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FULL RESEARCH PAPER

Fusarium head blight evaluation in wheat transgenic plants expressing the maize *b-32* antifungal gene

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Abstract The maize gene *b-32*, normally expressed in the maize (*Zea mays*) endosperm, encodes for a RIP (Ribosome Inactivating Protein) characterised by antifungal activity. Transgenic wheat plants were obtained via biolistic transformation, in which the *b-32* gene is driven by the *35SCaMV* promoter in association with the *bar* gene as a selectable marker. Plants were brought to homozygosity through genetic analysis of progeny and pathogenicity tests were performed on the fourth generation. Six homozygous b-32 wheat lines were characterised. All plants had a normal phenotype, not distinguishable from the control cv. Veery except for slightly smaller size, flowered and set seeds. Western blot analyses confirmed b-32 expression during the plant life cycle in the various tissues. Each line differed in the b-32 content in leaf protein extracts and the transgenic protein expression level was maintained at least up to 10 days after anthesis. Pathogenicity tests for Fusarium head blight (FHB) were performed on the b-32 transgenic wheat lines in comparison to the parental cv. Veery. Resistance to FHB was evaluated by the single floret injection inoculation method on immature spikes with spores of *Fusarium culmorum*. In all the transgenic lines, a similar reduction in FHB symptoms, not dependent on the level of b-32 protein, has been observed (20% and 30% relative to the control, respectively 7 and 14 days after inoculation). Percentage of tombstone kernels at maturity was also recorded; in all transgenic lines disease control for this parameter was around 25%. The data obtained indicate that maize b-32 was effective as in vivo antifungal protein reducing FHB symptoms in wheat lines expressing the maize RIP protein.

Keywords Antifungal-activity · Maize b-32 · *Fusarium culmorum* · Ribosome Inactivating Protein · *Triticum aestivum*

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Introduction

Fungal disease resistance is a major goal in plant breeding of cereal species since cereal diseases represent one of the major sources of quantitative

as well as qualitative damage for the grain. Fusarium species are a widespread group of pathogenic fungi on cereals in the world (Bottalico, 1998). They can infect various parts of the plant causing root, stem and ear rot in both small-grain cereals (wheat, rice, barley, triticale, oat, rye, sorghums and millets) and maize, with severe reductions in yield. In addition, several Fusarium strains are responsible for mycotoxin production, which can be found either in growing plants, or in stored seeds. Fusarium diseases are due to a co-occurrence of several Fusarium species usually referred to as a species complex (Parry, Pettitt, Jenkinson, & Lees, 1994). Fusarium strains considered pathogenic and responsible for the main damage to wheat in central Europe, have been described in Toth et al. (2005). Wheat head scab (WHS) or Fusarium head blight (FHB) is an important wheat disease (Parry, Jenkinson, & McLeod, 1995; McMullen, Jones, & Gallenberg, 1997; Dardis and Walsh, 2002) predominantly caused by *Fusarium graminearum* and *Fusarium culmorum*. Infected wheat spikes show characteristic premature death or blighting of spikelets; floret sterility and poor or absent grain filling are the causes of severe yield losses. In addition, mycotoxin production, such as deoxynivalenol (DON), nivalenol and zearalenon, are often associated with the disease occurence (Bottalico, 1998).

A major constraint in wheat cultivation, especially in those areas where climatic conditions promote disease development is the fact that no highly resistant cultivars are available (Rudd, Horsley, McKendry, & Elias, 2001). Due to their impact on the plant and on human and animal health, Fusarium pathogens have been thoroughly investigated and the search for resistant cereal genotypes via conventional breeding continues. Despite lacking complete resistance to FHB, the Chinese cultivar Sumai 3 and its derivatives are considered as the most effective sources of resistance so far discovered, and have been widely used in crosses (Bai and Shaner, 1994). Nevertheless, commercially acceptable FHB resistant cultivars have not yet been developed and the control of FHB by fungicides is ineffective. FHB resistance appears to be under polygenic control, making the development of resistant cultivars with suitable agronomic and quality traits a challenge. Molecular markers linked to FHB resistance have been discovered; however, because FHB resistance is quantitatively inherited, it is not expected that the use of markers will replace conventional phenotypic screening based on greenhouse or field methods in the near future (Van Sanford et al., 2001).

An alternative strategy is to use genetic engineering for the identification and cloning of defence genes and their introduction and exploitation into crop plants. Defence genes can be identified and cloned from plants, either crop species or wild relatives (Jones et al., 1994; Khush, Bacalangco, & Ogawa, 1990; Song et al., 1995), from microorganisms (Lorito et al., 1998), or different unrelated sources (Tossi, Sandri, & Giangaspero, 2000; Cary, Rajasekaran, Jaynes, & Cleveland, 2000; Zasloff, 2002; Coca et al., 2004, 2006). The introduction and expression of some of these genes has been shown to confer increased resistance to fungal plant pathogens, alone or in combination, and the approach has been successfully transferred from model plants such as tobacco (Jach et al., 1995) to crop plants, for example, cereal species (Bliffeld, Mundy, Potrykus, & Futterer, 1999; Chen et al., 1999; Krishnamurthy, Balconi, Sherwood, & Giroux, 2001).

In addition to the above, another class of plant proteins also displays antifungal properties. These are known as RIPs (Ribosome-Inactivating Proteins), and are specific N-glycosidases that inactivate the ribosome and block translation elongation by removing an adenine residue from the highly conserved stem-loop structure (SRL) in the large rRNA, which interacts with the elongation factors during protein synthesis (Hartley, Chaddock, & Bonness, 1996). Ribosome reconstitution experiments demonstrated that L3 is one of the first ribosomal proteins to be assembled into the ribosome and it is required for peptidyltransferase activity; the L3 binding site on ribosome has been localized to the SRL loop (Hampl, Schulze, & Nierhaus, 1981). In addition, Hudak, Dinman, and Tumer (1999) reported that Pokeweed antiviral protein (PAP, a RIP protein) and Fusarium mycotoxins tricothecenes inhibit protein synthesis by targeting

ribosomal protein L3 in order to depurinate the SRL loop of the large rRNA. Recently Di and Tumer (2005) have shown that the expression of a truncated form of L3 confers in tobacco resistance to PAP and to the Fusarium mycotoxin DON.

Cereal species contain RIPs in their grain endosperm such as barley (RIP30) and maize (b-32) that are weakly active or inactive against the producing cell's ribosomes, but which are active against alien eukaryotic ribosomes, such as the ones of pathogenic fungi (Nielsen and Boston, 2001; Motto and Lupotto, 2004). An accumulating body of data for the exploitation of plant RIPs as antifungal agents is in the current literature. Bieri et al. (2000) obtained transgenic wheat plants expressing RIP30; although the transgenic protein was effectively detected and its RIP activity maintained, its antifungal action against *Erysiphe graminis* was very small. Moreover, RIP30 driven by the strong constitutive *ubiquitin-1* promoter of maize was introduced in wheat and transgenic plants engineered with three single antifungal genes–*RIP30*, *Ag-AFP* and *chitinase-II*- then challenged for response to powdery mildew and rust. Significant reduction of infection was observed in Ag-AFP and Chi-II expressing plants, but not in RIP30-expressing wheat lines compared with the control (Oldach, Becker, & Loerz, 2001).

The maize b-32 defence properties against various biotic agents have been evaluated: antifungal activity was demonstrated in vitro and in vivo (Maddaloni et al., 1991, 1997) and its relative toxicity to insects has been proven (Dowd, Mehta, & Boston, 1998). Its expression in plant heterologous systems gave contrasting results. Expression of b-32 in transgenic tobacco resulted in enhanced tolerance to *Rhizoctonia solani* (Maddaloni et al., 1997), while its expression in rice did not result in increased resistance to *R. solani* and *Magnaporthe grisea* (Kim et al., 1999).

In order to further explore the antifungal activity of the maize *b-32* gene, six homozygous wheat lines expressing b-32 were raised and brought to homozygosity through genetic analysis of progeny. These plants showed co-integration of both *bar* and *b-32* genes in low gene copy number, estimated as one to three from Southern analyses. Expression of b-32 in leaf protein extract of the six lines was confirmed throughout the plant life cycle via immunoassay. Investigations of the same transgenic lines, made at the seedling level, had shown a significative increase in resistance to two fungal pathogens, *Blumeria graminis* f. sp. *tritici* and *Puccinia recondita* f.sp. *tritici* (Lupotto et al., 2003; Motto and Lupotto, 2004).

In this study the six wheat transgenic lines were challenged for response against *F. culmorum*, one of the major fungi responsible for wheat head scab or Fusarium head blight (FHB), in order to detect the potential protective role of b-32 against fungal diseases.

Materials and methods

Plant materials and growing conditions

The parental wheat (*Triticum aestivum)* genotype used was cv. Veery, considered a FHB-medium-sensitive genotype among currently cultivated wheats. Transgenic individuals were obtained from cv. Veery via biolistic transformation with the vector pSC1.b-32, in which the maize gene *b-32*–encoding for the RIP protein b-32–was driven by *35SCaMV* constitutive promoter and associated with the *bar* gene–encoding resistance to glufosinate ammonium–as a selectable marker (Lupotto et al., 2003). The six transgenic homozygous progenies analysed in the present work (V45-5, V45-8, V45-18, V45-19, V45-21 and V45-39) were obtained via genetic and molecular analysis up to the fourth generation. Plants were grown in a Class one containment-greenhouse under current national regulations for GMO plants (D.Leg.vo n°206 12.04.2001 according to EU Directive 98/81/CE) in pots in 1:5 mixture of washed sand: peat basic soil, and in a growth chamber at 21+/–2°C day and 18+/–2°C night temperatures, at 70% RH, under a 16 h photoperiod (about 6,000 lux) provided by fluorescent lights Osram 36W/21-840 Lumilux Plus. Plants were hand-watered every other day, and fertilized once a week with half-strength MS (Murashige and Skoog, 1962) basic salts from tillering to flowering stages. Each experimental unit consisted of a 15 cm tall pot containing five plants.

Six pots giving a total of 30 plants per treatment were used in each experiment.

Control of b-32 expression in transgenic plants

Samples of about 200 mg fresh leaves during various stages of development (a) IV leaf seedlings (6 weeks), (b) tillering (9 weeks), (c) ten days anthesis (11 weeks) were ground to fine powder in liquid nitrogen and homogenized in extraction buffer containing 50 mM Tris–HCl pH 6.8, 10 mM EDTA, and 0.1 mM phenyl methyl sulphonyl fluoride (PMSF), cleared twice with centrifugation at 14,000 rpm at 4°C for 10 min. Protein concentration in the total soluble extract was measured using the Bio-Rad Protein dye reagent. Bovine serum albumin was used as a standard. Polyclonal antibodies were raised in rabbits injected with the recombinant GST-b-32 expressed in *E. coli* (Triulzi, Lupotto, & Forlani, 2004). Antisera specificity and sensitivity was high, and amounts as low as 50 ng GST-b32 could be detected. Proteins (20 μg) were separated by 12% SDS-PAGE (Mini-PROTEAN II Cell, Bio-Rad), transferred to a nitrocellulose membrane (PROTRAN^{TM} Schleier and Schuell), probed with antiserum to GST-b32 (1:1,000), using horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG as secondary antibody (Bio-Rad). HRP staining was carried out with 0.009% H_2O_2 and 0.06% 4-chloro-1-naphtol. Protein amounts loaded were approximately 20 μg for total soluble leaf extract and 5 μg for the maize endosperm positive control. In western blots, the b-32 protein amount was estimated by densitometric measurements (Image Master 1D Elite Ver 3.01, Non Linear Dynamics Ltd.) of the signal strength given by the western blot bands in comparison to known amounts of purified GST-b-32 expressed in *E. coli*. Experiments of densitometric measurements were repeated twice; means and standard error bars are reported in the Results section of this paper.

FHB–artificial inoculation method

Resistance to scab was evaluated by a single floret injection inoculation method (Wang, Yang, & Xiao, 1982). Artificial inoculation was performed on the parental cv. Veery and transgenic plants at the early grain filling stage by puncturing the spike with a hypodermic needle and injecting spores of *F. culmorum*. The *F. culmorum* (IS-PAVE strain 1602, from V. Balmas, University of Sassari, Italy) strain was grown on PDA (Potato Dextrose Agar) medium in Petri dishes. Cultures were maintained at 26°C with 16 h light per 24 h day, up to complete development of mycelium on the agar surface. Conidial suspensions were prepared fresh just before inoculation. The plate surface was washed with 5 ml of sterile distilled water (SDW) and the conidial suspension was adjusted with SDW to the final desired concentration using a Bürker haemocytometer. Plants were inoculated with an injection of 20 μl of *F. culmorum* spore suspension in the central part of the spike. For optimization of the tests, three spore concentrations were tested: 10^5, 10^6, and 10^7 spores ml^{-1}. Control plants were inoculated with SDW. Following injection, spikes were covered with a transparent plastic bag for 48 h to provide conditions favouring disease development with high RH (relative humidity).

FHB–evaluation methods

A visual scale, based on percentage of infected spikelets, was chosen to estimate scab disease severity, as described by Stack and Mc Mullen (1998). Visual inspection for disease assessment was firstly performed on control cv. Veery at 7, 14, 21 and 28 days after inoculation, by means of spikelet observation and counting. FHB disease assessment was made 7 and 14 days after inoculation, counting white spikelets per inoculated spike and recording other visible symptoms of infection such as mycelial growth. The severity of symptoms was scored as the percentage of bleached spikelets of total number per spike. Each treatment was evaluated with ten plants. At maturity, spikes were manually harvested, glumes were removed, and scab-infected kernels (tombstones) were evaluated (McMullen and Stack, 1994). Ten replicates for each parameter were used in the experiments. The reduction in disease symptoms of transgenic lines relative to the control cv. Veery (disease control %) was calculated as reported in Krishnamurthy et al. (2001).

All data were analysed by ANOVA. All values were analyzed by the MSTAT-C-Programme, Michigan State University, Version 1991. Least significant difference (LSD) (Student's *t* test = $P \leq 0.05$) was used.

Results

Transgenic wheat lines characterization for b-32 expression

Transgenic lines of hexaploid wheat (*T. aestivum*) cv. Veery containing the maize gene *b-32* under the *35SCaMV* promoter and the *bar* gene for resistance to L-glufosinate as selectable marker, were obtained via microprojectile bombardment as previously described (Lupotto et al., 2003). The six homozygous lines at the fourth generation after transformation were used for the present investigation to evaluate FHB resistance in adult plants. In order to confirm that the b-32 expression was maintained during the following developmental stages, immunoblot assays were performed on plants at three different stages: (a) seedlings at the fourth leaf stage, (b) tillering, and (c) ten days after anthesis. The immunoblot of Fig. 1 shows the results: no bands were detectable for cv. Veery at any time, while the typical pattern with a double banding was visible in the V45-8 lanes in the three stages of development at comparable levels, and in the control maize endosperm W64A, as expected (Fig. 1–lane C). A similar pattern of b-32 expression was detected in immature spikelets and rachis (data not shown). These results confirmed a stable expression level of b-32 in green tissues of the transgenic lines throughout their development.

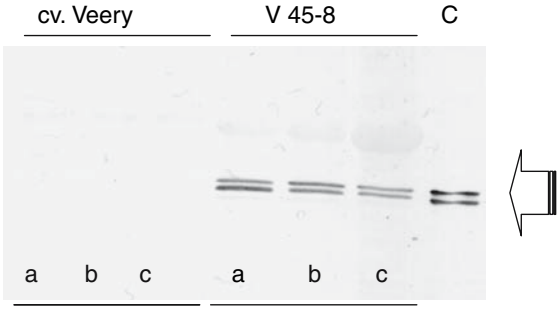

Fig. 1 Expression of b-32 in transgenic wheat plants of line V45-8 during various stages of development: (a) IVth leaf seedlings, (b) tillering, (c) ten days after anthesis, in comparison with the control non-transgenic cv. Veery. Lane C: maize inbred W64A endosperm extract as a control. Leaf extracts containing 20 μg total proteins were separated by 12% SDS-PAGE, and western blotting performed with polyclonal antibodies raised in rabbits injected with the recombinant GST-b-32 expressed in *E.coli*

The comparison of b-32 content in the protein leaf extracts at the heading stage was performed by means of immunoblot imagine scanner and is shown in Fig. 2 (means and standard error bars of two independent analyses). Differential b-32 expression in the various progenies was recorded. Line V45-8 showed the highest b-32 content, accounting for approximately 6 ng mg^{-1} total proteins; this line was chosen as a reference indicated as 100% for b-32 relative abundance in leaves. The other lines had lower b-32 levels: the highest amount was in V45-19 (87.8%) while V45-39, V45-5, and V45-21 had respectively 72.9, 62, and 57.4% b-32 content. Progeny V45-18 showed the lowest b-32 content (15.6%) compared to the reference line. As expected, control non-transgenic cv. Veery did not show any expression of cross-reacting proteins. All plants had a normal phenotype, not distinguishable from control cv. Veery except for slightly smaller size, flowered and set seeds.

Infection tests with *Fusarium culmorum*

A first set of experiments aimed at the identification of the most suitable conditions for artificial inoculation for the differentiation of the FHB response was performed on the control cv. Veery. For this purpose, three spore concentrations (10^5, 10^6, and 10^7 spores ml^{-1}) were tested. Control plants non-inoculated or inoculated with SDW remained disease-free throughout the experimental period. Symptoms were easily detectable when plants were covered with plastic bags in order to maintain suitable conditions for disease development. Inoculated plants were analyzed for FHB by visual inspection of the injected heads as shown in Fig. 3. No changes in colour or other morphological alterations were observed in glumes of control plants (Fig. 3-C). The severity

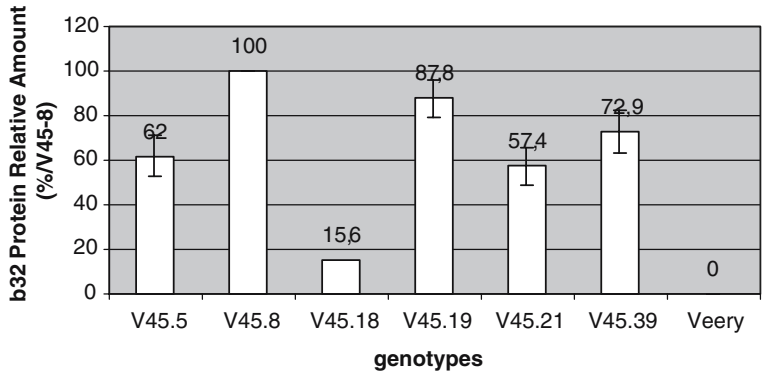

Fig. 2 Relative amount and comparison of b-32 amount in protein leaf extracts of transgenic lines at the heading stage. Histograms show the relative b-32 content with respect to V45-8 taken as 100% reference line. The b-32 amount was estimated by densitometric measurements (Image Master 1D Elite Ver 3.01, Non Linear Dynamics Ltd.) of the signal strength given by the western blot bands, in comparison to known amounts of purified GST-b-32 expressed in *E.coli*.

Fig. 3 Visual scale to estimate severity of Fusarium head blight following *F.culmorum* artificial inoculation. C: control plants inoculated with sterile distilled water; from right to left: 20-50-90-100%-infected spikelets/spike

of Fusarium scab disease was classified by the number of spikelets showing premature bleaching on emerging heads: in Fig. 3 disease severity in a range 20–100% infected spikelets is shown.

The highest spore concentration tested (10^7 spores ml^{-1}) induced severe FHB symptoms resulting in 70–80% infected spikelets 7 days after inoculation (Fig. 4). This experimental condition caused such an aggressive Fusarium attack that discrimination among transgenic lines was not possible. On the other hand, the lowest spore concentration (10^5 spores ml^{-1}) caused a weak fungal attack, not suitable for a clear differentiation of the FHB response.

Symptoms induced by 10^6 spores ml^{-1} concentration (30–40% infected spikelets) were not significantly different at any of the four scoring times. Therefore, an intermediate spore concentration of between 10^5 and 10^6 spores ml^{-1} would probably cause FHB symptoms of an intermediate intensity. For a reliable evaluation of the transgenic genotypes this preliminary experiment therefore supported the choice of 5×10^5 spores ml^{-1}, with scoring times of 7 and 14 days after inoculation.

Parental cv. Veery and the six transgenic lines expressing b-32 were artificially inoculated with *F. culmorum* in a containment greenhouse. The plants were analyzed for FBH by visual inspection of the injected heads. The severity of FHB reported as percent of infected spikelets/head, scored at 7 and 14 days after inoculation, was significantly (Student's t test = $P \leq 0.05$) lower in transgenic plants than in control cv. Veery plants, as shown in Table 1. Seven days after inoculation, the disease severity (percentage of wilted and bleached spikeletes/head) was 38.17% in the control cv. Veery, whereas in all transgenic lines the disease severity was not higher than 21.39% (LSD: 3.24). Fourteen days after inoculation, the

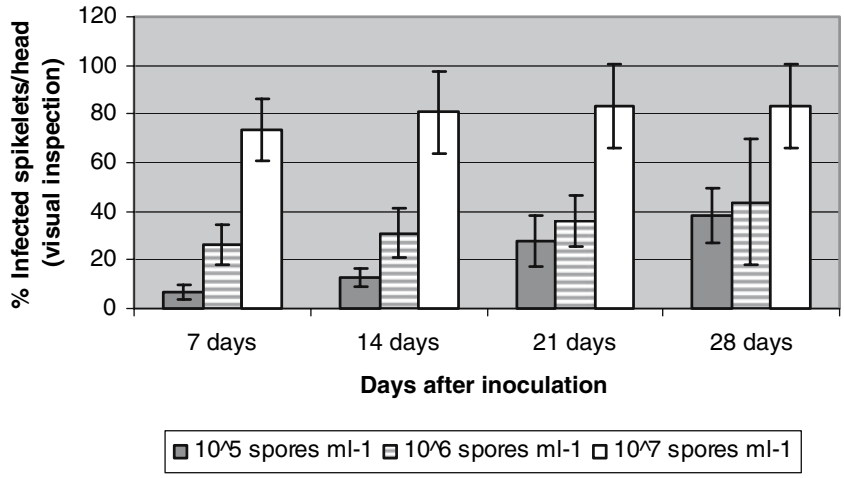

Fig. 4 Severity of Fusarium head blight reported as percent infected spikelets/head in cv. Veery after *F.culmorum* artificial inoculation with 10^5, 10^6, and 10^7 spores ml^{-1}

Table 1 Severity of Fusarium head blight reported as percent of infected spikelets/head after visual inspection (Disease severity %[b]) in cv. Veery and in transgenic lines, and suppression of Fusarium head blight disease symptoms (Disease control % relative to the control [c]) with b-32-expressing transgenic wheat lines, 7 and 14 days after inoculation

Wheat lines[a]	Seven days after inoculation		Fourteen days after inoculation	
	Disease severity (%)[b]	Disease control (%)[c]	Disease severity (%)[b]	Disease control (%)[c]
Veery	38.17 ± 4.45 a	–	51.87 ± 9.08 a	–
V45-5	18.26 ± 2.55 b	19.40 ± 4.34 a	18.26 ± 2.55 b	33.61 ± 9.06 a
V45-8	19.77 ± 3.40 b	18.04 ± 3.39 a	19.77 ± 3.40 b	32.10 ± 9.20 a
V45-18	18.90 ± 1.65 b	19.40 ± 5.08 a	20.74 ± 4.92 b	29.48 ± 9.63 a
V45-19	19.36 ± 3.32 b	18.89 ± 3.65 a	20.74 ± 2.64 b	29.48 ± 8.75 a
V45-21	18.44 ± 2.13 b	19.71 ± 3.12 a	18.44 ± 2.13 b	33.43 ± 8.55 a
V45-39	21.39 ± 2.82 b	16.92 ± 3.23 a	21.39 ± 2.82 b	30.37 ± 10.57 a
LSD[d]	3.24	n.s.[e]	5.28	n.s.[e]

[a] All plants are derivatives of wheat cv. Veery

[b] Percent of infected spikelets/head in cv. Veery and transgenic lines after *F.culmorum* artificial inoculation (5×10^5 spores/ml). Means with the same alphabetical letter are not significantly different (Student's t test = $P \leq 0.05$). All values were analyzed by MSTAT-C-Program (Michigan State University, Version 1991).

[c] Disease severity in Veery–Disease severity in transgenic lines, calculated individually for each replicate before statistical analysis

[d] Least significant difference (Student's t test = $P \leq 0.05$).

[e] not significant (Student's t test = $P \leq 0.05$)

frequency of wilted spikelets/head increased to 51.87% in the non-transformed control; in the transgenic lines the value remained stable around 20% (LSD: 5.28), thus confirming the resistance observed earlier. In all the transgenic lines, a similar reduction in FHB symptoms, not dependent on the level of b-32 protein, was observed. As reported in Table 1, in the six genotypes, no significantly different suppression of FHB disease symptoms by b-32 protein, was shown. Moreover, the reduction in FHB symptoms was around 20% relative to the control 7 days after inoculation and around 30% relative to the control, 14 days after inoculation; at both stages no significant (Student's t test = $P \leq 0.05$) differences between the six transgenic lines were observed (Table 1-Disease control %).

Another parameter used to assess scab disease severity in the tested genotypes was the percentage of tombstones (shrivelled, light-weight, dull greyish or pinkish in colour kernels). In Table 2 the severity of FHB reported as percentage of tombstones/total seeds recorded at maturity in cv. Veery control plants and in the transgenic lines,

are shown (Student's t test = $P \leq 0.05$). Independently from the differential b-32 content of the transgenic lines, the disease severity was equally reduced in all cases, being around 20% in the transgenic lines against 45.71% in control cv. Veery plants (LSD: 10.74). Also in this case, the reduction in FHB symptoms by b-32 was not significantly (Student's t test = $P \leq 0.05$) different in the six transgenic lines tested (Table 2-Disease control %).

Discussion

Transgenic approaches to combat Fusarium head blight in wheat and barley were recently reviewed in Dahleen, Okubara, and Blechl (2001). Various degrees of protection against FHB may be achieved by introducing *in planta* heterologous genes encoding for proteins with anti-Fusarium activity. A variety of anti-fungal genes have been isolated, and some of their products have been shown to inhibit Fusarium growth in vitro and *in planta* (McKeehen, Bush, & Fulcher, 1999). The effectiveness of an anti-fungal protein *in planta* will be determined in part by its expression levels in the crucial host tissues, and by the timing of its expression such that suitable levels accumulate before the host becomes most vulnerable to infection.

Table 2 Severity of Fusarium head blight reported as percent of tombstones/total seeds (Disease severity % [b]) in cv. Veery and in transgenic lines and suppression of Fusarium head blight disease symptoms (Disease control % relative to the control[c]) with b-32- expressing transgenic wheat lines

Wheat lines[a]	Maturity	
	Disease severity (%)[b]	Disease control (%)[c]
Veery	45.71 ± 9.46 a	–
V45-5	19.49 ± 6.99 b	27.60 ± 7.46 a
V45-8	18.26 ± 11.52 b	27.95 ± 11.70 a
V45-18	17.99 ± 10.24 b	29.00 ± 12.05 a
V45-19	16.64 ± 7.61 b	25.87 ± 11.08 a
V45-21	22.17 ± 10.12 b	23.54 ± 6.72 a
V45-39	19.11 ± 8.10 b	26.70 ± 3.35 a
LSD[d]	10.74	n.s.[e]

[a] All plants are derivatives of wheat cv. Veery

[b] Percent of tombstones/total seeds in cv. Veery and transgenic lines after *F.culmorum* artificial inoculation (5×10^5 spores/ml). Means with the same alphabetical letter are not significantly different (Student's t test = $P \leq 0.05$). All values were analyzed by MSTAT-C-Program (Michigan State University, Version 1991)

[c] Disease severity in Veery–Disease severity in transgenic lines, calculated individually for each replicate before statistical analysis

[d] Least significant difference (Student's t test = $P \leq 0.05$)

[e] Not significant (Student's t test = $P \leq 0.05$)

Our experiments aimed to evaluate the action of the protein RIP b-32 in transgenic wheat plants in supporting an increase of Fusarium head blight resistance. The six homozygous transgenic lines used for the present investigation were all phenotypically normal when compared to the parental non-transgenic cv. Veery, except for a slightly smaller size, in fully fertile and set seeds, thus confirming that expression of the exogenous RIP did not interfere with normal plant development (Lupotto et al. 2003). B-32 expression was maintained at comparable levels during various developmental stages of the transgenic wheat plants: at seedling stage, at tillering, and at least up to ten days after anthesis; no eventual endogenous b-32 expression was observed in control wheat plants. Detection of a typical pattern with a double banding is visible in the V45-8 line as in the control maize endosperm W64A. This pattern is characteristic for the maize b-32 protein, belonging to the type-3 class of RIPs, which are synthesized as an inactive precursor (pro-RIP) converted into an active form by partial proteolytic cleavage (Hey, Hartley, & Walsh, 1995). The comparison of b-32 amounts in protein leaf extracts of transgenic lines at the heading stage allowed the identification of lines with high (V45-8, V45-19), intermediate (V45-39, V45-5, V45-21), and low b-32 (V45-18) content in leaves. This is a useful range of expression for pathogenicity experiments, in order to evaluate an eventual differential response to fungal pathogen attack.

Resistance to FHB and its genetic control have been the subject of several studies; following the very innovative Schroeder and Christensen's (1963) definition, two well-established expressions of resistance to FHB are: Type I, resistance to primary infection; Type II, resistance to subsequent colonization after infection. These concepts have been reported many times in subsequent papers (Snijders, 1990; Meidaner,

1997; Stack, 2000; Rudd et al., 2001) and expanded by Mesterhazy (1995): in addition to the previous two FHB resistance components, Type III, resistance expressed in the developing kernel; Type IV, reduced accumulation of mycotoxins; Type V, tolerance as yield maintenance, have been introduced. A good practical indicator of Type I resistance is a reduced incidence of infection, in comparative trials using moderate airborne inoculum. Type II resistance has been the most studied in recent times: it is easy to detect and good sources of it are well documented. The single floret or single spikelet inoculation method selectively distinguishes this type of resistance (Wang and Miller; 1988; Chen, Bai, & Desjardins, 2000). Type III, kernel resistance, is reflected by a reduction in the occurrence of Fusarium-damaged kernels (tombstones) (Mesterhazy, 1995). Each of the above listed resistances is believed to be conditioned by multiple genes showing additive effects. The search for DNA markers for FHB resistance is actively underway in several laboratories (Bai, Kolb, Shaner, & Domier, 1999; Van Sanford et al., 2001). Much less is known about resistance of Type IV and Type V.

In our research FHB Type II resistance using the single floret inoculation method and one aspect of Type III resistance, analysing tombstone kernels abundance, were evaluated. Preliminary experiments aimed at the identification of the most suitable conditions of artificial inoculation for the differentiation of the FHB response. Significant differences (10-fold increase) in inoculum are needed to produce measurable changes (two-fold) in FHB, as previously reported (Stack, 1989). Therefore, in our study, an intermediate spore concentration between 10^5 and 10^6 spores ml^{-1} was expected to induce FHB symptoms gradually detectable for a reliable evaluation of the transgenic genotypes. FHB visual inspection of the injected heads did not show changes in colour or other morphological alterations in glumes of control water-inoculated plants of all genotypes. This observation indicates that the method *per se* does not create damage to the tested material. Glumes of the floret at the point of inoculation (PI) became slightly brown on the second-third day after inoculation, in all the genotypes tested. *Fusarium culmorum* hyphae and mycelium appeared on the spikes of control cv. Veery, on the fourth day after inoculation; no visible mycelium was noticed on the transgenic inoculated plants. In the control cv. Veery heads, infected spikelets first appeared to be water-soaked, but loss of chlorophyll later resulted in a faded straw colour. Despite the differential levels of b-32 in tissues, no significant difference in response to Fusarium artificial inoculation was observed among the six lines tested. This indication suggests that protection due to the presence of b-32 was not dependent on increasing levels of the RIP protein in the tissues, but that the lowest level of b-32 was effective. Protection may be due to the disease avoiding spread in all directions from the PI. Questions regarding the movement of Fusarium hyphae in inoculated wheat spikes and its relationship to cultivar resistance have been recently discussed in Argyris et al. (2005). The authors reported that movement of Fusarium both up and down the spike from the PI only occurred in susceptible genotypes, whereas the fungus was primarily localized around the PI for resistant or moderately resistant cultivars. This last point is important, considering that the infection of the rachis may also result in sterility of the spikelets above the infection and in consequent yield losses. Therefore, transgenic wheat plants constitutively expressing the anti-fungal maize b-32 protein showed a higher level of Type II resistance to FHB, reflected in reduced fungal colonization after artificial single spikelet inoculation.

As previously reported, Type III FHB resistance refers to the resistance expressed in the developing kernel and variation in the level of Fusarium-damaged kernels between cultivars, from that expected at a similar level of FHB symptoms in the spike. One aspect of Type III resistance, results in a reduction in the number of Fusarium-damaged kernels. In fact, FHB derived yield losses are also due to the formation of shrivelled, light test-weight kernels, dull greyish or pinkish in colour, resulting from infection during early kernel development (Stack, 2000). The inner part of infected kernels becomes floury and discoloured. These kernels are sometimes called tombstones because of their chalky, lifeless

appearance. Other scab-infected kernels may be more normal in size, if infection occurred late in kernel development; these latter ones, however, may have discolouration. Also in this case, all the transgenic wheat lines showed a reduced percentage of tombstones independently from the b-32 content. Therefore, the six transgenic wheat lines showed in addition to Type II, also a higher level of Type III resistance to FHB, at least for the reduction in the level of Fusarium-damaged kernels. Our results confirmed the data reported recently for FHB in wheat (Argyris et al., 2005): when visual ratings of FHB spikelet infection following point injection in the greenhouse was below 30%–as for the six transgenic lines tested in the present study–also the percentage of Fusarium seed infection remained below 30%. On the other hand, the authors mentioned above indicated that high visual ratings were poorly associated with the Fusarium presence in seed and other infected floral components.

An important issue in view of fungal protection against FHB is to verify the influence of the engineered anti-fungal b-32 protein in the containment of mycotoxins in the seed. In fact, one of the major concerns in FHB is that the Fusarium complex contains highly toxigenic strains (Bottalico, 1998).

Effective control measures are needed to reduce FHB disease in cereals and to prevent mycotoxins, especially DON, from entering the human and animal food chains. The DON resistance in some wheat cultivars correlates with their resistance to FHB, providing evidence that resistance to DON is a crucial component of resistance to FHB (Miller and Ewen, 1997). The studies reported in this paper indicate that wheat transformants expressing the RIP maize gene *b-32* have an increased resistance towards *F. culmorum*. The production of b-32 protein in leaves of six transgenic lines was evaluated at different developmental stages. In all the transgenic lines, a similar reduction in FHB symptoms, not dependent on the level of b-32 protein, was observed. Disease control by b-32 protein was observed as a reduction of visible FHB symptoms early after inoculation, and also at maturity, as a reduction in percentage damaged seeds.

The expression of antimicrobial proteins in plants or plant tissues, in which they are not normally produced, may have great potential to limit pathogen infection or growth. As reported in this study, maize b-32 was effective as in vivo antifungal protein in wheat, which normally does not produce this protein. One of the most devastating fungal diseases of wheat was therefore controlled at a significant level. These results confirmed, as previously shown in tobacco (Maddaloni et al., 1997), that the incorporation of maize *b-32* gene could be an effective tool in protecting crop plants against fungal diseases.

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