Transgenic wheat plants: a powerful breeding source

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Abstract

Plant breeders are always interested in new genetic resources. In the past, the sources have been limited to existing germplasm. Genetic engineering now provides the opportunity for almost unlimited strategies to create novel resources. As a first stage, the Applied Biotechnology Center (ABC) at CIMMYT developed a method for the mass production of fertile transgenic wheat (*Triticum aestivum* L.) that yields plants ready for transfer to soil in 13–14 weeks after the initiation of cultures, and, over the course of a year, an average production of 5–6 transgenic plants per day. CIMMYT elite cultivars are co-bombarded with marker gene and a gene of interest with co-transformation efficiencies around 25–30%. The reliability of this method opens the possibility for the routine introduction of novel genes that may induce resistance to diseases and abiotic stresses, allow the modification of dough quality, and increase the levels of micronutrients such as iron, zinc, and vitamins. The first group of genes being evaluated by the ABC are the pathogenesis related (PR) proteins, such as the thaumatin-like protein (TLP) from barley, chitinase, and 1–3 β-glucanase. Stable integration of the genes in the genome and inheritance in the progeny were determined by phenotypical analyses that challenged the plants against a wide range of pathogens. Using these genes, we have recovered more than 1200 independent events (confirmed by PCR and Southern blot analyses) that show responses to the pathogens that range from tolerance to hypersensitive reactions. The quantity and anti-fungal activity of the endogenous thaumatin-like proteins were analyzed in T_1 and T_2 progeny plants. Western blot analyses showed different protein patterns of the wheat endogenous TLPs. Preliminary results indicated that some patterns increased the resistance of transgenic wheat plants to *Alternaria triticina*. This relationship is being further investigated.

Introduction

Wheat fungal pathogens inflict losses of 5–10% on global wheat production of 600–700 million tons. Currently, epidemics of fungal pathogens are controlled through the application of chemicals, resistant varieties, and/or various agronomic techniques, such as tillage or crop rotations. Plant breeding is creating new cultivars with resistance to fungal diseases through the use of major genes and by pyramiding minor genes to give horizontal or durable resistance. These plant-breeding programs are based on the process of crosses, backcrosses, and selection, which is time-consuming and inadequate for coping with the rapid evolution of pathogenic microorganisms.

To combat this problem, approaches such as gene discovery and cloning, genetic engineering, and marker-assisted selection techniques can be used for the efficient production of elite plant germplasm. These approaches allow the rapid identification and isolation of genes, the transfer of traits from one species to another, and an acceleration of the selection process by identifying the studied traits in the progeny. One of the strategies to finding these traits (or genes) is the physiological characterization of the plant response to pathogen attack. Plants induce several defense reactions that initiate physiological mechanisms that play an important role in the overall expression of disease resistance (Legrand et al., 1987; Linthorst et al., 1990). Some of these hy-

drolytic enzymes have been shown to increase the resistance of plants against various pathogens *in vitro* (Kriehanaveni et al., 1997; Mauch et al., 1988; Sela-Burrlage et al., 1993). Another important part of the plant defense system, related to the PR proteins, is the induction of anti-fungal compounds called thaumatin-like proteins (TLPs); termed such for their similarity with thaumatins, a set of sweet-tasting proteins from the African shrub *Thaumatococcus daniellii* (Reimmann & Dudler, 1993). This study has focused on the effect of the antisense TLP gene on the pattern of expression of the endogenous wheat TLPs. We observed a reduction in the effects of *Alternaria* on the transgenic plants produced that could be related to different expression of the endogenous TLPs.

Materials and methods

Plasmid construction, transformation, selection, and regeneration procedures

Details of the transformation protocol for the mass production of transgenic wheat plants are described by Pellegrineschi et al. (1998, 1999).

Molecular and protein analyses

Plant DNA was extracted from young leaves using the Nucleospin DNA purification kit from Clontech and following the producer's protocol. All the Basta resistant plants were analyzed by PCR for the presence/absence of the *Bar* and *Hpt* genes. Two pairs of specific primers were used to detect the transgenes: for the *Bar* gene, forward primer 5′-GTCTGCACCATCGTCAACC-3′ and reverse primer 5′-GAAGTCCAGCTGCCAGA AAC-3′; for the *Hpt* gene, forward primer 5′-GCGTCTGCTGCTCCATACAAG-3′ and reverse primer 5′-ACCTGCCTGAAACCGAACTGC-3′. The PCR reactions were carried out in a total volume of 25 μl, comprising 10 ng of wheat genomic DNA, 50 mM KCl, 10 mm Tris-HCl (pH 8.8), 3 mM MgCl$_2$, 0.24 mM each dNTP, 0.04 units Taq DNA polymerase, and 0.16 μmol of each primer. For PCR analyses for both genes, DNA was denatured at 94 °C for 1 min. (hot start), followed by 30 amplification cycles (94 °C for 30 s., 64 °C for 2 min., 72 °C for 2 min.). Total genomic DNA was isolated from 1 g fresh weight leaf material using the Nucleon Phytopure Plant DNA Extraction Kit according to the manufacturer's instructions (Amersham Life Sciences). A

50-μg aliquot of DNA was digested overnight at 37 °C with the appropriate restriction enzyme. The digested DNA was fractionated in a 1.0% agarose gel, transferred to a positively charged nylon membrane (Boehringer Mannheim), and hybridized to digoxigenin (DIG)-dUTP labeled probes according to the manufacturer's instructions (Boehringer Mannheim). All Basta-resistant plants were analyzed for endogenous thaumatin expression by Western blot. Protein was extracted from young leaves, as described by Koziel et al. (1993). Protein concentration was determined using the standard Bradford assay (Bradford, 1976). Protein aliquots (20 μg per lane) were loaded and fractionated on a 1.5% SDS-polyacrylamide gel (Laemmli, 1970) before transfer to a nitrocellulose membrane. The membrane was incubated with a polyclonal rabbit anti-PR5 primary antiserum and then with an alkaline-phosphatase-conjugated goat anti-rabbit second antibody (Promega).

In vitro and *in vivo* *antifungal assay*

Alternaria, Fusarium, Helminthosporium, Pitium, and *Rhizoctonia* strains were obtained from the CIMMYT phytopathology laboratory. To obtain fresh spores of the pathogens, BS medium was inoculated with a small block of inoculated medium and cultured at 26 °C under a 16/8 hours (light/darkness) photoperiod. Conidia were harvested after 2 weeks by flooding the agar plates with sterile water and filtering through three layers of Miracloth. The concentration was adjusted at 10^6 conidia/ml. Assays were performed in 8-well dishes; the wells were filled with 5 ml of water/agar medium. Inoculated leaves were transferred to the multi-well dishes and incubated for 5 days. The damage of the pathogens was scored daily with an evaluation scale of 1 to 5, in which '1' indicates the least, and '5', the most chlorotic, according to the level of chlorosis and/or necrosis on the inoculated leaf. The *in vivo* test was performed only with *Alternaria triticina* because the tested plants showed resistance/tolerance only to this pathogen. The inoculum was applied by spraying the plants with a conidia solution (10^6 conidia/ml). The plants were left in high humidity for 72 h at 25–28 °C. After incubation, the plants were transferred to a biosafety growth-chamber at 28 °C with a photoperiod of 16 h of light/8 h darkness. After 10 days, samples of the infected leaves were collected and transferred to incubation medium for pathogen re-isolation.

Results

Transformation and regeneration

One hundred and eighty independent transformation experiments were conducted, using biolistic gun delivery on immature embryos of the Bobwhite and Baviacora wheat cultivars. Plantlets were obtained from the large number of PPT-resistant somatic embryos; 447 vigorously growing plants were selected and transferred to the biosafety greenhouse. For the preliminary screening of transformants, Basta herbicide was sprayed at the 5–6 leaf stage. PCR analyses for *Bar* and *Hpt* presence were carried out independently, and 100% of the resistant plants exhibited *Bar*. Only 113 were positive to *Hpt*.

Western blot analysis

Leaves from the plants with the 1.3-kb tlp-D34 DNA band diagnostic of the presence of a full-length copy of the tlp-D34 in their genomes were assayed for TLP expressions. Thirty plants had the expected 23-kDa TLP-D34 protein band as detected by the PR5 primary antibody. Comassie Blue staining of the protein gel was done with a standard concentration of BSA to estimate the approximate quantity of TLP produced by the transgenic plants. Seven transgenic lines had a clearly identifiable PR5 protein band pattern at apparent concentrations several times higher than the background proteins seen in this size range when compared to nontransgenic controls. Like the nontransformed control, other transgenic lines did not show an additional protein band. Protein bands of T_0 and T_1 plants of several other transgenic lines showed a high amount of protein.

Bioassays

We selected several lines for the fungal bioassay from the T_1 transgenic plant progenies that tested positive for the functional anti-TLP transgene in Western blots. With *Alternaria*, thaumatin-like antisense plants gave positive responses, indicating immunity in some cases. Inoculations with the other pathogens (*Fusarium, Helminthosporium, Pitium*, and *Rhizoctonia*) did not reveal any significant differences with respect to the controls. With the *A. triticina* inoculation, different levels of disease damage were observed, starting from the third day after the inoculation. The lesion areas on some of the transgenic plants were small or absent compared to those of the control plants. The plants that showed tolerance or resistance were selected for the in vivo assay. *In vivo*, the disease symptoms were evident in the controls 8 days after the inoculation. The selected transgenic plants either did not show any symptoms or else showed spots that rapidly necrotized. We were able to re-isolate the *A. triticina* from the necrotic spots from samples of the controls and transgenic plants.

Discussion

When plants are invaded by a fungal pathogen, major physiological changes are induced and plant defense mechanisms are activated. In previous studies, these plant enzymes, including PR proteins, were reported to increase in plants that were infected with pathogens. Currently, the common strategy utilized to increase the resistance/tolerance in wheat to fungal diseases has been based on the overexpression of genes related to PR plant response. This strategy produced some successes in inducing resistance to some fungal pathogens of tobacco and rice (Datta et al., 1999), enhancing resistance to *Botrytis cinerea* in grape (Derkel et al., 1998), and slowing the development of *Fusarium* in wheat (Chen et al., 1999). Our observations on the effects of the antisense thaumatin-like gene appears to contradict these results. In our experiments, the plants that should have overexpressed the barley thaumatin-like protein (construct 38/1) did not show any resistance. However, the phenotype observed in other studies, while not explained by the expression of the introduced transgene, could possibly be explained by gene inactivation (Hart et al., 1992; Montgomery et al., 1998; Mayer, 1995). Gene silencing is a widespread phenomenon in transformed plants (Beffa et al., 1993, 1996). It suppresses endogenous and/or transgene expression and may occur through a wide variety of mechanisms (Flavell, 1994; Meyer & Saedler, 1996; Itoh et al., 1997). In this study, we introduced a barley PR-5 antisense gene (thaumatin-like protein) into wheat. Molecular characterization revealed the presence of the coding sequence of the introduced gene and Western blot analyses revealed a modification of the PR 5 expression pattern on some of the transgenic plants. Bioassay data correlated these protein patterns with resistance to *A. triticina* in selected T_1 wheat lines. A few of the transformed lines did not show enhanced protein expression (CB629-3). This is likely due to suppression of the reintroduced gene, which has also been reported in earlier studies (Itoh et

al., 1997). The bioassays of *A. triticina* were under greenhouse conditions. Biomass of the plants, high humidity, optimum temperature, and genotype played important roles in determining the correct evaluation of the bioassay (Cu et al., 1996). Under greenhouse conditions, data were scored based on percentage density of *A. triticina* infection occurring on control plants. The mean of infection ranged from 0% to 19%, compared to 95–100% in the control plants. It is noteworthy that several transgenic lines were identified as having very limited infection compared to control plants.

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