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Optimization of an *in vitro* bioassay for wheat diseases

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Abstract

Alternaria triticina, Fusarium graminearum, Pyrenophora tritici-repentis, Bipolaris sorokiniana (Helminthosporium sativum); Pythium sp., and *Rhizoctonia* sp. were tested on the basis of being a representative group of important fungal pathogens in order to establish a reliable bioassay system to test eventual fungal resistance in transgenic plants. The inoculum was prepared on V-8 agar medium (in petri dishes) for *P. tritici-repentis* and *Pythium* sp., and on PDA (potato dextrose agar) medium for the other fungi. The cultures were incubated at room temperature for 7–10 days in a culture chamber with a constant standard illumination. The suspensions of conidia or micelia were prepared on sterile distilled water with a few drops of Tween 20 and scrapings of the fungi culture. The inoculum was homogenized by vortexing the suspension for a few seconds. The concentration used for the conidia solution was adjusted to 106 conidia/ml. Fresh leaf samples from adult plants (heading stage) were sterilized and then dipped in the inoculum suspension. The inoculated leaves were then transferred to water agar medium (1% agar), in 8-well rectangular multi-dishes, at room temperature. The level of resistance of the material to these pathogens was evaluated 4-7 days after inoculation. We believe that the routine use of this protocol (fast, reliable, and inexpensive) could allow the easy identification of resistant/tolerant plants to these diseases. In addition, because of the simplicity of this test, it can be useful for early screening of a large number of individuals, as required for transgenic plant screenings.

Introduction

Fungal pathogens are the causal agents of significant economical losses, decreasing yield and grain quality in several crops including wheat. These pathogens are involved in the development of important diseases in wheat: rusts, *Puccinia* spp.,; "fusarium head scab," *Fusarium;* "spot blotch," *Bipolaris sorokiniana (H. sativum);* "tan spot," *Pyrenophora tritici-repentis;* "*Alternaria* leaf blight," *Alternaria* sp.; and "leaf blotch," *Septoria tritici* (Stack 1999; Rajaram 1999). Some of them (e.g., *Fusarium* spp.) produce mycotoxins that contaminate food products and are dangerous for human and animal health. Other pathogens, such as *Pythium* sp., *Rhizoctonia* sp., and *Fusarium*, cause "damping off" or root problems.

Considerable efforts have focused on enhancing resistance in plants. More recently, advances in biotechnology offer new options for the development of fungal resistant crops. Pathogenesis related (PR) proteins have been identified that are component of plants' natural defenses against pathogen infections (Lamb et al. 1992; Xue et al. 1998; Krishnaveni et al. 1999). Wheat and a range of other crop plants have been transformed with antifungal genes in an effort to develop control measures against important fungal diseases. In some cases, the incorporation of PR genes in plants has enhanced resistance to a number of fungal pathogens (Broglie et al.1991; Nishizawa et al. 1999).

Among the PR-protein families, chitinases and glucanases are hydrolitic enzymes that degrade the polysaccharides chitin and β-1,3-glucan, which are the major components of the cell walls of many fungi (Wessels and Sietsma 1981). These enzymes have been extensively studied, mainly in tobacco (Legrand et al., 1987; Mauch, et al. 1988; Bol et al. 1990; Linthorst 1991; Dumas-Gaudot et al. 1996).

Another group of PR proteins with antifungal properties is PR-5, including the group of thaumatin-like proteins named such for their similarity with thaumatins, a set of sweet-tasting proteins from the African shrub *Thaumatococcus daniellii* (Reimmann and Dudler 1993; Hu and Reddy, 1995).

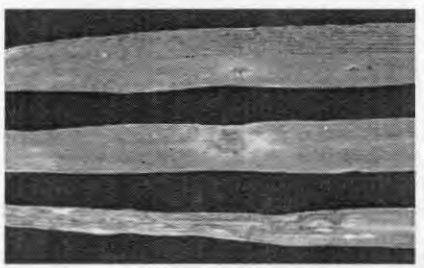
Studies of transgenic plants coming from different events generated by transformation with sense and antisense PR genes would be useful for identifying the possible role, regulation, and expression of these genes and the physiological functions of the corresponding proteins involved in the control of important fungal diseases in wheat. The overexpression or inactivation of these genes should serve to either enhance the tolerance/resistance or increase susceptibility of a wheat plant's response to fungal pathogens.

Most in vitro bioassays for testing antifungal activity involve the isolation and purification of the PR-proteins; the results obtained in vitro are not always reproducible in vivo. Others assays that involve the confrontation between several fungi and extracts of the sap of tolerant/resistant vegetative material do not consider the interaction between the host-pathogen, the events involved in the mechanism of infection (mainly in the early stages), and colonization of the tissue by the pathogen. Such study methods may be very useful for posterior studies after a general screening and will complement other data, such as molecular analysis.

We are proposing a very practical rapid and economical method to meet the need for an easy and rapid method of selection for tolerant/resistant wheat (including transgenic plants) to the attack of important fungal pathogens and for screening large numbers of plants.

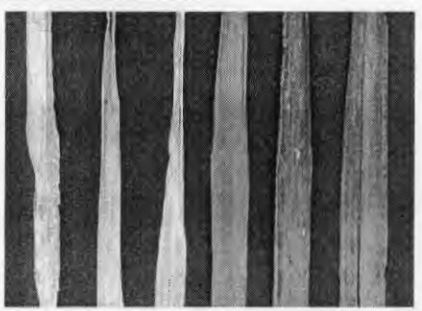
Materials and Methods

The fungi isolates were obtained from the collection of the Pathology Laboratory at CIMMYT; all of the samples were isolated from infected wheat. The plant material used was Bobwhite and Baviacora (as controls) and transgenic wheat plants with antifungal genes (PR genes), obtained from a previous project in the Applied Genetic Engineering Laboratory of the Applied Biotechnology Center at CIMMYT, Mexico.



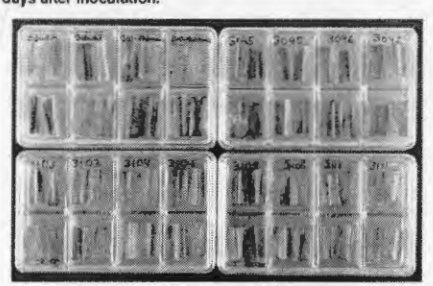
Effect of *A. triticina* in controls plants. 4, 8, 12, days from the top.

The inoculum was increased on V-8 agar medium, in petri dishes, for *A. triticina, P. tritici-repentis, B. sorokiniana,* and *Pythium* sp., and on PDA (Potato Dextrose Agar) medium for the *Rhizoctonia* sp. and *F. graminearum*. The cultures were incubated at room temperature for 7–10 days in a culture chamber with a constant standard illumination. The suspensions of conidia or micelia were prepared on sterile distilled water with a few drops of Tween 20 and scrapings of the fungi culture. The inoculum was homogenized by vortexing the suspension for a few seconds. The concentration used for the conidia solution was adjusted to the concentration required. Fragments (3 cm aprox.) of fresh leaf samples were sterilized with a solution of sodium hypochloride (0.1% final concentration) for 3 minutes, and rinsed 3 times in sterile distilled water. The leaf tissue was dipped in the inoculum suspension. The inoculated leaves were then transferred to a water agar medium (1% agar), in 8-well rectangular multi-dishes, at room temperature. The level of resistance of the material to these pathogens was evaluated 4–7 days after inoculation.

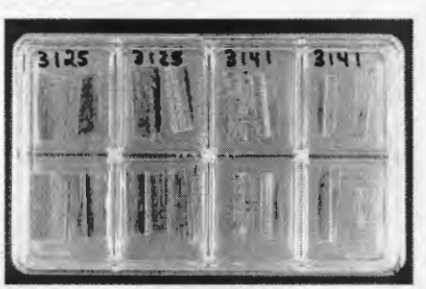


Effect of *A. triticina* in controls (left) and transgenic plants (right). 12 days after the inoculation.

The optimal concentration of inoculum of *A. triticina* was determined by inoculating the nontransgenic plants (Bobwhite and Baviacora cultivars) with different concentrations (10^4, 10^5, 10^6 conidia/ml). Two different phenological stages (5-leaf stage and spiking adult stage) were evaluated for their response to the attack of this pathogen. The level of resistance to these pathogens was evaluated 3–7 days after inoculation. The response to artificial inoculation with these pathogens in transgenic lines of wheat from To and T1 progeny was evaluated by an scale that considered the percentage of leaf area damaged and the type of lesion (necrotic or chlorotic, size of lesion)



In vitro bioassay of *A. triticina* controls top left and different transgenic lines.



Particular of biomass showing resistant and susceptible lines.

Results

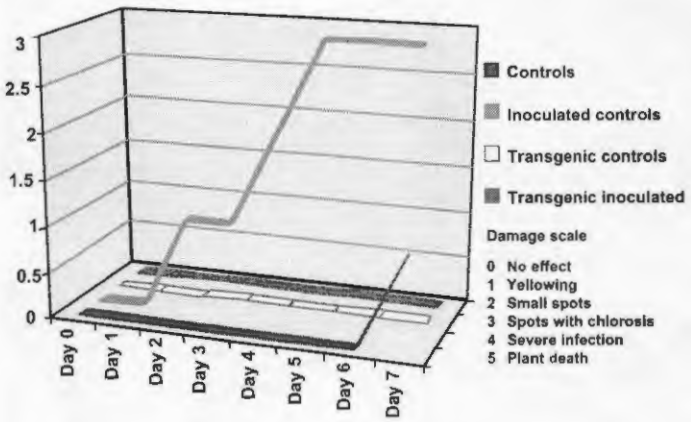
Several transgenic lines were identified in their tolerance/resistance to the attack of *A. triticina*, using an inoculum concentration of 10^6 conidia/ml under the incubation conditions mentioned earlier. Two different phenological stages (in the 5-leaf stage and spiking adult stage) were evaluated for their response to the attack of this pathogen, and although the wheat proved susceptible at both stages, it was most susceptible in the heading stage of adult plants.

After infection with the pathogens *F. graminearum, P. tritici-repentis, Rhizoctonia* sp., and *Phytium* sp. the detached leaves showed less or no resistance/tolerance than was shown by the control. This was in contrast to the inoculation with *A. triticina,* with which there was a clear difference between the controls and the transgenic plants. Although the infection with *B. sorokiniana* was successful, this pathogen was very aggressive in the colonization of the foliar tissue (95–100% foliar area infected by micelium) and no tolerant/resistant materials for this pathogen were detected for the transgenic lines tested.

Wheat transformation with PR genes generated a number of independent events that showed a range of responses to the pathogens, from increased susceptibility to tolerant (including hypersensitive reactions) and resistant reactions. Several lines and their progenies (that were Basta™ resistant) were selected for fungal bioassay. The presence and expression of the PR genes were confirmed by molecular tests (PCR and Western blot).

After *A. triticina* inoculation, different levels of disease damage were observed. The evaluation of the fungal infection was from the third day after inoculation. The damaged or lesion areas on some of the transgenic plants were small (less than 25% of leaf area damaged by chlorotic or necrotic lesions) or absent compared to those of the control (more than 50–75% of leaf area damaged). The plants that showed tolerance or resistance in the in vitro assay were selected and tested for the in vivo assay. In most cases, the in vivo results confirmed the in vitro results.

Several progenies from different events of the different PR gene constructs were challenged with *A. triticina*. Thaumatin-like antisense plants gave unexpected tolerance or resistance responses in some cases. These selected transgenic plants did not show any symptoms or showed small necrotic lesions as a hypersitive reaction. *A. triticina* was reisolated from the necrotic spots of both control and transgenic plant samples. Preliminary results of Western blots indicate that some band patterns of the wheat endogenous thaumatin-like proteins seem be related to increased resistance of transgenic wheat plants to *A. triticina*. However, this relationship is currently being investigated further (Pellegrineschi et al., In press)



In contrast to these results, susceptibility increased or remained the same as the positive control (inoculated nontransgenic plants) for most of the progenies from events of thaumatin-like protein sense, in which an overexpression of this PR protein and an enhanced tolerance/resistance response to the fungal attack was anticipated. Inoculations of transgenic wheat plants containing the β-1,3 glucanases transgene (sense and antisense) with the fungi, in most cases did not reveal any significant differences with respect to the controls.

Discussion

Because of the reliability of this phenotypic analysis, which challenged transgenic plants against a wide range of pathogens, it should be a very useful method for evaluating the effects of introducing genes coding for PR proteins into wheat.

This bioassay allows the rapid identification of transgenic plants or resistant wheat material with tolerance or resistance to important fungal pathogens such as *A. triticina* and the possibility of applying this method of selection to other, principally foliar, pathogens. It also allows an acceleration of the selection process by identifying the studied traits in the progeny through physiological characterization of the plant response to pathogen attack.

This assay was very reliable, economical, and reproducible for the screening of large numbers of plants. The preliminary results obtained from the in vitro bioassays were consistent with those obtained through in vivo bioassay and by molecular analysis for the presence and expression of the PR genes.

This antifungal assay has other advantages such as testing several fragments of leaves without kill the plant, and considering several repetitions (at least 8 fragments per plant)

Considering the reliability of results obtained with *A. triticina* this pathogen could be used as a model of a foliar disease for selecting tolerant/resistant material in a preliminary screening.

References

Beffa, R. S., Neuhaus, J. M. and Meins, F. Jr. 1993. Physiological compensation in antisense transformants: Specific induction of an "ersatz" glucan endo-1,3-β-glucosidase in plants infected with necrotizing viruses. Proc. Natl. Acad. Sci. 90: 8792-8796.

Beffa, R. S., Hoefer, R. M., Thomas, R. and Meins F. 1996. Decreased susceptibility to viral disease of β-1,3-glucanase-deficient plants generated by antisense transformation. Plant Cell 8:1001-1011.

Bol, J.F., Linthorst, H.J.M. and Cornelissen, B.J.C. 1990. Plant pathogenesis-related proteins induced by virus infection. Annu. Rev. Phytopathol. 28 113-138.

Broglie, K., Chet, I., Holliday, M., Cressman, R., Biddle, P., Knowlton, S., Mauvais, C.J. and Broglie, R. 1991. Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani.* Science 254: 1194-1197. 64.

Dumas-Gaudot, E., Slezack, S., Dassi, B., Pozo, M.J. Glaniazzi-Pearson, V. And Glaniazzi, S. 1996. Plant hydrolytic enzymes (chitinases and β-1,3-glucanases) in root reactions to pathogenic and symbiotic microorganisms. Plant Soil 185211-221.

Hu X. and Reddy, S.N. 1995. Nucleotide sequence of cDNA clone encoding a thaumatin-like protein from *Arabidopsis.* Plant Physiol. 107: 305-306.

Jach, G., Görnhardt, B., Mundy, J., Logemann, J., Pindorf, E., Leah, R., Schell, J., Maas, C.1995. Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco. Plant Journal., 8(1):97-109.

Jongedijk, E., Tigelaar, H., Van Roekel, J.S.C., Bres-Voloemans, S.A., Dekker, I., van den Elzen, P.J.M., Cornelissen, B.J.C., Melchers, L.S. 1995. Synergistic activity of chitinases and β-1,3-glucanases enhaces fungal resistance in transgenic tomato plants. Euphytica 85: 173-180.

Kauffmann, S., Legrand, M., Geoffroy, P. and Fritig, B. 1987. Biological function of 'pathogenesis-related' proteins: four PR proteins of tobacco have 1,3-*b*-glucanase activity. The EMBO Journal 6: 3209-3212.

Krishnaveni, S., Muthukrishan, S., Liang, D.H., Wilde, G. and Manicham. 1999. Induction of chitinases and β-1,3-glucanases in resistant and susceptible cultivars of sorghum in response to insect attack, fungal infection and wounding. Plant Science 144: 9-16.

Lamb, C.J., Ryals, J.A., Ward, E R. and Dixon, R. A. 1992. Emerging strategies for enhancing crop resistance to microbial pathogens. Biotechnology. 10: 1436-1445.

Legrand, M., Kauffmann, S., Geoffrey, P. and Fritig, B. 1987. Biological function of pathogenesis-related proteins: Four tobacco pathogenesis-related proteins are chitinases. Proc. Nat. Acad. Sci. USA 84: 5750-6754.

Linthorst, H.J.M., Van Loon, L.C., Van Rossum, C.M.A., Mayer, A., and Bol, J.F. 1990. Analysis of acidic and basic chitinases and their expression in transgenic tobacco. Mol. Plant-Microbe Interact. 3: 252

Linthhorst, H. J. M. 1991. Pathogenesis-related proteins of plants. Critical Reviews in Plant Sciences 10: 123-150.

Mauch, R., Mauch-Mani, B., Boller and T. 1988. Antifungal hydrolases in pea tissue. II. Inhibition of fungal growth by combinations of chitinase and b-1,3-glucanase. Plant Physiology 88: 936-942.

Nishizawa, Y., Nishio, Z., Nakazono, K., Soma, M., Nakajima, E., Ugaki, M. and Hibi, T. 1999. Enhanced resistance to blast (*Magnaporthe grisea*) in transgenic Japonica rice by constitutive expression of rice chitinase. Theor. Appl. Genet. 99: 383-390.

Pierpoint, W. S., Jackson, P. J. and Evans R. M. 1990. The presence of a thaumatin-like protein, a chitinase and a glucanase among the pathogenesis-related proteins of potato (*Solanum tuberosum*). Physiological and Molecular Plant Pathology 36: 325-338.

Rajaram, S. 1999. Historical aspects and future challenges of an international wheat program. In: *Septoria* and *Stagonospora* diseases of cereals: A compilation of global research. -The fifth International Septoria Workshop (September 20-24, 1999)-CIMMYT. Mexico.

Reimmann C. and Dudler, R. 1993. cDNA cloning and sequence analysis of a pathogen-induced thaumatin-like protein form rice (*Oryza sativa*). Plant Physiol. 101:1113-1114.

Sela-Buurlage, M., Ponstein, A.S., Bres-Vloemans, S., Melchers, L., van den Elzen, J.M. and Cornelissen, B.J.C. 1993. Only specific tobacco (*Nicotiana tabacum)* chitinases and *b*-1,3-glucanases exhibit antifungal activity. Plant Physiol. 101: 857-863.

Stack, R. W. 1999. Return of an old problem: *Fusarium* head blight of small grains In: APS net features (May 1-May 31).

Wessels, J.G.H. and Sietsma, J.H. 1981. Fungal cell walls: a survey. In: encyclopedia of plant physiology, N.S., vol 13B: Plant carbohydrates II, pp 352-394. Tanner, W., Loewus, F.A. eds. Springer, Berlin Heidelberg New York.

Xue,L., Charest, P.M. and Jabaji-Hare, S.H. 1998. Systemic induction of peroxidases, 1,3-β-glucanases, chitinases and resistance in bean plants by binucleate *Rhizoctonia* species. Phytopathology 88 (4): 359-365.

Zhu, Q., Maher, E. A., Masoud, S., Dixon, R. A. And Lamb, C. J. 1994. Enhanced protection against fungal attack by constitutive co-expression of chitinase and glucanase genes in transgenic tobacco. Bio/Technology 12: 807-812.