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

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Fungal diseases resistance is a major goal in plant breeding of cereal species, since they 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). Wheat head scab (WHS) or Fusarium head blight (FHB) is an important wheat disease (Parry *et al*., 1995; McMullen *et al*., 1997; Dardis and Walsh, 2002) predominantly caused by *Fusarium graminearum* Schwabe and *Fusarium culmorum* Sacc. Infected wheat spikes show characteristic premature death or blighting of spikelets; florets sterility and poor or absent grain filling are the cause of severe yield losses. In addition, mycotoxin production, such as deoxynivalenol (DON), nivalenol and zearalenon, are often associated with the disease occurence (Bottalico, 1998). Commercially acceptable FHB resistant cultivars have not yet been developed and the control of FHB by fungicides is ineffective. Molecular markers linked to FHB resistance have been discovered, however, because FHB resistance is quantitatively inherited, it could not be expected that the use of markers will replace conventional phenotypic screening based on green-house or field methods in the near future (Van Sanford *et al*., 2001).

An alternative strategy is offered by genetic engineering by means of the identification and cloning of defence genes and their introduction and exploitation into crop plants. Defence genes can be identified and cloned from plants (Jones *et al*., 1994; Song *et al*., 1995), from microrganisms (Lorito *et al*., 1998), or different unrelated sources (Zasloff, 2002; Coca *et al*., 2006). The introduction and expression of some of these genes have been shown to confer increased resistance to plant fungal pathogens (Jach *et al*., 1995; Bliffeld *et al*., 1999; Chen *et al*., 1999; Krishnamurthy *et al*., 2001). A class of plant proteins, known as RIPs (Ribosome-Inactivating Proteins), also display antifungal properties; RIPs 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 *et al*., 1996). Some cereal species, like barley and maize contain RIPs in their grain endosperm – respectively RIP30 and 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). 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 *et al*., 1998).

In order to further explore the antifungal activity of the maize b-32, six homozygous wheat lines (V45-5, V45-8, V45-18, V45-19, V45-21 and V45-39) were obtained via biolistic transformation, in which the b-32 gene was driven by the 35SCaMV promoter in association to the *bar* gene as a selectable marker; plants were raised and brought to homozygosity through genetic analysis of progeny (Lupotto *et al*., 2003). In this study the six wheat transgenic lines and the parental cv. 'Veery' were challenged for response against *Fusarium culmorum,* in order to detect the potential protective role of b-32 against fungal diseases.

Expression of b-32 in leaf protein extract of the six lines was confirmed throughout the plant life cycle via immunoassay. 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 an useful range of expression for

pathogenicity experiments, in order to evaluate a possible differential response to fungal pathogens attack. As expected, cv. 'Veery', used as non-transgenic control, did not show any expression of cross-reacting proteins. All plants had normal phenotype, not distinguishable from control cv. 'Veery' except for a slightly smaller size and reduced set seeds. Pathogenicity tests for Fusarium head blight (FHB) were performed on the b-32 transgenic wheat lines in comparison to the parental cv. 'Veery', in a containment green-house. Resistance to scab was evaluated by a "single floret injection inoculation method" (Wang *et al*., 1982). Artificial inoculation was performed on the parental cv. 'Veery' and transgenic plants at the early grain filling stage puncturing the spike with a hypodermic needle and injecting spores of *Fusarium culmorum*. Plants were inoculated at the right time with an injection of 20 µl of *F. culmorum* spore suspension ($5x10^5$ spores/ml) in the central part of the spike. Control plants were non-inoculated and inoculated with distilled sterile water. Following injection, spikes were covered with a transparent plastic bag for 48 hours to provide proper conditions favouring disease development with high relative humidity. A visual scale, based on percentage of infected spikelets, has been chosen to estimate scab disease severity, as described by Stack and Mc Mullen (1998). FHB disease assessment was made at 7 and 14 days after inoculation, counting white spikelets per inoculated spike. Each treatment was evaluated with ten plants. As shown in Fig. 1, in all the transgenic lines, a similar reduction in FHB symptoms, not dependant on the level of b-32 protein, has been observed (in all transgenic lines, % infected spikelets was around 20% both 7 and 14 days after inoculation, compared to the values around respectively, 40% and 50% observed in the control).

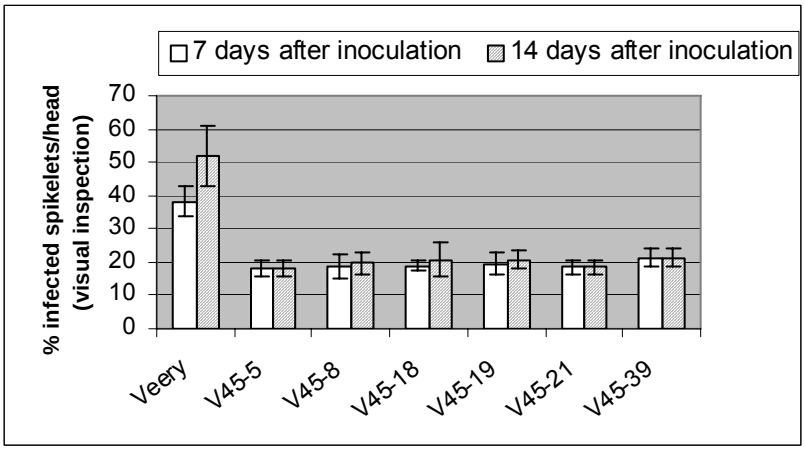

Fig. 1. Percent of infected spikelets/head in cv. 'Veery' and transgenic lines 7 and 14 days after *F. culmorum* artificial inoculation ($5x10^5$ spores/ml).

Another parameter used to attest scab disease severity in the tested genotypes was the percentage of "tombstones" (shriveled, light weight, dull greyish or pinkish in colour kernels; McMullen and Stack, 1994).

In Fig. 2 the percentage of tombstones/total seeds recorded at maturity in cv. 'Veery' control plants and in the transgenic lines, are reported. Independently from the differential b-32 content of the transgenic lines, the disease severity, was equally reduced in all cases, being the percentage of tombstones around 20% in the transgenic lines against 45% in control cv. 'Veery' plants.

The studies reported in this paper indicate that wheat transformants expressing the RIP maize gene *b-32* have an increased resistance towards *Fusarium culmorum*. Disease control by b-32 protein was observed as reduction of visible FHB symptoms, early after inoculation, and also at maturity, as a reduction in damaged seeds percentage. The FHB protection was not dependent on increasing levels of the RIP protein in the tissues, but also that the lowest level of b-32 was effective.

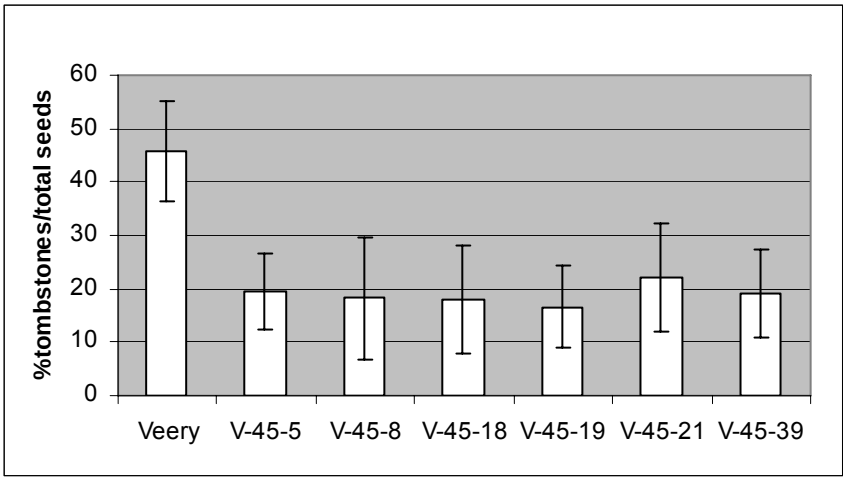

Fig. 2. Percent of tombstones/total seeds, at maturity, in cv. 'Veery' and transgenic lines after *F. culmorum* artificial inoculation ($5x10^5$ spores/ml).

Transgenic approaches to combat Fusarium head blight in wheat and barley were recently reviewed in Dahleen *et al*. (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 *et al*., 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 in part by the timing of its expression so that suitable levels accumulate before the host becomes most vulnerable to infection. The expression of antimicrobial proteins in plants or plant tissues in which they are not normally produced, may have a greater 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 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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