Research article

Claviceps purpurea expressing polygalacturonases escaping PGIP inhibition fully infects PvPGIP2 wheat transgenic plants but its infection is delayed in wheat transgenic plants with increased level of pectin methyl esterification

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ABSTRACT

Claviceps purpurea is a biotrophic fungal pathogen of grasses causing the ergot disease. The infection process of C. purpurea on rye flowers is accompanied by pectin degradation and polygalacturonase (PG) activity represents a pathogenicity factor. Wheat is also infected by C. purpurea and we tested whether the presence of polygalacturonase inhibiting protein (PGIP) can affect pathogen infection and ergot disease development. Wheat transgenic plants expressing the bean PvPGIP2 did not show a clear reduction of disease symptoms when infected with C. purpurea. To ascertain the possible cause underlying this lack of improved resistance of PvPGIP2 plants, we expressed both polygalacturonases present in the C. purpurea genome, cppg1 and cppg2 in Pichia pastoris. In vitro assays using the heterologous expressed PGs and PvPGIP2 showed that neither PG is inhibited by this inhibitor. To further investigate the role of PG in the C. purpurea/wheat system, we demonstrated that the activity of both PGs of C. purpurea is reduced on highly methyl esterified pectin. Finally, we showed that this reduction in PG activity is relevant in planta, by inoculating with C. purpurea transgenic wheat plants overexpressing a pectin methyl esterase inhibitor (PMEI) and showing a high degree of pectin methyl esterification. We observed reduced disease symptoms in the transgenic line compared with null controls. Together, these results highlight the importance of pectin degradation for ergot disease development in wheat and sustain the notion that inhibition of pectin degradation may represent a possible route to control of ergot in cereals.

1. Introduction

Claviceps purpurea is a biotrophic, non-appressorium forming fungal pathogen of grasses and cereals, causing the ergot disease. The infection process of C. purpurea on rye flowers is accompanied by pectin degradation and polygalacturonase (PG) activity represents a pathogenicity factor [1]. Fungal PG activity is controlled by specific protein inhibitors called polygalacturonase inhibiting proteins (PGIPs) localized in the apoplast [2]. The effectiveness of PGIP in limiting host tissue colonization by fungal [3–10] and bacterial [11,12] pathogens has been shown in various plant species. In wheat, the overexpression of the Phaseolus vulgaris PGIP2 (PvPGIP2) caused a significant reduction of symptoms following the infection of Bipolaris sorokiniana [8] or

Abbreviations: NS, null segregant; HD, honeydew; HG, homogalacturonan; GaA, galacturonic acid; PG, polygalacturonase; PGIP, polygalacturonase inhibiting protein; CWDEs, cell wall degrading enzymes; PMEI, pectin methyl esterase inhibitor.
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Fusarium graminearum [9], indicating 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.

The activity of fungal PG is also affected by the level of pectin methyl esterification. Pectin is composed mainly of homogalacturonic acid (HG), consisting of a linear homopolymer of 1,4-linked α-D-galacturonic acid (GalA), synthesized in the Golgi apparatus and secreted into the cell wall in a highly methyl esterified form [13]. The de-methyl-esterification of the C6 linked methyl ester group of HG is mainly controlled by pectin methyl esterases (PMEs, [14]) whose activity is regulated by an endogenous inhibitor protein called pectin methyl esterase inhibitor (PMEI, [15]). The level of methyl esterification of cell wall pectin can greatly influence cell wall structure and properties with consequences on several physiological processes [14,16] including the capacity of the plant to respond to pathogens [14,17]. Specifically, PG activity secreted by several fungal pathogens is reduced on highly methyl esterified pectins [18–20]. It has been also reported that pectin epitopes recognized by the monoclonal antibody LM7, which recognize epitopes of HG composed by three or four contiguous unesterified GalA with adjacent or flanking methyl esterified residues are more sensitive to fungal PG and pectate lyase [21,22]. We recently reported that the activity of PGs from B. sorokiniana and F. graminearum is inefficient on highly methyl esterified pectin [23]. According with these results, we demonstrated also that transgenic wheat plants with a high degree of pectin methyl esterification and a low number of epitopes recognized by the monoclonal antibody LM7 showed a reduced disease symptom caused by these pathogens [23].

Because of the importance of PG in the infection process of the biotrophic C. purpurea [1], we tested whether the accumulation of increased level of PGIP or a high degree of methyl esterification of the cell wall pectin in wheat transgenic plants can affect pathogen infection and ergot disease symptom development. To this end, we used transgenic wheat lines expressing the bean PvPGIP2 or the pectin methyl esterase inhibitor from Actinidia chinensis (AcPGMEI) that caused an increase in the level of methyl esterification of the cell wall pectin. Both these transgenic wheat lines have been previously demonstrated to have an improved resistance against the necrotizing fungal pathogens F. graminearum and B. sorokiniana [8,23].

2. Results

2.1. Pvpip2 transgenic wheat plants are fully infected by Claviceps purpurea

A conidial suspension of a C. purpurea strain isolated from wheat plants was used to inoculate 20–40 individual florets of each ear of the transgenic (T) bread wheat line MJ82-23a expressing Pvpip2, hereafter called Pvpip2 plants (for a detailed characterization of this line see Refs. [8]), and the corresponding Null Segregant (NS) plants (“null” genotype that lost the transgene by segregation). Forty-five ears of each transgenic and control plants were inoculated, for a total of 900–1800 florets for each genotype. In the infected ears of both transgenic and control plants, honeydew (HD) was detectable only at 8 dpi and sclerotia were formed at about 20 dpi. Ergot disease symptoms were evaluated both by assessing the HD production on a scale from 1 to 4 and by determining the average number of sclerotia per spike in mature plants. The HD production (Fig. 1) showed different mean values (significant at \(P \leq 0.05\)) between transgenic and NS plants with the maximum difference at 9 dpi, when the average HD score was 2.86 ± 0.19 and 3.31 ± 0.16 for the transgenic and NS plants, respectively. The difference in mean number of sclerotia per spike between transgenic and control plants was non-significant (19.11 ± 1.17 and 17.55 ± 1.32 sclerotia/spike for Pvpip2 plants and NS plants, respectively).

To verify whether pathogen infection was hampered in the early stage of infection, the progression of the pathogen on wheat carpsel was also analyzed by fluorescence microscopy of infected ovaries bisected longitudinally and stained with aniline blue fluorescent dye. The analysis was done on 50 ovaries of each transgenic and control sample excised at 3, 4, 5 and 7 dpi. The analysis was performed also at 1 and 2 dpi but no infection was observed on any carpels at this stage. On the basis of the severity of the infection of the ovary, we grouped the symptoms observed in the following five classes from uninfected to the sphecacelium stage, when the pathogen produces spore and the ovary structure is no longer visible (Fig. 2a). These analyses revealed no significant difference in the rate of infection between transgenic and control plants from 1 dpi through 7 dpi, when the fungus begins to produce spores in most of the ovaries and the honeydew starts to extrude from the floret (Fig. 2b).

2.2. Transcript accumulation, heterologous expression and activity of cppg1 and cppg2

The lack of ergot symptom reduction in the Pvpip2 plants can be due to different causes, including an inadequate level of PvPGIP2 in the wheat transgenic tissues attacked by C. purpurea or the inability of PvPGIP2 to inhibit the activity of the palyogalacturonases (PGs) secreted by this pathogen. To investigate this further, we first tested the inhibitory activity of total protein extracts from different tissues, including ovary and stigma hairs which are the primary sites of infection of C. purpurea. Agarose diffusion assays, performed using a PG of Fusarium phyllophilum (FpPG) that is inhibited by PvPGIP2 but is insensitive to the endogenous wheat PGIP [8], showed that total protein extracts from all tissues of the transgenic plants, but not of that of control plants, including ovary and stigma hairs, inhibited this PG to completion (Fig. 3) indicating the presence of PvPGIP2 activity in these tissues.

To verify the capacity of PvPGIP2 to inhibit the PG activity of C. purpurea, we firstly verified that both pg genes contained in C. purpurea, cppg1 and cppg2, are expressed during wheat spike infection as already observed in infected rye spikes [24]. RT-PCR analysis performed by using primers specific for cppg1 or cppg2 [24] on total RNA extracted from infected wheat spikes showed the expression of both genes (data not shown). Based on this result,
primers spanning the complete coding region were developed and used to amplify both genes by RT-PCR. The amplification product corresponding to **cppg1** and **cppg2** were cloned and characterized. Nucleotide sequences of these genes are identical to those reported in databases (CpPG1, accession number CAA71246 and CpPG2, accession number CAA71247). CpPG1 and CpPG2 share about 91% and 95% identity at the amino acid and nucleotide level, respectively. Removal of the predicted N-terminal signal peptide (at alanine 19) generates mature proteins of 350 amino acids (theoretical molecular mass of 36188 Da, predicted pI = 9.08) for CpPG1 and 351 amino acids (theoretical molecular mass of 36656 Da, predicted pI = 6.72) for CpPG2. Both proteins contain also three or four potential N-glycosylation sites (at asparagine 69 for CpPG1 and at asparagine 217/218, 280/281 and 314/315 for CpPG1 and CpPG2, respectively).

Both **cppg1** and **cppg2** were expressed in *Pichia pastoris* and the purified proteins showed on SDS-PAGE a single band with an apparent molecular weight of about 32–33 kDa ([Supplementary Fig. 1](#supplementarymaterial)). Neither purified CpPG1 and CpPG2 were inhibited by purified PvPGIP2 in agarose diffusion assays ([Fig. 4a](#figure4a)). Inhibition assays

![Fig. 2.](#figure2) Progression of wheat ovary infection by *C. purpurea*. a) Classes of wheat ovaries recognized following infection with *C. purpurea*. **NI** = Non Infected; **LI** = Lightly Infected; **HI** = Heavily Infected, when the hyphae do not reach the base of the ovary; **AS** = Almost Sphacelium, when the hyphae ramify through the host tissue but the host cellular structure and the ovary is still visible, and **S** = Sphacelium, when the fungus is in the sphacelial spore-producing stage and the ovary structure is no longer visible. b) Percentage of the different classes of infected ovaries by *C. purpurea* in transgenic (T) and null segregant plants (NS) at 3, 4, 5, and 7 dpi. No significant differences in the rate of infection between transgenic and NS plants were observed.

![Fig. 3.](#figure3) Agarose diffusion assay of crude protein extract from Pvpgip2 transgenic (T) and null segregant (NS) plants. The assay was performed using 0.0011 reducing units of *Fusarium phyllophylum* endopolygalacturonase (FpPG). 1, Purified FpPG. Purified FpPG plus crude protein extract (1 mg) from: 2, leaf of T; 3, stigma hair of T; 4, ovary of T; 5, leaf NS; 6, boiled T. The lack of the halo indicates the inhibition of FpPG activity.
with crude protein extracts from transgenic and null segregant plants showed that both PGs were partially inhibited to a similar extent, probably by the activity of the endogenous wheat PGIP (Fig. 4a). Conversely, the same amount of total protein extract from Pvgpgip2 plants, but not that from null segregant plants, inhibited to completion FpPG, that is inhibited by Pvgpgip2 but is insensitive to the endogenous wheat PGIP (Fig. 4a).

Altogether, these results highlight that the lack of in vitro PG inhibition by Pvgpgip2 is consistent with the absence of the expected improved resistance of Pvgpgip2 plants against C. purpurea.

2.3. The activity of CpPG1 and CpPG2 is strongly reduced on methyl esterified pectin

Since PG activity can be affected by the degree of pectin methyl esterification, we tested the activity of CpPG1 and CpPG2 on highly methyl esterified pectin (70–75%). Agarose diffusion assays using polygalacturonic acid (PGA) or 70–75% methyl esterified pectin as substrate showed almost no hydrolytic activity of both PGs on methyl esterified pectin compared to that observed on PGA (Fig. 4b).

2.4. Reduced ergot symptoms on Acpmei wheat plants

Since cell wall pectin methyl esterification can influence plant resistance to fungal pathogens, transgenic durum wheat line MJ15-69 expressing AcPMEI, hereafter called Acpmei plants, and possessing a degree of methyl esterification of the cell wall pectin increased by more than 50% compared to control plants (for a detailed characterization of this line see Refs. [23]), were infected with a conidial suspension of C. purpurea. Two independent tests were performed by inoculating individual florets (12–27) of each ear of Acpmei plants and corresponding null segregant plants. In the infected ears of both transgenic and control plants, honeydew (HD) was detectable at 5 dpi and sclerotia were formed at about 20 dpi. As with the Pvgpgip2 transgenic inoculations, ergot disease symptoms were evaluated both by assessing the honeydew production on a scale from 1 to 4 (Fig. 5a) and by the average number of sclerotia per spike in mature plants. The HD production (Fig. 5a)
was significantly lower ($P < 0.01$) in Acpmei plants at 5 dpi (Fig. 5a) but not significant at later stage of infection. At 5 dpi the average HD score was $1.59 \pm 0.40$ and $3.18 \pm 0.32$ for the transgenic and NS plants, respectively. Moreover, the average number of sclerotia per spike in mature plants was significantly lower ($P < 0.01$) in the transgenic plants ($5.00 \pm 0.57$ and $7.30 \pm 0.61$ sclerotia/spike for Pvpip2 plants and NS plants, respectively). To further analyze this last result, we monitored the progression of the infection and determined the percentage of diseased florets on the total number of inoculated florets per spike (Fig. 5b). All time points analyzed showed a significantly ($P < 0.01$) reduced number of infected florets in the transgenic line compared to the control plants (Fig. 5b). For example, at 5 dpi the transgenic plants showed only about 5% of infected spikelets, whereas in the NS plants these were about 15% (Fig. 5b). The same extent of reduction in spikelet infection rate was maintained in the transgenic plants compared to NS plants in subsequent time points (Fig. 5b).

3. Discussion

The potential of PGIP to confer a broad-spectrum disease resistance has attracted the attention for several years. This capacity of PGIP resides in its ability to inhibit polygalacturonases (PGs) that are cell wall degrading enzymes (CWDEs) secreted by most fungal pathogens during the initial stage of infection [25]. The efficacy of PGIP in improving disease resistance has been even demonstrated in wheat, a crop species with a low pectin content in its cell wall, against the necrotizing fungi Bipolaris sorokiniana and Fusarium graminearum [8,9]. In this study, we extended our exploration of the inhibition of CWDEs strategy for disease control...
in wheat to a biotrophic interaction by verifying the efficacy of overexpressed heterologous PGIP activity to protect wheat against the fungal pathogen *C. purpurea*, by using the same wheat transgenic plants that showed reduced leaf blotch and fusarium head blight symptoms (FHB) caused by *B. sorokiniana* and *F. graminearum*. We verified first that during wheat infection *C. purpurea* secretes both cppg1 and cppg2, as reported during the infection of rye [24]. Subsequently, we demonstrated that the ergot symptoms develop almost to the same extent in both PvPpgip2 transgenic and control plants. This result was unexpected because PvPpgip2 is a particular efficient inhibitor, strongly conserved across bean germplasm [26] and capable of inhibiting several different fungal PGs [27,28]. Transgenic plants expressing it have been shown to manifest an improved resistance against a number of fungal pathogens [6,8–10]. Recently, PvPpgip2 has been shown to be effective in improving tobacco plant resistance against a fungus and two Oomycota pathogens and its efficacy has been further demonstrated in field experiments [10].

In the attempt to explain the lack of resistance of the PvPpgip2 wheat plants, we demonstrated that neither Cppg1 nor Cppg2, produced by *C. purpurea* are inhibited by PvPpgip2, suggesting that the lack of improved resistance of the transgenic PvPpgip2 plants is due to incapacity of PvPpgip2 to inhibit both Cppg2s. A similar result has been obtained in transgenic tomato plants expressing PvPpgip1. Also in these experiments a correlation between the limited capacity of PvPpgip1 to inhibit in vitro the PGs of *Fusarium oxysporum f. sp. lycopersici*, *Botrytis cinerea*, and *Alternaria solani* and the lack of resistance against these pathogens was reported [29].

Taken all together, these results support the notion that PG inhibition by PGIP is a prerequisite to exploit the properties of PGIP to improve plant resistance. Indeed, in the traditional assumption, a direct interaction between PG and PGIP is necessary for both limiting the hydrolytic activity of PG and to favour the release of elicitor active oligogalacturonides [30]. However, some recent findings indicate that the direct inhibition of PG by PGIP, as determined in *in vitro* assays, is dispensable to obtain a protective effect by PGIP. Joubert et al. [31], showed a reduction of symptoms caused by the activity of BCPG2 on plant tissue when co-infiltrated with PvPpgip1, although no interaction between PvPpgip1 and BCPG2 was detected in the *in vitro* assays. In agreement with these results, previous experiments showed that PvPpgip2 interacts with the pectin component of the cell wall [32], suggesting a possible role of PGIP in shielding the pectin component from PG activity. Recently, it has been also reported that the expression of PvPpgip1 in transgenic tobacco plants caused some modifications in the cellulose-xyleloglucan components of the cell wall, suggesting that these subtle cell wall modifications can also contribute to the observed improved resistance of the PvPpgip1 transgenic tobacco plants against *B. cinerea* [33]. In this view, the slightly reduced HD production in the PvPpgip2 transgenic plants compared to NS plants could indicate an effect of PvPpgip2 on pathogen growth, however, its contribution does not produce a noticeable phenotypic effect on ergot hodyenw symptoms nor in the resulting number of developed sclerotia.

A more clear effect on ergot symptoms was obtained by inoculating *C. purpurea* on Acpmei plants that possess a higher degree of pectin methyl esterification as compared to control plants. These experiments showed a significant reduction of HD symptoms at 5 dpi, similar to results obtained with the same Acpmei plants when challenged with *B. sorokiniana* and *F. graminearum* [23]. In all these infection experiments, the reduction of disease symptom development in the Acpmei plants compared to control plants is particularly evident in the early stage of infection. In the infection experiments with *C. purpurea* the reduced ergot symptoms were evident both as a delay in the onset of HD symptoms and also as reduced number of infected spikelets, indicating that a high degree of pectin methyl esterification can prevent or at least delay the establishment of *C. purpurea* infection. These results correlate with the strong reduction of activity showed by both PGs of *C. purpurea* on pectin with a high degree of methyl esterification, supporting the importance of PG activity for the development of the ergot disease in wheat, as already demonstrated in rye [11].

In conclusion, these results suggest that the lack of resistance of PvPpgip2 transgenic wheat plants against *C. purpurea* is due to the lack of inhibition of both PGs of this pathogen by PvPpgip2, thus reinforcing the notion that direct inhibition of PG by PGIP is of primary importance to exploit its protective role. We demonstrated also that the PG activity of *C. purpurea* is negatively affected by the pectin methyl esterification and that wheat transgenic plants possessing a high degree of pectin methyl esterification showed reduced ergot symptoms at early stage of disease development, thus indicating the importance of PG activity for the development of the ergot disease in wheat and suggesting the possibility to exploit genotypes with a high degree of pectin methyl esterification to improve wheat resistance against the ergot disease.

### 4. Methods

#### 4.1. Fungal growth, cloning and expression of cppg1 and cppg2

*C. purpurea* isolate O3–20.1 was cultured on potato dextrose agar (PDA; Fluka, BioChemika).

*C. purpurea* pg genes were isolated from total RNA obtained from wheat spikes infected with *C. purpurea* isolate O3–20.1 at 14 days post inoculation (dpi). Total RNA extraction was performed using “RNeasy Plant mini” Kit (Qiagen GmbH, Milano, Italy), starting from no more than 100 mg of wheat spike tissue. cDNA was generated using ImProm-II Reverse Transcriptase (Promega, Italy) according to the manufacturer’s protocol, using oligo dT primer. The following primer pair 5′-ATGTTATCTGCTCAGTTT-3′ and 5′-TTAACAAT TAACCTGGCC-3′ was used to amplify both cppg genes by PCR from the synthesized cDNA. The amplified product was cloned into the pGEM-T plasmid vector (Promega, Milano, Italy) and used to transform *Escherichia coli* DH5α. Transformed colonies were screened using specific primer pairs: 5′-GGGTAGCTTTGGTGCTGGGC-3′ and 5′-CCTAGTG AGTGCTGGCC-3′ for cppg1 and 5′-GGGTAGCTTTGGTGCTGGGC-3′ and 5′-CTAGTGCTCCACGGCG-3′ for cppg2 as described by Tenberge et al. [24]. Positive colonies were selected to isolate the plasmids containing cppg1 or cppg2 and then the recombinant plasmids were sequenced. The deduced products of cppg1 and cppg2 were analyzed for the presence of a signal peptide cleavage site (predicted by SignalP program, http://www.cbs.dtu.dk/services/SignalP/) and N-glycosylation sites (predicted by ScanProsite, http://www.expasy.org/).

The coding region of cppg1 or cppg2 corresponding to the deduced mature proteins were amplified with Pfu DNA polymerase (Promega, Milano, Italy) using the following primer pairs 5′-A TGCGAATCCCGCACTTGCC3′ and 5′-ATGGCTCTACCAATCAGA TAACAACCGCC-3′ to introduce EcoRI/XbaI restriction sites (under lined). The PCR products were ligated into the EcoRI and XbaI sites of the pPICZαA vector (Invitrogen, Invitrogen Milano, Italy) and used to transform *Pichia pastoris* strain X-33 by electroporation using the BTX ECM 630 electroporator (Harvard Apparatus Company). The settings for *P. pastoris* were 1500 V, 400Ω, 25 μF. Transformants were selected on YPD agar medium supplemented with 100 μg/ml zeocin (Invitrogen, San Giuliano Milanese [MI], Italy). Some positive colonies were grown in 20 ml of BMGY medium (Invitrogen, San Giuliano Milanese [MI], Italy) at 28 °C with shaking at 250 rpm until the cells were in log-phase (approximately 20 h), harvested by centrifugation at 3000 g for 5 min and resuspended in 100 ml BMGY medium (Invitrogen, San Giuliano Milanese [MI], Italy) for 48 h. The same conditions were used for the December 2010 batch.
Milanese (MI), Italy). Methanol was added every 24 h to a final concentration of 0.5% (v/v). After three days, cells were pelleted by centrifugation at 10,000 g for 15 min and the supernatant of the culture was assayed for PG activity.

The secreted recombinant PGs from cultures of *F. phyllophilum* were purified by ammonium sulphate precipitation and affinity chromatography on Concanavalin A-Sepharose. After ammonium sulphate precipitation (80% saturation), the precipitated was dissolved in 5 ml of 50 mM sodium acetate, pH 6.0, containing 1 M NaCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$ and 1 mM MnCl$_2$, dialyzed overnight against the same buffer and loaded onto a column containing 750 µl of Concanavalin A-Sepharose. After binding, the column was washed exhaustively to remove non-specifically bound proteins (until OD$_{280}$ < 0.01) and eluted with 750 µl of the same buffer amended with 0.25 M methyl alpha-D-mannopyranoside. The most active fraction was used in the successive assays.

4.2. Enzymatic activity and inhibition assays

Crude protein extracts containing PGIP activity were obtained from tissues of transgenic or control plants, as previously described [27]. Protein concentrations were determined with the Coomassie plus-the-better Bradford assay kit (Pierce, Rockford, IL, USA).

Endo-polygalacturonase (PG) of *F. phyllophilum* (previously classified as *Fusarium moniliforme* [34]) was kindly provided by Prof. G. De Lorenzo, University of Rome La Sapienza, Italy). Enzymatic activity of PGs and inhibitory activity of PGIP were measured using agarose diffusion assay in the presence of 20 mM Na acetate, pH 4.6 as described by [4]. This assay uses polygalacturonic acid (PGA) or pectin as substrate and inhibition of PG activity by PGIP results in a size-reduction of clearing zones (halo) or even in the absence of halo. PG activity was expressed as agarose diffusion PG units (APU), with 1 APU defined as the amount of enzyme that produced a halo of 0.5-cm radius (external to the inoculation well of 0.5-cm radius) after 18 h at 30 ºC.

4.3. Fluorescence microscopy

Ovaries from infected transgenic and control plants were collected up to 7 dpi, longitudinally bisected and incubated with 1 M KOH overnight. After washing extensively with water, ovaries were stained with 0.05% aniline blue (w/v) in 0.067 M K$_2$HPO$_4$ for 2 h at room temperature [35] and then observed with a microscope (Zeiss Axioscop 20) equipped for epifluorescence microscopy (mercury short arc lamp HBO 50 W). We grouped the symptoms observed in the following five classes: Non Infected (NI), Light Infected (LI; when the hyphae do not reach the base of the ovary), Heavy Infected (HI; when the hyphae reach the base of the ovary), Almost Sphacelium (AS; when the hyphae ramify through the host tissue but the host cellular structure and the ovary is still visible) and Sphacelium (S; when the fungus is in the sphacelium stage producing spore and the ovary structure is no longer visible).

4.4. Plant growth and infection assays

Wheat seeds were surface sterilized with sodium hypochlorite (0.5% vol/vol) for 20 min and then rinsed thoroughly in sterile water. Plants were vernalized at 4 ºC for 2 weeks and grown in a climatic chamber at 18–23 ºC with a 14-h photoperiod (300 µE m$^{-2}$ s$^{-1}$).

The transgenic bread (*Triticum aestivum* cv Bobwhite) wheat line J82–23a expressing *Pvpgip2* [8], the transgenic durum (*T. durum* cv Seveo) wheat MJ15-69 expressing *Acpmei* [23] and the control plants (Null Segregant plants, the corresponding “null” genotype that lost the transgene by segregation) were used for the infection experiments.

Individual florets of each ear of wheat transgenic and control plants at the Zadoks stage 59 were inoculated with a conidia suspension of a single isolate (D3–201) of *C. purpurea*. Conidia concentration in the honeydew from infected wheat spikes was estimated using a Thoma hemocytometer adjusting the final concentration to 1 x 10$^5$ conidia/ml.

The infection experiments were performed by inoculating individual florets of each ear of both transgenic and control plants. A single experiment was performed with the *Pvpgip2* transgenic and control plants using 45 plants each, whereas two separate infection experiments were performed with the *Acpmei* transgenic and control plants by using 13 plants of each genotype for each experiment.

Disease symptoms were evaluated by assessing the honeydew (a sugary fluid containing millions of conidia produced by the fungus at the sphacelium stage) exuded from inoculated florets based on a scale from 1 to 4 [36] and by the average number of sclerotia (the over-wintering structures of the fungus which replace the normal grain) per spike. For the *Acpmei* transgenic and control plants, disease symptoms were evaluated, in a time-course manner, also by assessing the percentage of diseased florets on the total number of inoculated florets per spike.

Data from infection experiments were analyzed statistically applying the Student’s t test.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at [http://dx.doi.org/10.1016/j.plaphy.2013.10.011](http://dx.doi.org/10.1016/j.plaphy.2013.10.011).

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