Eur J Plant Pathol (2009) 124:471–482
DOI 10.1007/s10658-009-9434-2

The *Zea mays* b-32 ribosome-inactivating protein efficiently inhibits growth of *Fusarium verticillioides* on leaf pieces *in vitro*

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Received: 5 August 2008 / Accepted: 29 January 2009 / Published online: 15 February 2009

Abstract In maize endosperm, a cytosolic albumin, b-32, with a molecular weight of 32 kDa is synthesised in temporal and quantitative coordination with the deposition of storage proteins. This protein has homology with several previously characterised Ribosome-Inactivating Proteins (RIPs). To verify if the maize plant expressing b-32 in various tissues has an increased tolerance to fungal pathogens, transgenic plants were obtained through genetic transformation using a chimeric gene containing the *b-32* coding sequence downstream of a constitutive *35SCaMV* promoter. A set of four independent homozygous progenies expressing b-32, were selected for a detailed analysis of b-32 expression in leaves and for pathogenicity tests. A differential b-32 content in leaf protein extracts was recorded in the transgenic progenies. Proteomic investigations on protein leaf extracts were carried out; the overlapping of the two-dimensional electrophoresis maps demonstrated the presence in a transgenic progeny, of additional spots, identified as b-32 and as a protein for herbicide resistance, in comparison to the negative control. Transgenic progenies were tested in bioassays to evaluate the response to Fusarium attack in leaf tissues. Preliminary experiments supported the choice of bioassay parameters for a reliable evaluation of transgenic progenies. The negative control was most susceptible to *Fusarium verticillioides* attack, compared to transgenic progenies. The data obtained indicate that maize b-32 was an effective antifungal protein by reducing Fusarium infection progression. Additionally, the reduction in Fusarium attack symptoms was related to b-32 concentration in leaf tissues.

Keywords Antifungal protein · Maize · Plant defence · Transgenic plant

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Introduction

Fungi of the genus Fusarium are widely distributed pathogens of maize, causing diseases of seedlings,

roots, stalks and kernels (Bottalico 1998; Reid et al. 1999). In addition to their effects on yield, Fusarium species can affect grain quality, producing a number of toxic compounds, including fumonisins (Munkvold 2003), involved in human and animal health (CAST 2003). Therefore, the development of maize plants carrying resistance to *Fusarium* ssp. (Bottalico 1997; Lew et al. 1997) as well as resistance to mycotoxin production is highly desirable.

Although sources of resistance to Fusarium ear rot were identified (Gendloff et al. 1986), their genetic basis is not well understood as the methods for evaluating hybrids are unsatisfactory (Zhang et al. 2006). Pyramiding resistance genes through marker-assisted selection appears cumbersome to handle; in addition, quantitative trait loci (QTL) account for no more than 44% of the phenotypic variance for Fusarium ear rot tolerance (Perez-Brito et al. 2001).

Plants respond to attack by pathogenic fungi by mobilising a complex network of active mechanisms such as the production of antimicrobial compounds, enhanced strengthening of cell walls, stimulation of lytic enzyme syntheses, and production of various antifungal proteins (Punja 2001). Plants also constitutively accumulate high levels of proteins that are either toxic or inhibitory to pathogens, including Ribosome-Inactivating Proteins (RIPs). Evidence supporting a defensive role for plant RIPs has been recently documented (reviewed in Motto and Lupotto 2004). Collectively, these studies indicate that RIPs are cytotoxic enzymes that catalytically inactivate eukaryotic, and in some cases prokaryotic, ribosomes by inhibiting protein synthesis by virtue of their N-glycosidase activity. RIP site-specific de-adenylation interrupts factors EF1 and EF2 and blocks protein synthesis at the translocation step (for review, see Nielsen and Boston 2001). Moreover, plant RIPs inhibit ribosomes from phylogenetically-distant species including animals, insects, and fungi (Dowd et al. 1998; Krawetz and Boston 2000) suggesting that RIPs have defensive functions in plants. Therefore, their targeting properties and expression patterns have been recently engineered to produce transgenic plants with increased protection against fungi and viruses and immunotoxins for chemical use (reviewed by Motto and Lupotto 2004).

In maize endosperm, a cytosolic albumin with a molecular weight of 32 kDa, termed b-32 is synthesised in temporal and quantitative coordination with the deposition of storage proteins (Soave et al. 1981; Hartings et al. 1990). Endosperm-derived native b-32 was shown (i) to enzymatically inactivate ribosomes, for its capacity to specifically modify rRNA inhibiting protein synthesis *in vitro* (Walsh et al. 1991; Maddaloni et al. 1991), and (ii) to inhibit the growth of *Rhizoctonia solani* mycelia in an *in vitro* bioassay (Maddaloni et al. 1997). Similarly, Balconi et al. (2007) found that maize RIP-b-32 protein was effective in wheat transgenic lines as an anti-fungal protein by reducing Fusarium head blight (FHB) symptoms. By contrast, Kim et al. (1999) reported that although maize b-32 was expressed at various levels in leaf as well as seed tissues of transgenic rice lines, no challenge was detected against plant pathogens. To further explore the antifungal activity of the maize b-32, transgenic maize plants containing the *b-32* coding sequence under a constitutive *35SCaMV* promoter were obtained through genetic transformation. Accordingly, in this study, four homozygous maize transgenic lines with differential ectopic expression of b-32 were challenged, in comparison with plants expressing b-32 only in the endosperm, for a molecular characterisation of the transgene. Moreover, we have analysed protein profiles and the response of the different progenies against *F. verticillioides* by leaf tissue colonisation bioassays.

Materials and methods

Plant materials and growing conditions

The vector pSC1b32, containing the maize gene *b-32*, driven by the 35SCaMV constitutive promoter and the *ubi1-bar* cassette (phosphinothricin acetyltransferase) conferring gluphosinate herbicide (Basta) resistance as a selectable marker (Fig. 1A), was used to transform protoplasts from maize suspension cell line HE-89 (Morocz et al. 1990) using the polyethylene glycol (PEG) method. Basta treatment was done by spraying seedlings with L-glufosinate 4 g l^{-1} at the IV-leaf stage. The presence of the transgene in segregating plants was assessed through resistance to gluphosinate and by PCR screening (data not shown). A set of four homozygous Basta-resistant transgenic progenies (SM 3.4, SM 16.1, SM 19.4, SM 20.2), and Basta-sensitive progeny (SM 20.4), as a negative control, were raised to maturity in a Class 1

containment-greenhouse according to the Italian guidelines for biosafety-GMO-plants (D.Leg.vo n°206 12.04.2001 according to EU Directive 98/81/CE), and then transferred in pots in a 1:5 mixture of washed sand: peat basic soil, to a growth chamber at 25°C/21°C day/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 watered daily, and supplemented weekly with half-strength standard (Balconi et al. 2007) basic salts from tillering to flowering stages. Each experimental unit consisted of a 30 cm high by 32 cm wide pot containing two plants. Ten pots for a total of twenty plants per progeny were considered in each experiment. At flowering stage, leaf tissues were harvested, frozen in liquid nitrogen and stored at −80°C until used for molecular analyses, or for pathogenicity tests. Mature seeds of the transgenic progenies were used for endosperm analysis. The B73 inbred line was included, as a control, for leaf tissues and endosperm molecular analyses.

Southern blot analysis

DNA was extracted from leaf tissue using CTAB (1M TrisHCl ph 7.5, 5M NaCl, 0.5M EDTA pH 8.2% CTAB) as the extraction buffer and digested with EcoRI-HindIII. Restricted DNAs were separated on 0.9% agarose gels, transferred to nylon membranes (Hybond-NX, Amersham, UK) and hybridized with ^{32}P-labelled DNA probes. Probes were obtained by different digestions of pSC1b-32, as follows (Fig. 1A): *b-32* probe: EcoRV-HindIII (934 bp); *Nos-bar* probe EcoRI-BamHI (870 bp). Labelling was performed using the Rediprime II Random Prime Labelling System (Amersham). Hybridisation was carried out over night at 65°C. Membranes were washed with 2xSSC/0.1% SDS and with 0.1xSSC/0.1% SDS at 65°C. A 1 Kb molecular weight marker, (Invitrogen) was used.

SDS-PAGE

Total protein extracts for immunoblots were obtained from leaf and endosperm (collected by removing the pericarp and dissecting the embryo from mature seeds) by grinding the leaf tissues in liquid nitrogen to a fine powder and adding the extraction buffer (20 mM Tris HCl pH 8, 100 mM NaCl, 1 mM EDTA pH 8, 0.5% Nonidet P40, 2% ß-mercaptoetanol, 0.2 mM PMSF). Extraction was carried out at 4°C for 30 min followed by centrifugation at 14,000 rpm for 30 min at 4°C. Protein concentrations were determined by Bradford's (SIGMA) with BSA (bovine serum albumin) as standard. Leaf and endosperm extracts containing 5 µg total proteins were separated by 12% SDS-PAGE. Western blotting analyses were performed in triplicate; 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 *Escherichia coli* (previously reported by Balconi et al. 2007).

Two-dimensional electrophoresis (2DE) and protein identification

For 2DE, proteins were extracted from leaf tissues of one of the transgenic progenies (SM 20.2) and the negative control (SM 20.4) following the method of Porubleva et al. (2001) with slight modifications. The differences were the addiction of a protease inhibitor (Complete, Roche) during the leaf grinding and the omission of the Orange G dye in the buffer. The supernatant containing the proteins was cleaned with the ReadyPrep 2-D Cleanup Kit (Bio-Rad) and the protein concentration calculated with a 2-D Quant Kit (Amersham Biosciences). Large two-dimensional electrophoresis (2DE) gels were set up to control the expression of proteins in transgenic plants. Three hundred and fifteen µg of proteins were solubilised in 300 µl of a solution containing 7 M urea, 2 M thiourea, and 4% CHAPS, 40 mM TRIS, 0.12% *v/v* DeStreak Reagent (GE Healthcare), 0.5% Bio-Lyte Ampholyte 3–10 (Bio-Rad) and loaded onto a 3–10 pI NL 17 cm strip (Bio-Rad). After a passive re-hydration for 14 h, IEF was performed on a Protean IEF Cell (Bio-Rad) for 60,000 Vhr. IEF proteins were then reduced and alkylated as already described (Pessione et al. 2003). SDS-PAGE was carried out on a ready-to-use gradient gel 8–16%T, (Bio-Rad) using the Protean II Xi Cell (Bio-Rad). Gels were automatically stained using the Processor Plus (Amersham Biosciences) with freshly prepared Blue Coomassie Colloidal stain home-made. 2-DE gels were digitised with a GS-800 Densitometer (Bio-Rad). Overlapping and warping of the images was performed with the use of Progenesis PG 220 software (Nonlinear Dynamics) using pink colour for SM 20.2

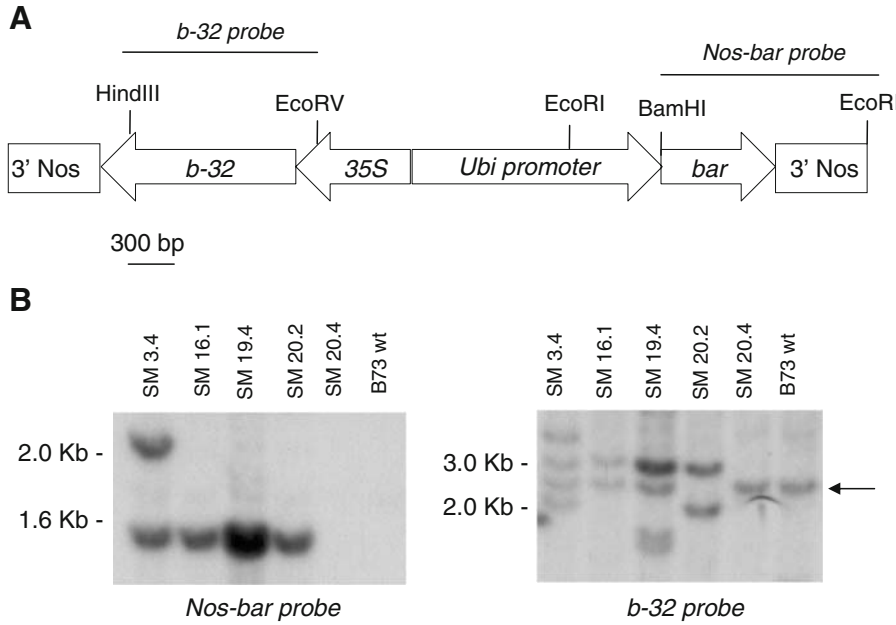

Fig. 1 **A** Schematic diagram of plasmid pSC1b-32. The *b-32* gene is inserted between the *35SCaMV* promoter and *nos* terminator; the *bar* gene is inserted between the maize *ubiquitin (ubi)* promoter and *nos* terminator. **B** Southern-blot analysis of genomic DNA from transgenic progenies (SM 3.4, SM 16.1, SM 19.4, SM 20.2), negative control (SM 20.4) and the B73 inbred line, in combination with the *b-32* (934 bp) (left panel) and *nos-bar* (870 bp) (right panel) probes. Molecular weights of hybridising bands were estimated using 1 Kb molecular weight marker (Invitrogen)

and green colour for the negative control. Protein identification was achieved through the 'peptide mass fingerprinting' (PMF) strategy (Pappin et al. 1993) using a MALDI TOF analysis. The procedure was performed as described elsewhere (Pessione et al. 2003). Mini 2DE were set up for immunoblotting analysis. After the 2-D Cleanup procedure, 100 µg of proteins were solubilised in 125 µl of the above mentioned solution, and loaded onto a 3–10 NL pI 7 cm strip (Bio-Rad). After passive re-hydration for 14 h, IEF was performed for 10,000 Vhr and IEF strips were then treated as already described. SDS-PAGE was carried out on a ready-to-use gradient mini-gel 8–16%T, (Bio-Rad) using the Mini-PROTEAN 3 cell (Bio-Rad). Products were transferred onto a nitrocellulose membrane using the X Cell II Blot Module (Invitrogen). Immunostaining was performed as described above. Membranes were digitised with GS-800 Densitometer (Bio-Rad).

In vitro Fusarium leaf tissue colonisation bioassay

Leaf tissues, collected at flowering, were surface-sterilised and square segments, (1 cm side), were dissected according to Yates and Jaworski (1998). Four leaf squares were plated on PDA (Potato Dextrose Agar) in Petri dishes and inoculated with spores of *F. verticillioides*. The Fusarium strain (MRC826 from Dr. Scholten- PRI-Wageningen, The Netherlands) was grown on PDA medium in Petri dishes; cultures were maintained at 26°C with a photoperiod 16 h light/8 h dark, up to complete development of mycelium on the agar surface. Conidial suspensions were prepared just before inoculation. The plate surface was washed with 5 ml of sterile distilled water (SDW) and the conidial suspension adjusted with SDW to the final desired concentration using a Bürker haemocytometer. Leaves were inoculated placing a 5 µl spore suspension drop in the centre of the square. For optimisation of the bioassay four spore concentrations (10^4, 10^5, 10^6 and 10^7 spores ml^{-1}) were tested on the negative control progeny (SM 20.4). For each experimental trial three replicates were used. Progression of infection was detected at different days after inoculation (DAI), monitoring: (i) the radial growth of mycelia as a function of the fungal colony diameter around inoculated leaf squares (ii) the extension of the

infection on leaf surfaces as well as mycelial colour. Control experiments were performed on non-inoculated and sterile water-inoculated sterile leaf squares. The percent of radial growth inhibition in inoculated leaves of transgenic progenies compared to that of negative control SM 20.4, was calculated as reported in Krishnamurthy et al. (2001).

Statistical analysis

Data were subjected to standard analysis of variance (ANOVA) (MSTAT-C-Programme, Michigan State University, Version 1991). Means were compared using the protected least significant difference (LSD) at the 0.05 probability level.

Results

Transgenic maize progeny characterisation

The integration patterns of the *b-32* transgene were determined by genomic Southern-blots, using *EcoRI-Hind III* double digestions, as appropriate enzymes to estimate the transgene copy number.

Four Basta-resistant progenies (SM 3.4; SM 16.1; SM 19.4; SM 20.2), one Basta-sensitive progeny (SM 20.4) and the B73 inbred line, were analysed using a *Nos-bar* (resistance gene) and *b-32* probe (Fig. 1A).

As reported in Fig. 1B, (left panel), *Nos-bar* probe detected the presence of 1-2 hybridising bands (ranging in size from 1.6 to 2.0 Kb) in all the Basta-resistant progenies (a very prominent band dominated the SM 19.4 lane); no bands were detected with genomic DNA from B73 inbred line and from the negative control progeny.

The *b-32-probe* detected the presence of the b-32 endogenous gene in the control B73 inbred line and in the negative control progeny SM 20.4 (as indicated by arrow in Fig. 1B; right panel); a band at the same position was present in transgenic progenies SM 3.4, SM 16.1, SM 19.4, and at a slightly different position in the SM 20.2 progeny, most likely due to a recombination event involving the endogenous gene and transgene, indicating that several insertion events has occurred. In addition to the endogenous b-32 band (native gene), all transgenic progenies contained a few additional bands corresponding to insertions of the transgene (2-3 hybridising fragments, ranging in size from 3 to 1.8 Kb). The unique hybridisation patterns observed indicated that each progeny resulted from independent transformation events.

Control of quantitative b-32 expression in transgenic progenies

As shown in Fig. 2, the B73 inbred line and the negative control SM 20.4 progeny, showed b-32 expression in the endosperm (pattern with a double band was visible; lanes 1 and 3, respectively); in addition, these materials did not exhibit any expression of cross-reacting proteins in leaf tissues (lanes 2 and 4, respectively). However, SM 20.2 transgenic progeny showed detectable bands both in leaf and endosperm tissues (lanes 5 and 6). Furthermore, the other three transgenic lines (SM 3.4, SM 16.1, SM 19.4) showed an immunoblot pattern similar to that observed in the SM 20.2 (data not shown).

A comparison of b-32 protein amounts in leaf extracts at the flowering stage was performed by immunoblot imagine scanning and revealed a differential b-32 expression among the various progenies (Table 1). Progenies SM 3.4, SM 16.1 and SM 20.2 each showed a b-32 content accounting, respectively, for approximately 39.8, 36.4 and 19.9 ng μg^{-1} of total proteins. These values were significantly higher (LSD: 2.4) than observed in progeny SM 19.4 (1.2 ng μg^{-1} of total proteins). As expected, SM 20.4, i.e. the negative control, showed non-detectable b-32 content (n.d.) in leaf tissues.

Proteomic experiments

Proteomic experiments were performed on protein leaf extracts of one of the transgenic lines expressing a high b-32 level (SM 20.2) and were compared to the negative control progeny (SM 20.4).

In Fig. 3A the 2DE map overlapping clearly showed the presence of additional spots in SM 20.2 progeny (pink colour) in comparison to SM 20.4 progeny which was Basta-sensitive and b-32 western-negative. These spots were cut from gels and digested with trypsin to allow protein identification by PMF. Both induced b-32 spots (n° 1, 2, and 3) and herbicide resistance spots (n° 4 and 5) were successfully identified. Protein identification and sequence coverages are summarised in Table 2.

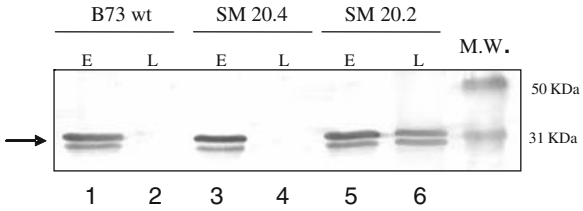

Fig. 2 Western-blot analysis of b-32 expression at protein level. Endosperm and leaf tissue, respectively, of inbred line B73 (lanes 1 and 2), negative control line SM 20.4 (lanes 3 and 4), and transgenic line SM 20.2 (lanes 5 and 6), were tested for the presence of b-32 protein

Western blot analysis of SM 20.2 progeny (Fig. 3B) showed a positive reaction in the area of the spots already identified as b-32 and a faint tail of reaction at a slightly lower molecular weight, and more basic pH corresponding to the double banding pattern visible in Fig. 2.

In vitro Fusarium leaf tissue colonisation bioassay

Fusarium verticillioides growth at 7 DAI on SM 20.4 leaf squares inoculated with different spore concentrations, in comparison with the sterile water-inoculated leaf square as the control, is shown in Fig. 4A. Both the control and non-inoculated leaves (data not shown), did not show any mycelial growth. The lowest spore concentration (10^4 spores ml^{-1}) induced a weak fungal attack, without covering the leaf surface; a concentration of 10^6 spores ml^{-1} allowed white mycelia to completely cover the leaf surface, while an inoculation with 10^7 spores ml^{-1} induced a heavy colonisation of leaf tissue by pink mycelia.

In Fig. 5, the mean values of *F. verticillioides* colony diameter extending from SM 20.4 leaf squares, at 2, 3, 4 and 7 DAI with 10^4, 10^5, 10^6 and 10^7 spores ml^{-1}, are shown. Using 10^4 and 10^5 spores ml^{-1} for inoculation, fungal growth on the leaves was gradually detectable for 7 days following the inoculation (ranging from 2 mm, 2 DAI to 22 mm, 7 DAI). A similar trend, although with wider average colony diameter, (ranging from 7 mm, 2 DAI to 27 mm, 7 DAI), was observed inoculating leaf squares with 10^6 spores ml^{-1}. In the case of the highest spore concentration (10^7 spores ml^{-1}) a very rapid growth around the cut edges was already observed 2 DAI (colony diam around 10 mm). Later than 7 DAI, in the case of 10^6 and 10^7 spores ml^{-1}, the colony diam (> 25 mm) was too large to be correctly recorded because of colony confluence around the leaf squares plated on the same Petri dish. *In vitro* leaf tissue colonisation bioassays, similarly conducted on a transgenic Basta-resistant progeny lacking the transgenic b-32 protein, showed a response not significantly different from that observed on the negative control SM 20.4 progeny (Basta-sensitive) (data not shown).

The following bioassay parameters, i.e. (i) spore suspension containing 10^6 spores ml^{-1}, (ii) 3-4-7 DAI as the detection time, were adopted for pathogenicity experiments including, together with the negative control SM 20.4, also individuals of the four transgenic progenies. Results shown in Table 3, indicate that fungal colony diameters measured around the inoculated leaves of SM 20.4, were, at all detection times, significantly (Student's t test = $P \leq 0.05$) larger than those observed in all four progenies expressing b-32. In more detail, for the negative control SM 20.4, average colony diam was: at 3 DAI, 7.6 mm in comparison to transgenic progenies, range 2.70–5.3 mm (LSD: 0.8); similarly at 4 DAI, 11.3 mm in comparison to 6.4–9.3 mm (LSD: 0.9); and at 7 DAI, 25.0 mm in comparison to 12.3–18.9 mm (LSD: 0.9). In addition, the fungal colony diameters measured around the inoculated leaves of SM 19.4 were, at all detection times, significantly (Student's t test = $P \leq 0.05$) larger than those observed in the other three progenies expressing b-32.

As shown in Table 3, the suppression of Fusarium leaf colonisation (growth inhibition, % relative to the

Table 1 Relative amounts and comparison of b-32 amounts in protein leaf extracts of transgenic progenies and of negative control (SM 20.4), at the flowering stage

Progenies	ng b-32 μg^{-1} of total proteins
SM 20.4	n.d. (non-detectable)
SM 3.4	39.8±1.2
SM 16.1	36.4±1.9
SM 19.4	1.2±0.03
SM 20.2	19.9±1.3
LSD 0.05	2.4

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*. Means and standard errors of three independent assays are given

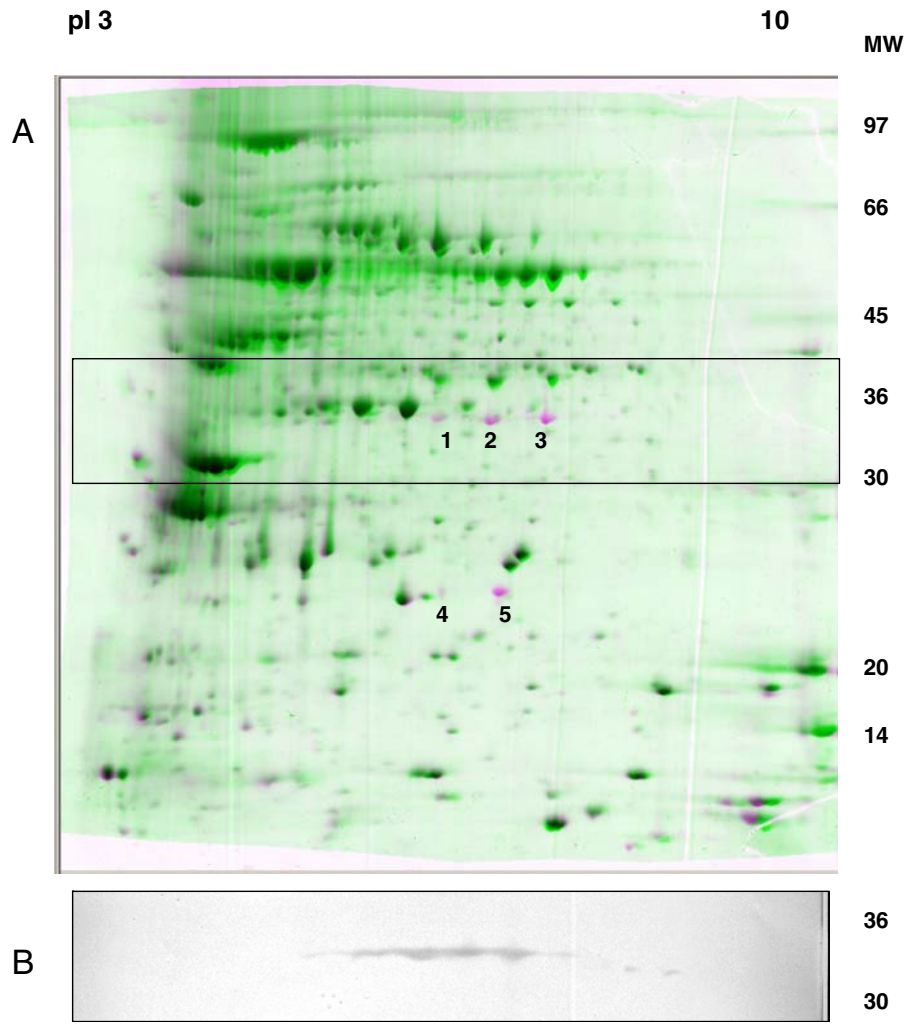

Fig. 3 **A** Overlapping of the 2DE gels from SM 20.4 and SM 20.2 progenies. Spots 1, 2, 3, 4 and 5 (in pink colour) clearly are not present in the negative control (SM 20.4), and were selected for PMF identification. **B** Western Blotting of a 2DE gel of SM 20.2 progeny with b-32 antisera

control) in the SM 19.4 progeny was significantly (Student's t test = $P \leq 0.05$) lower than that observed in the other three transgenic progenies, at all detection times. In particular, Fusarium growth inhibition was at 3 DAI, 30.6% in SM 19.4 progeny, in comparison to 61.9–64.2% in the other three transgenic progenies (LSD: 12.7); similarly, at 4 DAI, 15.8% in comparison to 40.5–43.0% (LSD: 8.4); at 7 DAI 23.0% in comparison to 47.3–50.8% (LSD: 3.3).

For each genotype, observations on Fusarium attack and the progression of infection on the leaf segments, were recorded by complete photographic documentation. As reported in Fig. 4B, at 10 DAI, an extension of mycelial growth on leaf surfaces was observed for SM 19.4 progeny, but to a lesser extent than that observed for the negative control SM 20.4; on the other hand, in the case of the SM 20.2 (and SM 3.4, SM 16.1, data not shown), a reduced growth on leaf surfaces was observed.

Discussion

The effectiveness of an anti-fungal protein *in planta* may be predicted, in part, by its expression levels in crucial host tissues, and by the timing of expression, since suitable levels should accumulate before the host becomes most vulnerable to infection. In this context, the maize endosperm albumin b-32, as a RIP has been the subject of extensive studies aimed at investigating and at exploiting its action as a defence protein against fungi (Maddaloni et al. 1991, 1997; Balconi et al. 2007). The role of b-32 in defence against pathogens was also suggested by an increased

Table 2 Identification of proteins from selected spots and sequence coverage by PMF using MALDI-TOF MS (showed as italics amino acids)

Spot n°	Name and accession number*	mass (kDa) observed	% coverage	Sequence coverage
1, 2, 3	b-32 protein Q41776	37	46	MAETNPELSDLMAQTNKKIVPK *FTEIFPVEDVNYPYSAFIASVR* KDVIKHCTDHK*GIFQPVLPP* *EKKVPELWFYTELK*TR *TSSITLAIRMDNLYLVGFR* TPGGVWWEFGKAGDTHL LGDNPRWLGFGGR *YQDLIGNKGLETVTMGR* AEMTRAVNDLAKKKKMATLE EEEVQMQMQMPEAAELAAAA AAADPQADTKSKLVKLVVMVC EGLRFNTVSR*TVDAGFNSQHGVTLTVTQGK* QVQKWDRISK*AAFEWADHPTAVIPDMQK* LGIKDKNEAARLVALVK *NQTTAAAAAATAASADNDDDEA*
4, 5	Gene bar, Herbicide-resistant P16426	25	51	MSPERRPADIRR*ATEADMPAVCTIVNHYIE* *TSTVNFRTEPQEPQEWTDDLVR* LRERYPWLVAEVDGEVAGIAYAGPWKAR *NAYDWTAESTVYVSPRHQRTGLGSTLYTHLLK* SLEAQGFK*SVVAVIGLPNDPSVRMHEALGYAPR* GMLRAAGFKHGNWHDVGF WQLDFSLPVPPRPVLPVTEI

*Swiss-Prot and TrEMBL entries

susceptibility of *opaque-2 (o2)* mutant kernels (in which the level of b-32 is greatly decreased), to fungal attack (Loesch et al. 1976; Warren 1978) and insect feeding (Gupta et al. 1970). In addition, the results for pure inbred lines, and their isogenic *o2*-mutants, tested in field experiments with the Silk Channel Inoculation Assay (SCIA) on adult plants, showed that the *o2* mutants were significantly more susceptible to the *F.verticillioides* attack than the pure lines (Balconi et al. 2005).

In the current study we have evaluated the action of the protein RIP b-32 in transgenic maize plants expressing b-32 in various organs and tissues in supporting an increase in Fusarium leaf colonisation resistance. Four independent transgenic maize progenies constitutively expressing the *b-32* gene were produced, with co-integration of both *bar* and *b-32* genes. All plants had normal morphology, when compared to the negative control (data not shown), and were fertile, thus confirming that the ectopic expression of the b-32 RIP did not interfere with normal plant development, as previously observed for wheat (Balconi et al. 2007). The evaluation, in the B73 inbred line and in the negative control, of b-32 expression at the protein level, in endosperm and leaf tissues, confirmed the endosperm-tissue specificity of this gene. However, the transgenic progenies also showed b-32 protein expression in leaves, with a similar doublet pattern also observed in the endosperm. Proteomic analyses of leaf extracts clearly showed the presence of both the b-32 and herbicide-resistance enzymes; these were the only significant variations detected between the transgenic and the Basta-sensitive progeny protein profiles.

The identification of progenies with a differential b-32 expression in the leaves was useful for setting up pathogenicity experiments, aimed at evaluating a possible differential response to a Fusarium attack in leaf tissue colonisation bioassays. Lauren and Di Menna (1999) described patterns of Fusarium infection and mycotoxin contamination in leaf and ear sections of maize plants. The authors reported that Fusarium infection occurred first in leaf axil fractions, later than in leaf blades and stalks and then in the

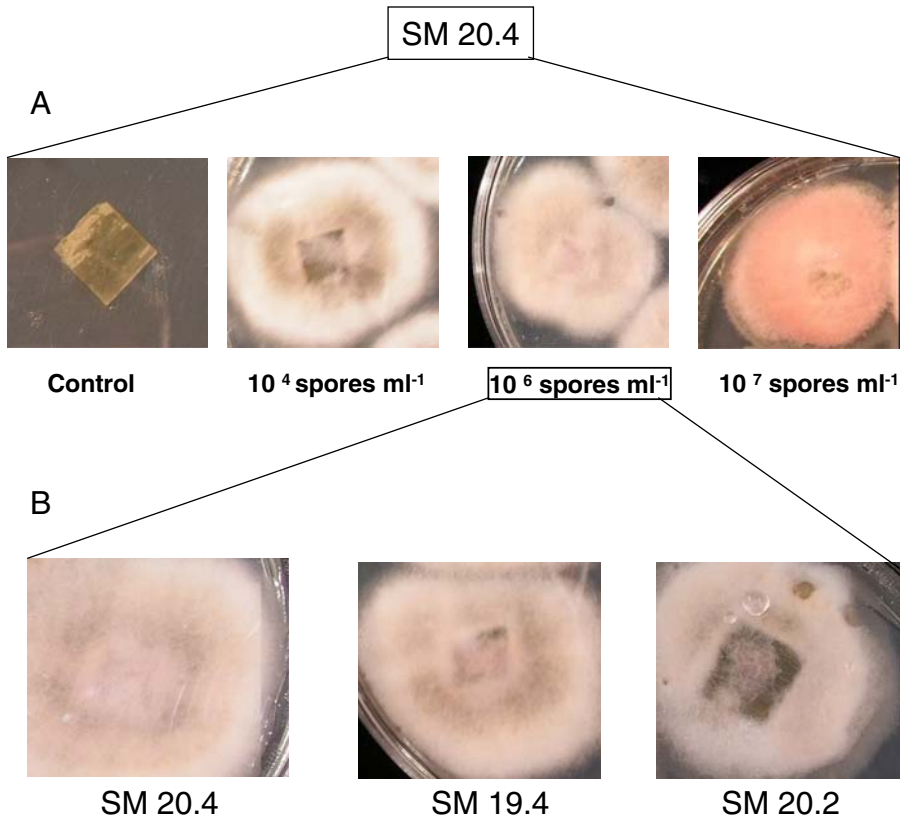

Fig. 4 Progression of *F. verticillioides* infection: **A** at 7 days after inoculation (DAI) with different spore concentrations (from left to right: 10^4, 10^6 and 10^7 spores ml^{-1}) in SM 20.4 progeny. C: control leaf tissues inoculated with SDW. **B** at 10 days after inoculation (DAI) with 10^6 spores ml^{-1}, in SM 20.4, SM 19.4 and SM 20.2 progenies

rachis and peduncle and finally in kernels. In this respect, ectopic expression of b-32 in maize may result in a wider defence mechanism for other tissues.

Significant differences (10-fold increase) in inoculum are needed to produce measurable changes (two-fold) in artificial inoculation protocols, as previously reported (Balconi et al. 2007). Therefore, in our study, a range 10^4–10^7 spores ml^{-1}, as spore concentrations, was applied to the negative control, the progeny not expressing b-32 in leaf tissues, to identify the most suitable inoculation protocol to induce symptoms gradually detectable for a reliable evaluation of the transgenic progenies. Non-inoculated and sterile water-inoculated leaves, did not show any mycelial growth, or any changes in colour or other morphological alterations; hence, the protocol adopted for leaf tissue surface-sterilisation was effective in eliminating all external contamination and therefore useful for the pathogenicity bioassay.

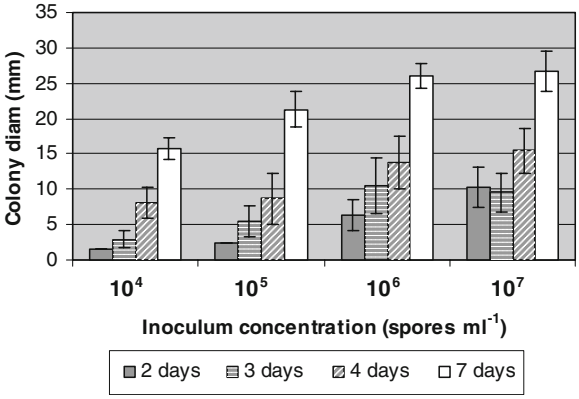

Fig. 5 Effect of different *F. verticillioides* spore concentrations (10^4, 10^5, 10^6 and 10^7 spores ml^{-1}) on *in vitro* SM 20.4 progeny leaf colonisation at 2, 3, 4 and 7 days after inoculation (DAI)

The transgenic progenies expressing b-32 in leaf tissues were less susceptible than the negative control, when evaluated for their response to *F. verticillioides* attack, showing significantly reduced colony diameter around the inoculated leaves. Similar results were reported concerning blast inoculation assays conducted on detached leaves of transgenic rice plants expressing the antifungal AFP protein (Coca et al. 2004). A good correlation between the b-32 content in leaves and the level of resistance to Fusarium attack

Table 3 Severity of Fusarium leaf tissue colonisation as mycelial radial growth (colony diameter[t]) in the negative control (SM 20.4 progeny) and in the transgenic progenies, and suppression of Fusarium leaf colonisation (percent of radial growth inhibition, relative to the control[u]) in the b-32- expressing transgenic progenies, 3, 4 and 7 days after *F.verticillioides* (10^6 spores ml^{-1}) inoculation

Progenies	Days After Inoculation (DAI)					
	3 DAI		4 DAI		7 DAI	
	Colony diam (mm)[t]	Growth inhibition (%)[u]	Colony diam (mm)[t]	Growth inhibition (%)[u]	Colony diam (mm)[t]	Growth inhibition (%)[u]
SM 20.4	7.6±0.5	–	11.3±0.4	–	25.0±0.0	–
SM 3.4	2.7±0.9	64.0±11.3	6.4±0.8	43.0±6.8	12.3±0.9	50.8±3.5
SM 16.1	2.9±0.6	61.9±7.7	6.7±0.6	40.5±4.1	12.8±1.3	47.3±3.7
SM 19.4	5.3±0.7	30.6±7.8	9.3±0.9	15.8±5.1	18.9±0.9	23.0±3.2
SM 20.2	2.7±0.5	64.2±7.9	6.5±0.5	42.1±4.5	12.6±0.5	49.8±2.0
LSD 0.05	0.8	12.7	0.9	8.4	0.9	3.3

[t] Triplicate samples measured 3, 4 and 7 days after *F. verticillioides* leaf tissue inoculation (10^6 spores ml^{-1}) in the negative control (SM 20.4 progeny) and in the transgenic progenies. All values were analysed by MSTAT-C-Programme (Michigan State University, Version 1991)

[u] Percent of radial growth inhibition compared to negative control, SM 20.4 progeny, calculated individually for each replicate before statistical analysis

was observed. In the case of progenies with high b-32 content in the leaves, in addition to a reduced mycelial growth around the cut edges of the leaves, a very weak growth on leaf surfaces was observed in comparison with progeny exhinbiting the lowest b-32 content in leaves. The expression of the herbicide-resistance protein (Basta-resistance, enzyme) did not interfere in any mechanism involved in *F. verticillioides* maize leaf colonisation and the increased resistance to Fusarium colonisation, observed in the transgenic progenies must be attributed to the expression of b-32, excluding any additional transgenic effects.

Even though the two low molecular weight peptides, reported to be the activated form of RIP (Walsh et al. 1991), were not detected in the transgenic leaf tissues, the expression of b-32 protein (reported to be the 32 kDa proenzyme, pro-RIP) supported the increase in Fusarium leaf colonisation resistance. This result agrees with previous studies on tobacco, which showed that transformed plants were more tolerant to *R. solani* infection than the negative control, even though no low molecular weight immuno-reactants were detectable with b-32 antiserum (Maddaloni et al. 1997).

In all tested transgenic progenies and in the negative control, 100% of inoculated leaf squares showed Fusarium colonisation (data not shown) suggesting, as expected, that b-32 protein does not prevent Fusarium attack, but rather promotes the reduction of mycelial growth on the colonised tissue. As previously reported for FHB, b-32 crop protection may be due to preventing the disease from spreading in all directions from the point of inoculation (PI) (Balconi et al. 2007).

An important issue in fungal protection against maize fusariosis is to verify the influence of the engineered anti-fungal b-32 protein in the containment of mycotoxins (fumonisins) in the plants infected by *F. verticillioides* (Duvick 2001). Because most mycotoxin problems develop in the field, strategies are needed to prevent infection of growing plants by toxigenic fungi (Munkvold, 2003). The expression of antifungal proteins in plants or plant tissues, in which they are not normally expressed, may be very useful to reduce pathogen colonisation and growth; in this context, a reduction of *F. verticillioides* infection in maize leaves and stalks, could be very useful for limiting the fungal infection from spreading to the exposed silks and consequently to reduce grain fumonisin contamination (Lauren and Di Menna, 1999).

The studies reported in this paper confirmed, as previously reported for tobacco and wheat (Maddaloni et al. 1997; Balconi et al. 2007), that the incorporation of the maize *b-32* gene and the

ectopical expression of b-32 protein, could represent an effective and reliable tool in crop disease management programmes.

Acknowledgements Part of the work received financial support from the EU-funded project SAFEMAIZE (ICA4-CT2000-30033) in FP5 and the MIPAAF-funded project AFLARID. Thanks are due to F. Forlani for the immunoblot image scanning assistance, to M. Lauria for Southern-blot analyses assistance, to N. Berardo for the support in statistical analysis, to A. Conti for the critical reading of the manuscript and to M. Maddaloni for scientific advice.

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