European Journal of Plant Pathology **108:** 699–703, 2002.

Saccharomyces cerevisae and *Arabidopsis thaliana*: Useful model systems for the identification of molecular mechanisms involved in resistance of plants to toxins

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Key words: mycotoxin, deoxynivalenol, drug efflux, ribosomal protein L3, trichothecene

Abstract

Secondary metabolites produced by pathogens during the infection process are thought to play a role as pathogenicity or virulence determinants in many plant diseases. Baker's yeast and the plant *Arabidopsis thaliana* are attractive models for elucidating molecular mechanisms of resistance to toxic substances. For the *Fusarium* mycotoxin deoxynivalenol, the following resistance mechanisms were identified in yeast: (1) reduced toxin uptake due to the ABC transporter protein Pdr5p (molecular efflux pump), (2) detoxification by the acetyltransferase Ayt1p, and (3) modification of the ribosomal target by amino acid changes in the ribosomal protein L3 (Rpl3p). *PDR5*-like genes exist in plant genomes as large gene families and could play an important role as a first line of defence against a broad range of toxic metabolites. Amino acid alterations in the highly conserved *RPL3* genes could likewise play a role in trichothecene resistance in plants. The knowledge obtained using model systems should be valuable in biotechnological approaches to disease control and marker-assisted resistance breeding.

Abbreviations: ABC – ATP binding cassette; PDR – pleiotropic drug resistance.

Introduction

Plant pathogenic fungi frequently produce toxins in infected plants. These products can cause adverse health effects when high levels are present in food or animal feed. Mycotoxins are frequently phytotoxic, and for obvious reasons toxin production is suspected to contribute to the virulence of pathogens. A well characterized example is the interaction of *Cochliobolus carbonum* with its host plant maize. Resistance against the pathogen is caused by neutralization of the fungal pathogenicity factor (HC toxin) by the product of the *HM1* disease resistance gene, which encodes a specific detoxification enzyme (Pannaccione et al., 1992; Johal and Briggs, 1992). For most toxins, the situation is less clear. Often only circumstantial evidence is available (e.g. correlation of the amount of toxin produced with virulence of the pathogen). On the other hand, plant breeders are frequently confronted with a situation where quantitative differences in toxin resistance are observed between cultivars and these differences are correlated with resistance to the pathogen (Buiatti and Ingram, 1991). For most agriculturally important pathosystems, the molecular basis of toxin resistance in the respective host plants is not known.

Fusarium head blight (FHB, scab) of small-grain cereals is an agriculturally important disease (Parry et al., 1995; McMullen et al., 1997) causing severe yield losses and unacceptable contamination of the harvested grain with deoxynivalenol (DON). The mycotoxin DON is a representative of the class of trichothecenes, a highly diverse class of toxins acting as inhibitors of eukaryotic protein biosynthesis. Many 'pathogenicity-related' proteins are specifically induced in plants by

pathogens and seem to play a central role in the defence response of plants (Kombrink and Somssich, 1997). Toxins with the potential to interfere with the expression of defence related proteins are therefore obvious candidates for virulence factors (affecting the kinetics or severity of disease development). Evidence that the production of trichothecenes contributes to the virulence of *Fusarium graminearum* on wheat has been obtained in laboratory and field studies using gene disruption mutants of the fungus (Proctor et al., 1995; Desjardins et al., 1996).

No complete resistance is known in the wheat breeding material, but quantitative differences exist and are inherited in a complex fashion. Toxin resistance seems to be a relevant component of *Fusarium* resistance (Grausgruber et al., 1998). Testing of a large number of wheat cultivars representing the full spectrum of available *Fusarium* resistance revealed a strong correlation between toxin resistance (determined using a seed germination assay, Lemmens et al., 1994), and field resistance, indicating that about 40% of the variation can be explained by differences in toxin resistance (Lemmens et al., unpublished data).

Our research goal is to identify molecular mechanisms responsible for differences in trichothecene resistance in plants, and for this purpose we have employed the yeast *Saccharomyces cerevisiae* and the plant *Arabidopsis thaliana* as model systems.

Results and discussion

Identification of candidate trichothecene resistance genes: Drug efflux pumps

Resistance to a toxic compound can be achieved in several ways: reduced net uptake, detoxification (and sequestration), and modification of the drug target. Using yeast it was found that basal resistance was determined by the level of expression of the ABC (ATP binding cassette) transporter protein encoded by the *PDR5* (pleiotropic drug resistance) gene (Adam and Lemmens, 1996). Mutants containing a deleted *PDR5* gene showed growth inhibition by DON, 3-acetyldeoxynivalenol (3-AcDON), nivalenol, T-2 toxin and all other trichothecenes tested, while wild-type yeast strains tolerated high levels of these mycotoxins.

PDR5 is one of nine members of the (NBF-TMS_6)2 subclass of ABC genes present in the yeast genome (Decottignies and Goffeau, 1997). Characteristic features of *PDR5*-like genes are the internal duplication and the presence of the ATP binding sites (NBF, nucleotide binding folds Walker A and Walker B) which are located N-terminally of the predicted six transmembrane spanning domains (TMS). This structure is shown in Figure 1A. It differs from the structure of the *MDR1* related ('P-glycoprotein' like) and vacuolar *MRP*-like proteins, which have also been identified in plants (Dudler and Hertig, 1992; Lu et al., 1997). Pdr5p and other proteins of this class are located in the plasma membrane, and most likely confer resistance by acting as 'molecular efflux pumps', removing toxic substances using the energy of ATP hydrolysis.

The finding that the ABC transporter Pdr5p confers resistance to trichothecenes in yeast could become relevant for efforts to increase *Fusarium* resistance of crop plants by biotechnology. First, *Agrobacterium*-mediated transformation has been used to introduce the yeast *PDR5* gene into tobacco. Increased resistance to DON was observed in several of the regenerated plants using a leaf disc regeneration assay. On the other hand, there is evidence that *PDR5*-like genes are present in plant genomes as large gene families. For instance, database searches revealed the existence of 15 genes with *PDR5*-like topology and high sequence similarity in the *Arabidopsis* genome (see Figure 1A). The nomenclature proposed in 1999 at the 9th International Congress on Molecular Plant–Microbe Interactions and the COST 835 meeting in Rome is to name these genes *AtPDRxy*, with *y* counting the number of *PDR5*-like genes on *Arabidopsis* chromosome number *x* (Mitterbauer et al., 2000). The sequence similarity between the yeast *PDR5* gene and the various plant homologues is, for instance, high in a protein fragment (see Figure 1B), which is characteristic of the *PDR5*-like subclass of ABC transporters (for review see: Decottignies and Goffeau, 1997). Our group currently works on the inducible overexpression of *PDR5*-like genes in *Arabidopsis* and on the expression of the *Arabidopsis* cDNAs in yeast mutants with the goal to learn more about the role and substrate specificity of these plant *PDR* genes.

ABC transporter proteins with their broad but distinct substrate specificity could have an important, currently disregarded role in plant defence, as a first line protection against toxic metabolites produced by plant pathogens. Over-expression of foreign ABC transporter protein genes in plants using the tools of plant biotechnology seems a straightforward strategy. Nevertheless, the finding that plant genomes already contain a bounty of *PDR* genes raises the question: why has natural selection (or selection by plant breeders)

A)

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AtPDR11(11)   ---GRTVVCTIHQPSIDIFESFDELLLMKRGGQVIYAGSLGHHSQKLVEYFEAVEGVPKINDGYNPATWMLD---
AtPDR12(8)    ---GRTVVCTIHQPSIDIFEAFDELMLMKRGGQVIYAGPLGQNSHKVVEYFESFPGVSKIPEKYNPATWMLE---
AtPDR13(7)    ---GRTVVCTIHQPSIDIFEAFDELLLMKRGGHVIYSGPLGRNSHKVVEYFESFPGVPKIPEKYNPATWMLE---
AtPDR14(12)   ---GRTVVCTIHQPSIDIFEAFDELFLLKRGGEEIYVGPLGHESTHLINYFESIQGINKITEGYNPATWMLE---
AtPDR21(3)    ---GRTVVCTIHQPSIDIFEAFDELLLMKRGGQVIYGGKLGTHSQVLVDYFQGINGVPPISSGYNPATWMLE---
AtPDR22(4)    ---GRTIVCTIHQPSIDIFESFDELLFMKRGGELIYAGPLGQKSCELIKYFESIEGVQKIKPGHNPAAWMLD---
AtPDR23(5)    ---GRTIVCTIHQPSIHIFEAFDELVLLKRGGRMIYSGPLGQHSSCVIEYFQNIPGVAKIRDKYNPATWMLE---
AtPDR24(6)    ---GRTVVCTIHQPSIDIFESFDELLLMKRGGQVIYAGTLGHHSQKLVEYFEAIEGVPKIKDGYNPATWMLD---
AtPDR31(1)    ---GRTVVCTIHQPSIDIFEAFDELLLLKRGGQVIYAGPLGQNSHKIIEYFQAIHGVPKIKEKYNPATWMLE---
AtPDR32(9)    ---GRTIVCTIHQPSIDIFEAFDELVLLKRGGRMIYTGPLGQHSRHIIEYFESVPEIPKIKDNHNPATWMLD---
AtPDR33(10)   ---GRTVVCTIHQPSIDIFESFDELFLLTRGGEEIYVGPIGHHSSQLIEYFEGIRGVGKIKEGYNPATWALE---
AtPDR41       ---GRTVVCTIHQPSIDIFETFDELILMKDGGQLVYYGPLGKHSSKVIKYFESIPGVPKVQKNCNPATWMLD---
AtPDR42(2.2)  ---GRTVVCTIHQPSIDIFETFDELILMKNGGQLVYYGPPGQNSSKVIEYFESFSGLPKIQKNCNPATWILD---
AtPDR43       ---GRTVVCTIHQPSIDIFEAFDELILMKNGGKIIYYGPLGQHSSKVIEYFMRIHGVPKLKENSNPATWILD---
AtPDR44       ---GRTVVCTIHQPSIDIFEAFDELILMKNGGKIIYYGPLGQHSSKVIEYFMSIPGVPKLKENSNPATWILD---
ScPDR5        ---GQAILCTIHQPSAILMQEFDRLLFMQRGGKTVYFGDLGEGCKTMIDYFE-SHGAHKCPADANPAEWMLE---
```

B)

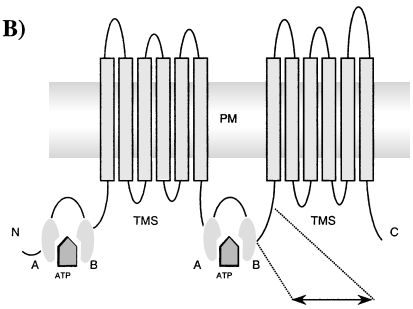

Figure 1. (A) Alignment of protein sequence fragments predicted from *Arabidopsis thaliana* genomic sequences and yeast Pdr5p. The numbers in brackets indicate the *PDR* gene numbering according to Sanchez-Fernandez et al., 2001. See note added in proof for details. (B) Model of the structure of *PDR5*-like ABC transporter proteins. Indicated are the ATP-binding folds (A, Walker A; B, Walker B) and the transmembrane spanning domains. The location of the sequence shown in (A) is indicated by the arrow.

not come up with such a simple solution? In the case of *PDR5*, over-expression leads to increased sensitivity against yet another toxic *Fusarium* metabolite. Thus, much basic research remains to be done in this area to avoid unwanted side effects in transgenic crop plants.

Detoxification

Mutants of yeast that are extremely sensitive to toxins are a valuable tool to study detoxification reactions. For instance, over-expression of the yeast *AYT1* gene, encoding a putative trichothecene 3-O-acetyltransferase, suppressed DON sensitivity of Δ*pdr5* mutants (unpublished data). The yeast *AYT1* gene is highly related to the *Tri101* gene found in *Fusarium* (Kimura et al., 1998). Detoxification of trichothecenes *in planta* could be an attractive approach. Yet, the identified acetyl-transferase, which converts DON into 3-AcDON, does not seem to be a prime candidate for a biotechnological application. The toxicity of 3-AcDON towards mammals is only slightly lower than that of DON. Furthermore, most *Fusarium* strains seem to initially produce acetylated compounds. Plant carboxylesterases, in addition to fungal enzymes, may play a significant part in the conversion of the acetylated precursors to DON. Consequently, the effectiveness of over-expression of an acetyl-transferase in plants could be severely limited by futile cycling. We are therefore studying additional candidate detoxification enzymes from yeast and other sources, which hopefully will lead to a more pronounced and irreversible detoxification.

Target modification

Trichothecene-sensitive Δ*pdr5* mutants of yeast were also employed to identify alterations of the ribosomal target of trichothecenes, which confer toxin resistance. To this end, a saturation mutagenesis of the gene encoding ribosomal protein L3 was performed. A library of mutagenized plasmids was transformed

into a toxin sensitive host strain which contains solely a conditional copy of the gene encoding ribosomal protein L3 (*pGAL1-RPL3,* which is not expressed in glucose medium). Transformants resistant to deoxynivalenol were isolated and the mutations in *RPL3* characterized by DNA sequencing (Raditschnig et al., unpublished data).

Like other ribosomal protein genes, *RPL3* is extremely well conserved in evolution, and it is an attractive biotechnological approach to introduce the corresponding amino acid changes (which confer semi-dominant toxin resistance in yeast) into a plant *RPL3* homologue. On the other hand, the knowledge of the spectrum of mutations conferring resistance in yeast could also be useful for screening *Fusarium* resistant germplasm and for the development of molecular markers for plant breeding. Ribosomal resistance has, for instance, been suggested for the highly *Fusarium* resistant wheat cultivar Frontana (Miller and Ewen, 1997).

In summary, we have tried to use the wealth of 'genomics' information already available for yeast and *Arabidopsis* to gain insights into an important aspect of the problem of mycotoxins in agriculture. The identified mechanisms of trichothecene resistance – especially drug efflux pumps and mutations of the ribosomal target – are most likely relevant for crop plants. Knowledge of candidate resistance mechanisms and genes may be advantageous especially in the case of *Fusarium* diseases, where many important crop plants (wheat, durum wheat, barley, rye, corn) are affected, and when breeding for increased *Fusarium* resistance is pursued in parallel.

Acknowledgements

This work was supported by the Austrian Academy of Sciences (APART Fellowship to GA), the Austrian Science Foundation (FWF P-11551) and the City of Vienna (Hochschuljubiläumsstiftung H-239/99).

Note added in proof:

In their work on the *Arabidopsis thaliana* ABC protein superfamily Sanchez-Fernandez et al. (2001) claim that 13 *PDR5*-like ABC transporters exist, numbering the genes *PDR1* to *PDR13* (see also: http://www.arabidopsisabc.net/). According to our analysis *PDR11* and *PDR13* are the same genomic sequence from different BACs, spliced differently. We also arrive at a slightly different splicing pattern for *PDR3*, *PDR6*, *PDR7*, and *PDR10*. We have identified a cluster of 4 tandemly repeated genes on chromosome IV, only the second gene has been recognized by Sanchez-Fernandez et al. but spliced incorrectly '*PDR2.2'*. We have cloned and sequenced the full length cDNA (Mitterbauer, 2000; and unpublished data).

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