall implicated in host defence, several evidences had been reported on the involvement of the pectin structure. The degree of pectin methylation was correlated with the resistance of tomato cultivars against *Pseudomonas syringae* pv. *tomato* and *Pseudomonas syringae* pv. *apii* (Venkatesh B., 2002). Similarly, a high percentage of methylated and branched pectins had been correlated with the resistance against *E. carotovora* subsp. *Atroseptica* infection in potato (McMillan et al., 1993). The degree of pectin methyl esterification can be controlled by increasing the level of the protein inhibitor PMEI, located in the apoplast and inhibiting the activity of pectin methyl esterase (PME), the enzyme responsible for removing the methyl ester group from newly synthesized pectin (Siedlecka A et al., 2008). Volpi et al. (2011) demonstrated that the overexpression of the AcPMEI from *Actinidia chinensis* in durum wheat cv. Svevo increased the degree of pectin methylesterification and reduced significantly the infection of *B. sorokiniana* and *F. graminearum.* A possible explanation of the correlation between increased resistance and higher level of pectin methylesterification is that highly methylesterified pectins are less susceptible to the action of PG and pectate lyase secreted by pathogens (Willats et al., 2001b). The stronger barrier encountered by the pathogens delays the pathogen entry and allows the plant to set up a more effective response.

Since the individually overexpression of PGIP and PMEI in wheat determined a reduction of disease symptom, it is conceivable that their pyramiding can limit further disease symptom. This possibility is reinforced further if the pyramiding included also an additional cell wall glycosidase inhibitor such as the TaxiIII or Xip-III that target a different polysaccharide component of the cell

wall. Different approaches can be used to combine the multiple transgenes in a single plant including sexual crossing between plants carrying separate transgenes (Ma et al., 1994; Bizily et al., 2000), sequential retransformation (Lapierre et al., 1999), and co-transformation with multiple plasmids (Chen et al., 1998; Ye et al., 2000) or with single plasmids on which several transgenes are linked (Bohmert et al., 2000; Zhong et al., 2007). Substantial progress has been made in recent years with the development of *Agrobacterium*-mediated transformation systems for monocot species including rice (Hiei et al. 1994; Cheng et al., 1998), maize (Ishida et al., 1996), barley (Tingay et al., 1997), wheat (Cheng et al., 1997), and sorghum (Song et al., 2004). However, particle bombardment is still the method of choice for production of transgenic plants, particularly for monocot species, because it is not only variety-independent transformation but also simple and efficient. Hadi et al., 1996 demonstrated the use of particle bombardment to produce transgenic plants with multiple genes by co- transformation. They successfully co-transformed soybean with 12 different plasmids and found that the majority of transgenic soybean clones had integrated with all 12 plasmids. Similarly, Chen et al., 1998 showed that up to 13 of the 14 plasmids could be simultaneously introduced into the rice genome. In wheat, co-transformation with multiple plasmids by particle bombardments had been demonstrated by Campbell et al., 2000 in *T. aestivum* cv. Bobwhite. They used 1:0.5:0.5 molar ratio for three different plasmids each containing RNase L or 2-5A synthetase genes, and neomycin phosphotransferase II (NPT II) gene as selectable marker for transformation. On the basis of the inheritance of the two genes of interest, the authors demonstrated that the genes were linked.

We verified two approaches to produce multi-stacking in wheat: I) co-transformation with multiple plasmids and II) sexual crossing between plants carrying separate transgenes. The multiple co-transformations were performed by particle bombardment and included the four different genes: (1) *Pvpgip2, Acpmei, Taxi-III* and *bar* as selectable marker in durum wheat. (2) *Pvpgip2, Acpmei, Xip-III* and *bar* as selectable marker in bread wheat. In durum wheat co-transformation (batch MJ56) a total of twelve T_0 plants were regenerated. Among them, seven plants showed 58% co-transformation frequency for all four transgenes. Similarly, in bread wheat transformation (batch MJ65) a total of eighteen T_0 plants were regenerated. Among them, five plants showed 27% co-transformation frequency for all four transgenes. These result are in agreement with the previously reported co-transformation frequencies ranging from 20 to 80% for multiple plasmids co-transformation experiments performed in bean (Araga˜o et al., 1996), rice (Chen et al., 1998; Maqbool and Christou 1999), in wheat (Campbell et al., 2000), and in maize (Brettschneider et al., 1997). In the few attempts reported for multiple co-transformations in wheat, a maximum of three

transgenes, including the marker gene, have been reported (Campbell et al., 2000). In these experiments it was observed 36% co-transformation frequency for the three transgenes. In our experiment we found a slightly lower percentage of co-transformation frequency but using four different transgenes, confirming the feasibility and effectiveness of this approach. Our experiments represent also the first attempt to perform multiple co-transformations in durum that showed an higher of co-transformation frequency than in bread wheat. A wide range of co-transformation frequencies reported is likely due to the differences in transformation protocol, selection and regeneration for individual plant transformation experiments. Among the twelve T_0 transgenic durum wheat plants in batch MJ56, seven were produced with *Pvgpip2* (58.0%), eight with *Acpmei* (67.0%), seven with *Taxi-III* (58.0%) and all the twelve contained bar (100%) gene. Similarly, amongst the eighteen T_0 transgenic bread wheat plants in batch MJ65, five were produced with *Pvgpip2* (27%), ten with *Acpmei* gene (55.0%), eight with *XipIII* gene (55.0%) and all the eighteen plants contained *bar* gene (100%). In all the co-transformation experiments, larger number of plants with *bar* gene was observed, as *bar* gene is the selectable marker for transformed tissues. So we could expect a high frequency of events containing *bar* gene. The co-integration frequency of the genes from non-selected plasmids (*Pvpgip2, Acpmei Taxi-III* and *Xip-III* gene) with selected plasmid (*bar*) was above 58% and 27% in durum wheat and bread wheat respectively. Consequently, no preferential integration was observed for any of these specific genes. Results from southern blotting confirmed the accuracy of the PCR detection system in a subset of progeny and integration of transgene. Further, T_1 and T_2 segregation analysis was performed to predict the type of linkage between the four transgenes and their inheritance behaviour. The T_1 generation analysis revealed that 70% and 63% of the progenies were with four transgenes in durum and bread wheat, respectively. All the transgenic lines showed significant association between the four transgenes during segregation and fit the 3:1 ratio. This indicated that all the four transgenes were tightly linked. Similarly, the T_2 segregation analysed in three lines of durum wheat was performed and about 72% of the progenies were with four transgenes during segregation and fit 3:1 ratio, confirming that all the four transgenes were tightly linked. Other papers also reported the insertion of multiple genes or fragments thereof into a single locus including in rice (Kumpatla et al., 1997; Kohli et al., 1998, and Agrawal et al., 2005), maize (Register et al., 1994), bean (Aragao et al., 1996), and wheat (Campbell et al., 2000).

In the multistacking approach through classical crossing between plants carrying separate transgenes, we performed two crossing experiments: First cross was produced between transgenic lines over expressing PGIP or PMEI. Second cross was performed between the transgenic lines over

expressing PGIP/PMEI and TAXI-III. In cross between PGIP and PMEI plants, a totally of five plants were obtained. All these five plants contained both the transgenes, exhibited high expression of *Pvpgip2* and *Acpmei* and showed high inhibition activity of their encoded products. Further, two F_2 PGIP/PMEI plants from F_1 S10-05-1 were crossed with T_1 line MJ30-23-1 over-expressing *Taxi-III.* A total of sixteen F_1 seeds were obtained and only two F_1 seeds, M011-1-4 and M011-1-5, contained all the three transgenes. F_1 M011-1-4 gave high expression for three transgenes, which was revealed through their high inhibition activity. However, there was silencing of *Acpmei* in F_1 MO11-1-5 line. Then F_2 segregation analysis was performed on seeds of F_1 M011-1-4 line. Among the 32 seeds analyzed, only three seeds showed all the three transgenes - *Pvpgip2, Acpmei* and *Taxi-III.* By analyzing the two approaches-multiple plasmid co-transformation by particle bombardment and crossing between plants carrying separate transgenes. It could confirm that co-transformation is a better approach as above 70% of the progenies were with three transgenes of interest and also tightly linked. On the contrary, in the crossing method only 10% of the segregating progeny contained all three transgenes of interest.

Co-expression analysis at protein level for the three transgenes was performed. For durum wheat the three T_1 generation lines MJ56-5, MJ56-16a and MJ56-16b were used. All the plants of the three lines showed silencing event for *Pvpgip2*. In the same plants *Acpmei* was expressed in most of the plants and *Taxi-III* was expressed in around 30%. Observed co-expression for *Acpmei* and *Taxi-III* was around 30%. Similarly, in T_2 generation co-expression frequency for AcPMEI and TAXI-III in MJ56-5-10 line showed 83% and 47% in both lines: MJ56-16a-12, MJ56-16b-1. Several evidences had been reported on gene silencing events following co-transformation. Anand et al. (2003) performed co-transformation in bread wheat cv Bobwhite using *chitinase* and *glucanase* genes and observed the silencing event for transgenes. Similarly, Chen et al. (1998) also showed the silencing event with one gene in rice which was co-transformed with fourteen transgenes.

Progenies from both multiple co-transformation and crossing of transgenic plants showed silencing of specific transgenes. Transgenic durum wheat lines showed complete silencing of Pvpgip2 and partial silencing of *TaxiIII*. The specificity of complete suppression of *Pvpgip2* is quite surprising because occurred both when multiple transgenes were present in a single plant (eg. *Pvpgip2, Acpmei*, *Taxi-III* and *bar* genes). Similarly when the transgenic plants contained only *Pvpgip2* or combined with another transgene (eg. *Acpmei*) showed silencing event. We do not know the reason underlying this specific event, however, the lack or reduced expression of transgenes is explained with the accumulation of high number of transgenes in single genotypes. In our experiments, all the constructs contained the maize Ubiquitin promoter that is a widely used promoter in wheat

transformation and gives good constitutive expression throughout the plant. The presence of multiple transgenes driven by the same constitutive promoter in a single plant may result in homology-dependent gene silencing (HDGS) (Meyer et al., 1996), especially when the promoter is also highly active (Butaye et al., 2005) as the Ubiquitin promoter (Wu et al., 2002). HDGS may act when multiple copies of the transgene are inserted at one locus, but also when present at unlinked sites. It is also believed that this phenomenon can occurs either at the transcriptional (transcriptional gene silencing; TGS) or post-transcriptional level (post-transcriptional gene silencing; PTGS). This silencing phenomenon can represents a limit in both multiple co-transformation and crossing approaches used. Probably multiple co-transformation can produce better results if performed using *Agrobacterium tumefaciens* as transformation system in place of the biolistic system because single or low copy number of integrated transgenes can be obtained. Also, the pyramiding of multiple transgenes by crossing can produce better results if performed by using transgenic lines carrying low copy number of transgenes. Lower the copy number can cause, however a problem in obtaining the required level of transgene expression. This possibility has been demonstrated in durum wheat transgenic plants expressing *Acpmei*. Transgenic lines expressing a low level of AcPMEI did not modify the level of pectin methylesterification and did not improve resistance to *Bipolaris sorokiniana*. Only when an adequate level of AcPMEI accumulated, the degree of pectin methyl esterification increased and the transgenic plants showed improved resistance to fungal pathogens (Volpi et al., 2011). Other possible strategies can be adopted to avoid or limit silencing events and obtain the required level of transgene expression. For example, the use of transgenes under control of different promoters or the use of Matrix attachment regions (MARs). The first approach can be particularly effective with the use of induced or tissue-specific promoters, whereas flanking transgenes with MARs could decrease the probability of silencing because they may stabilize transcription (Brouwer et al., 2002).

Those T_2 generation plants showing co-expression of AcPMEI and TAXI-III were used for infection experiments on leaf tissue with fungal pathogen *B. sorokininana*. Leaf tissue infection experiments with *B. sorokiniana* revealed that three lines T_1MJ56-5, T_1 MJ56-16a, and T_1 MJ56-16b showed 73%, 73% and 69% reduction in symptom severity respectively. This increased level of protection is higher than overexpression of AcPMEI (about 55%) in durum wheat was reported by Volpi et al. (2011). It is probably because of the co-presence of TAXI-III. However, the possibility that a higher level of PMEI activity in these transgenic plants is responsible for the higher protection cannot be ruled out.

For bread wheat, T_1 MJ65-28 line was used co-expression analysis, interesting *Pvpgip2* was expressed in some plants and 60% co-expression frequency was observed for PvPGIP and AcPMEI.

The inhibitory activity of XIPIII in these plants has not been determined because of limiting wheat material and the lack of information about the inhibiting activity of XIPIII.

Leaf tissue infection experiments of T_1 MJ65-28 line with *B. sorokiniana* showed 54.0% reduction in symptom severity. This level of leaf symptom reduction is similar to that was observed in wheat overexpressing AcPMEI from *Actinidia chinensis* (Volpi et al., 2011). Although we do not know if these plants express XIPIII, these results indicate that the pyramiding of PvPGIP2 and AcPMEI does not determine an increase in protection against *B. sorokiniana*. Probably the contribution that a reduced degradation of the pectin of the cell wall can give to increase the resistance to *B. sorokiniana* is already maximum with the individual components, PGIP or PMEI, and therefore their combination does not have an effect on the increase of wheat resistance.

5. Conclusion:

- We successfully transformed durum and bread wheat with 4 different transgenes.
- Co-transformation produced tightly linked transgenes in both durum and bread wheat.
- Co-transformation approach produced 70% of the progenies with tightly linked transgenes.
- Crossing approach produced highly segregating progeny and only 10% of the progeny are with pyramided transgenes.
- When all three were pyramided, both co-transformation and crossing approaches in durum wheat showed silencing of one glycosidase inhibitor gene. Only one plant from the crossing approach showed activity of all the three inhibitors. Probably high copy number of Ubiquitin promoter caused homology-dependent silencing.
- Three co-transformed durum wheat lines expressing both AcPMEI and TAXI-III showed about 70% protection against leaf blotch disease caused by *B. sorokiniana*. This result shows slightly higher protection than that overexpression of AcPMEI (about 55%) in durum wheat was reported by Volpi et al. (2011).

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