Optimization of an in vitro bioassay for wheat diseases
Salgado M.M., A. Pellegrineschi, M. Mezzalama, S. McLean, and D. Hoisington

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, Fusarium spp.; "Tilletia head scab," Fusarium; “spot blotch,” Bipolaris sorokiniana (M. sativum); "race spot," Pyrenophora triticicrepentis; "Alternaria leaf blotch," Alternaria sp.; and "leaf blotch," Septoria tritici (Black 1999; Rajaram 1998). 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 "capping 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 (Brogie et al. 1991; Nishizawa et al. 1999).

Among the PR-protein families, chitinases and glucanases are hydrolytic enzymes that degrade the polysaccharide chitin and 1,3-glucan, which are the major components of the cell walls of many fungi (Weselsky and Sietmann 1981). These enzymes have been shown to be effective in controlling disease (Lengyel et al. 1987; Mauch, et al. 1989; Boi et al. 1992; Lindhorst 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 thaumatin, a set of sweet-tasting proteins from the African shrub Thaumatomucuna dianthi (Weismann 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 function of the corresponding proteins involved in the control of important fungal diseases in wheat. The overexpression or inactivation of these genes should serve to enhance the tolerance/resistance or increase susceptibility of wheat plant's response to fungal pathogens.

Most in vitro bioassays for testing anthifungal 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 fungal species 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 for tolerances' resistant wheat (including transgenic plants) to the attack of important fungal pathogens and for screening large number of plants.

Materials and Methods
The fungal isolates were obtained from the collection of the Pathology Laboratory at CIMMYT of the samples of wheat (Triticum sativum) from infected wheat. The plant material used was Bobwhite and Blavacora (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.

The inoculum was increased on V-8 agar medium, in petri dishes, for A. tritici, R. oryzae, B. sorokiniana, and Pythium sp., and on PDA (Potato Dextrose Agar) medium for the Rhizoctonia sp. and R. graminis. 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 homogenized by vortexing 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 6-well rectangular multi-dishes, at room temperature. The level of resistance of the material to these pathogens was evaluated 4-7 days after inoculation.

The optimal concentration of inoculum of A. tritici was determined by inoculating the nontransgenic plants (Bobwhite and Blavacora) with different concentrations (10", 10", 10" conidia/ml). Two different phenological stages (5-leaf stage and spikeless stage) were considered for each inoculation. The response to artificial inoculation with these pathogens in transgenic lines or wheat from T0 and T1 progeny was evaluated by an scale that considered the percentage of leaf area damaged and the type of lesion (neurotic or chlorotic, size of lesion).

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

Particular of biomass showing resistant and susceptible lines.

Effect of A. tritici in controls. 4, 8, 12 days from the top.

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Results

Several transgenic lines were identified in their tolerance/resistance to the attack of A. triticina, using an inoculum concentration of 10⁵ 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. tritic-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 Basalt™ 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 vitro results confirmed the in vivo results.

Several progenies from different events of the different PR genes were challenged with A. triticina. Thaumatin-like class disease genes 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 hypersensitive 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 to be related to differences with respect to the controls. Inoculation of these different lines with A. triticina was successful, this pathogen was very aggressive in contrast 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 follar, 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 for a fungal disease for selecting tolerant/resistant material in a preliminary screening.

References


