Transgenic expression of the TRI101 or PDR5 gene increases resistance of tobacco to the phytotoxic effects of the trichothecene 4,15-diacetoxyscirpenol

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Abstract

Mycotoxins are fungal secondary compounds that are toxic to vertebrates. Their presence in food and feeds, as the result of fungal disease in crops, can present a danger to animal or human health. Many mycotoxins have also been shown to be phytotoxic and in some cases, such as with trichothecenes produced by the wheat head blight fungus Fusarium graminearum, mycotoxins may act as virulence factors. Antibiotic-producing organisms, including fungi, protect themselves from their own toxins by metabolic alteration of the compound, modification of the target site of action or by exporting the compound to the extracellular space. We have tested the effectiveness of adapting two of these strategies, metabolic alteration and extracellular transport, to protect plant cells from the deleterious effects of the trichothecene 4,15-diacetoxyscirpenol (DAS). Tobacco plants were transformed with either the Saccharomyces cerevisiae gene PDR5, which encodes a multi-drug transporter, or with the Fusarium sporotrichioides gene TRI101, which encodes a trichothecene 3-O-acetyltransferase. Both genes conferred significant increased tolerance to DAS as measured by a sensitive seed germination assay. Expression of PDR5 or TRI101 in a seed-specific manner in crop plants such as wheat could lower the incidence of head blight as well as reduce mycotoxin levels within the seed. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Trichothecenes are cyclic sesquiterpene epoxide mycotoxins produced by several fungal species belonging to the genus Fusarium and related genera [1]. Trichothecenes are potent inhibitors of protein synthesis in eukaryotic cells [2] and act as virulence factors in plant diseases such as wheat head scab due to their phytotoxic properties [3]. Trichothecenes are also of considerable interest because of their potential occurrence in animal feeds and human foods and the risk to health and agricultural productivity that their presence creates.

Mycotoxin-producing fungi have developed strategies to protect themselves from their own toxins [4]. In the case of Fusarium graminearum, for instance, trichothecene 3-O-acetyltransferase (TRI101) converts the highly toxic trichothecenes into considerably less toxic derivatives [5]. Another protection strategy is to transport toxins out of the cell via membrane transporter pumps [6]. Either of these mechanisms could be adapted through the genetic engineering of crop plants to...
increase the resistance against *Fusarium*-caused diseases such as wheat head scab by increasing the plant’s tolerance to the trichothecene virulence factors. As a first step in applying these strategies, we tested the effects of expressing either TRI101, a *Fusarium sporotrichioides* gene encoding trichothecene 3-O-acetyltransferase, or PDR5, a yeast gene encoding a multidrug resistance transporter [7], on the tolerance of transgenic tobacco to trichothecene inhibition of germination and plantlet development.

2. Materials and methods

2.1. Construction of vectors and plant transformation

The PDR5 [8] and TRI101 genes [5] were amplified by *Pfu* polymerase using templates of *Saccharomyces cerevisiae* genomic DNA (generously provided by J. Golin, The Catholic University of America) and *F. sporotrichioides* cDNA, respectively. The amplified products were subsequently cloned into the pBI121 plasmid (Clontech) in place of the β-glucuronidase gene, as previously described [9]. A vector control plasmid, a pBI121 derivative that lacks the β-glucuronidase gene, was also generated and transgenic tobacco plants containing one of the three constructs (TRI101, PDR5, or vector control) were produced [10].

2.2. Growth conditions and seed germination assay

Tobacco cell suspension cultures were propagated as before [9], but with the modification that culture volumes were scaled down from 100 to 20 ml and culture flasks were inoculated with 1 ml of 7-day-old cells rather than 4 ml. Tobacco seed germination studies were performed under aseptic conditions. Tobacco seeds were surface sterilized by incubation for 15 min in 10% (v/v) Clorox containing 0.1% Tween-20. Subsequently, the seeds were rinsed four times with sterile distilled water and then distributed onto 60-mm diameter petri plates containing MS salts and 30 g/l sucrose (pH 5.8). Plates, containing an average of 240 seeds, were incubated for 2 weeks under 16 h light/dark cycle, with average temperatures of 26°C (day) and 22°C (night). Afterwards, the plates were photographed, then the seeds were tallied by hand for germination (radicle emergence) using a dissecting microscope.

2.3. Trichothecene preparations

The trichothecenes 4,15-diacetoxyscirpenol (DAS) and 3,4,15-triacetoxyscirpenol (TAS) and (deoxynivalenol) DON (see Fig. 1) were prepared as described previously [11], dissolved in acetone to 0.1 M and added to the media, after it had been autoclaved, to final concentrations indicated. Preliminary experiments revealed that equivalent amounts of acetone alone (0.02% v/v) did not affect germination or plantlet development.

2.4. RNA blot analysis

Tobacco cell RNA blot analysis was performed as described previously [12], using the full length PDR5 gene or the TRI101 cDNA as probes.

3. Results

Deoxynivalenol (DON) is a major trichothecene product of *F. graminearum*, the primary fungal species responsible for head scab in wheat, while DAS is generally produced by *Fusarium poaeae* and *Fusarium equiseti*, species regarded as minor fungal components of wheat head scab infection [13]. To develop a suitable test system for measuring phytotoxicity in our model transgenic (tobacco)
Fig. 2. Effects of DAS and TAS on the growth of tobacco suspension cells. Initial fresh weight of cells was approximately 0.075 g per flask. Trichothecenes were added at day 0 and cells were incubated for 7 days. Data points represent the means of two samples. Error bars indicate the range of the individual samples.

While DAS effectively bleached leaf discs and prevented plantlet regeneration when cultured on regeneration medium, seed germination was determined to be a more reliable and sensitive bioassay for monitoring trichothecene phytotoxicity and was used therefore to test the effects of PDR5 or TRI101 expression on plant tolerance of trichothecenes.

It has been proposed that some of the trichothecene-producing Fusarium fungi possess the ability to protect themselves from their own toxins by acetylating the trichothecene ring at the three position [5]. To see if 3-O-acetylation also renders Fusarium trichothecenes less phytotoxic, we compared the relative inhibitory effects of DAS versus TAS on tobacco suspension cell culture growth (Fig. 2). Acetylation of the 3-O position on the trichothecene ring of DAS appeared to strongly reduce its toxicity as measured by the fresh weight gain of the culture over 7 days. While alternative interpretations of the data are possible, such as a reduced uptake of TAS by the cells, these results encouraged us nevertheless to pursue the transgenic approach toward reducing trichothecene phytotoxicity in tobacco.

The TRI101 or PDR5 genes were incorporated into separate plant expression cassettes and moved into tobacco via Agrobacterium-mediated transformation. A number of kanamycin-resistant tobacco cell lines were recovered for each transformant, as well as for the vector control, and all were tested in a preliminary T1 seed germination screen for increased trichothecene resistance (data not presented). The most promising lines, (TRI1011, TRI1013 and PDR56), along with two vector control lines (P1 and P3), were characterized further. In all three experimental lines, RNA blot analysis revealed high level expression of TRI101 or PDR5 genes, while the vector control lines lacked either transcript (Fig. 3).

With non-transformed tobacco seeds, DAS was shown to be an effective inhibitor of germination, with an I0.5 of approximately 6 µM. (Fig. 4). Moreover, DAS also appears to impede shoot morphogenesis as most of the seeds that did germinate in the presence of DAS did not proceed beyond the radicle stage (Fig. 5). In the presence of lower DAS concentrations, a few of the germinating seedlings produced chlorophyll within the rudimentary shoot zone while others produced plantlets with thick, stubby roots and small, curled
cotyledons and leaves (Fig. 6). In rare instances, DAS-treated seeds produce normal plantlets, which were devoid of chlorophyll.

Tobacco seed germination was significantly less susceptible to inhibition by DAS when the plants were transformed with either the TRI101 or PDR5 genes when compared with the non-transformed controls (Fig. 4). In fact, for all three experimental lines, germination was remarkably unaffected by DAS concentrations up to 10 μM. In contrast, the germination was inhibited by DAS in the vector control transformants to the same degree as in non-transformed control.

The resistance of the PDR5 and TRI101 transformants was reflected not only in the higher percentages of germinating seeds, but also in the higher numbers of seedlings containing chlorophyll and that had proceeded with morphological development (Fig. 5). In this regard, the PDR5 and two TRI101 seed lines were only slightly less vigorous in appearance when grown in the presence of 5 μM DAS than any of the lines grown without DAS. In contrast, the few non-transformed or vector control seedlings that did develop green shoots in the presence of 5 μM DAS were highly reduced in size compared with seedlings not challenged with DAS.

4. Discussion

The role of mycotoxins in plant disease has not been established unequivocally [1]. However, there is evidence, both from gene disruption [3] and UV-induced mutations [14] of trichothecene biosynthesis that trichothecenes serve as virulence factors in some plant diseases such as wheat head scab. Consistent with this role, a number of trichothecenes have been shown to be phytotoxic [1,13]. In the case of tobacco, which was chosen here for study due to the ease of genetic transformation, DAS appears to be an effective inhibitor of seed germination, root and shoot growth, fresh mass accumulation in cultured cells, and of regeneration from leaf discs. All of these effects have been reported to occur in other plants as well. For instance, trichothecenes decrease the mitotic indices of roots from germinating rye, wheat, triticale and field beans [15], inhibit seed germination, reduce root growth and inhibit leaf mass increases in wheat seedlings [16], inhibit root and shoot growth in germinating maize embryos [17] and reduce the fresh mass accumulation and regeneration potential of anther-derived wheat callus [18]. As trichothecenes are known inhibitors of protein synthesis, all of these far-ranging phytotoxic effects could be explained by the inhibition of plant cell protein synthesis, although, in fact, little is known about the specific details of the mode of action of these compounds in tobacco or any other plant [13]. Interestingly, we found DAS to be more phytotoxic than DON toward tobacco, whereas DON was far more inhibitory than DAS toward wheat [16]. This
Fig. 5. Representative plates of the experimental and control tobacco seed lines on various concentration of DAS. The seed lines are, starting from the top row, non-transformed control, P1 vector control, P3 vector control, \( PDR5_{5-6} \), \( TRI101 \), \( TRI101 \). The DAS concentrations, starting from the left column, are 0, 5, 10, and 20 \( \mu \text{M} \).
observation supports the view that species-specific tolerance toward particular mycotoxins is based on differences in the uptake, translocation, metabolism, and target sensitivity [13].

Transgenic approaches to increase plant disease resistance have typically involved the expression of antifungal proteins aimed at inhibiting pathogen invasion into the plant tissues (e.g. [19]). Here, since trichothecene production is a virulence factor, we have taken the approach that a reduction in the intracellular levels of toxin should diminish pathogen ingress by removing or decreasing the amounts of fungal virulence factors. In our preliminary test of this approach, we found that the transgenic expression of either the PDR5 or the TRI101 gene significantly increased the resistance of germinating tobacco seeds to DAS inhibition. Since we have not determined biochemically the fate of DAS in either of these transformants lines, we assume that the increases in resistance is due to the expected mechanisms of the two gene products (e.g. toxin export or chemical modification, respectively). As these genes do confer these two unrelated capabilities to the plant however, it is encouraging to see that both strategies appear to be viable ways to reduce the phytotoxic effects of mycotoxins in planta. Whether the reduction of effective intracellular trichothecene levels in vivo can also reduce the pathogenesis of mycotoxin-producing plant diseases remains to be tested and in this regard, transformation of wheat and barley with these same two genes is in progress in collaborating laboratories. Regardless of their role in plant disease, the presence of mycotoxins in feeds and foodstuffs is a health concern for both animals and human beings, and approaches which seek to reduce mycotoxin levels within plant tissues could, at the least, improve the nutritional quality of plant-based foods and feeds.

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References


