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The *Fusarium* mycotoxin zearalenone inhibits Hsp90 ATPase activity and is inactivated *in planta* by glucosylation and sulfatation

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Abstract

The *Fusarium* mycotoxin zearalenone (ZON) is well known for its estrogenic activity in animals. The role of ZON in plant-pathogen interaction was previously unclear. We have identified a prominent target for zearalenone: heat shock protein 90 (Hsp90). Zearalenone and, more strongly, β-zearalenol (bZOL) inhibit Hsp90 ATPase activity *in vitro*. ZON was found to be rapidly converted into ZON-4-O-glucoside and ZON-4-sulfate in *Arabidopsis* and other plants. Both conjugates do not have inhibitor activity in the Hsp90 *in vitro* assay. Hsp90 plays a prominent role in plant pathogen interaction and is necessary for disease resistance. Yet previous results with *Fusarium* gene disruption mutants deficient in ZON biosynthesis indicate that ZON is not a relevant virulence factor. Possible reasons for these conflicting results are discussed.

Keywords

Fusarium graminearum, mycotoxin, zearalenone

Introduction

The resorcylic acid lactone zearalenone is produced by many *Fusarium* species (e.g. members of the *F. graminearum* species complex, *F. pseudograminearum*, *F. culmorum*, *F. equiseti*, *F. crookwellense*/*cerealis*, *F. semitectum*). ZON received most scientific attention due to its ability to bind with high affinity to the estrogen receptor protein in animals and humans and to act as powerful xenoestrogen (KUIPER-GOODMAN et al. 1987). Due to this hormone activity regulatory limits in food are low (e.g. 20 µg kg^{-1} in infant food (EC 1881/2006 and 1126/2007)). In comparison, high concentrations (easily exceeding 20 mg kg^{-1}) can occur in infected plant material, especially in corn. Plants do no have an estrogen receptor, and it was previously unclear whether ZON has biological functions in plants. We have used the model system *Arabidopsis thaliana* to elucidate the biological function of ZON.

Material and methods

A. thaliana seedlings (ecotype Columbia) grown in liquid MS medium were treated with 50 µM ZON and gene expression was monitored using the Affymetrix ATH1 Gene chip (for details see WERNER 2005). The yeast heat shock protein 90 homolog (product of the *HSP82* gene) was expressed in *Escherichia coli* and affinity purified using a 6×HIS tag. Further purification was achieved by gel filtration (Sephadex G25) and ion exchange (Resource Q1). ATPase activity of the purified protein was measured by a nonradioactive phosphate release assay based on malachite green formation (ROWLANDS et al. 2004). ZON and ZOL conjugates were purified by preparative HPLC from *Fusarium* cultures (ZON-4-sulfate) or from ZON treated yeast cultures expressing an *Arabidopsis* glucosyltransferase (ZON-4-O-glucosid and ZOL-glucosides) as previously described (POPPENBERGER et al. 2006, BERTHILLER et al. 2009a). Radicicol was purified from rice cultures of *Nectria radicicola* MA1224.

Results and discussion

Treatment of *Arabidopsis* with ZON led to an at least 2-fold change in gene expression (WERNER 2005, WERNER et al., unpublished) of 495 genes after 2 hours. The strongly upregulated genes included multiple candidate genes with a role in ZON detoxification, such as drug efflux pumps (e.g. *AtPDR12* induced 14×), 9 genes encoding glutathione-S-transferases (GSTs) and 3 genes coding for UDP-glucosyltransferases (UGTs). Strongly induced were also several genes encoding small heat shock proteins. In contrast, 46 genes with a predicted role in cell wall related functions (remodeling/reinforcement) were strongly downregulated, especially peroxidases, consistent with a negative role of ZON in cell wall mediated plant defense. ZON furthermore was found to be able to suppress the short-root phenotype triggered by a mutation in a cellulose biosynthetic gene leading to constitutive overproduction of ethylene and jasmonic acid (WERNER et al. unpublished). Consistent with the transient transcriptome response, rapid metabolization of ZON into glucose and sulfate conjugates (and further unknown metabolites) was observed (BERTHILLER et al. 2006).

The finding that ZON induced 7 genes (6.4× to 47×), which code for small heat shock proteins was intriguing. Examples are shown in *Figure 1*. Several of the yeast genes showing the highest similarity to the ZON induced small

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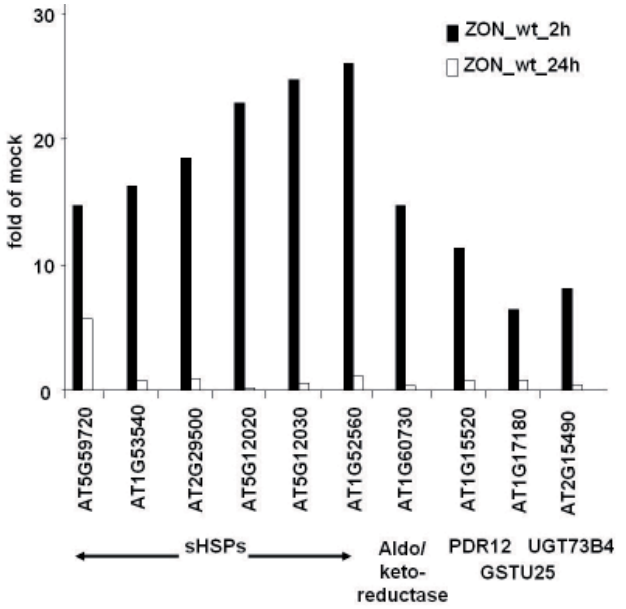

Figure 1: **Expression analysis of ten highly ZON induced genes in *Arabidopsis* Col-0 (wt). These include several small heat shock proteins (sHSPs), an aldo/keto reductase, an ABC transporter family gene (*PDR12*), a glutathione-S-transferase (*GSTU25*) and a glycosyltransferase (*UGT73B4*). Data are extracted from microarray experiments and presented relative to the respective mock controls.**

HSPs from *Arabidopsis* were found to be Hsp90 interactors in systematic screens. In *Arabidopsis* specifically one of seven HSP90 genes, the *AtHSP90-1* gene was upregulated 3.7× by treatment with ZON. Furthermore, we noticed the structural similarity of zearalenone with radicicol, a known inhibitor of Hsp90 ATPase (for review see SGOBBA and RASTELLI 2009), which is produced by *Nectria radicicola* (MIRRINGTON et al. 1965) and other fungi.

To test the hypothesis that ZON is an inhibitor of Hsp90 ATPase, we expressed the bakers yeast Hsp90 gene (*ScHSP82*) in *E. coli* and purified the 6×HIS tagged protein. The intrinsic ATPase of the purified protein was strongly inhibited by radicicol (positive control, IC_{50}=1,5 µM). Also ZON and, even stronger, bZOL inhibited in a concentration dependent manner, with IC_{50} values of 8.6 and 49 µM for bZOL and ZON, respectively. We also tested the available ZON-conjugates for inhibitor activity. ZON-4-sulfate is a prominent side product in many *Fusarium* strains (PLASENCIA and MIROCHA 1991) and also a ZON metabolite in *Arabidopsis* (BERTHILLER et al. 2006). ZON-4-O-glucosides and ZOL-4-O-glucosides were produced using genetically engineered *Saccharomyces cerevisiae* expressing the glucosyltransferase *AtUGT73C6* (POPPENBERGER et al. 2006). Also at the highest concentration tested (150 µM) no inhibition of Hsp90 ATPase activity was observed, demonstrating that the formation of the conjugates is a detoxification reaction of plants. This was also supported by the observation that ZON in high concentrations is toxic for yeast strains with deletions of several ABC transporters, and that expression of the glucosyltransferase protects against toxicity *in vivo*.

The finding that ZON and its biosynthetic precursor bZOL (KIM et al. 2005) are Hsp90 inhibitors suggests that ZON could play a role as suppressor of plant defense. Hsp90 is necessary for the stability of many client proteins such as signal transduction components, and has been shown to be essential for disease resistance. The highly pathogen inducible and also ZON inducible cytosolic HSP90.1 gene is specifically required. Several disease resistance gene products were shown to directly interact with Hsp90, e.g. the tomato *I2* resistance gene product (against *F. oxysporum*). Interference with *HSP90* expression (by virus induced gene silencing) or with Hsp90 activity using inhibitors (geldanamycin, radicicol) showed that Hsp90 is required for function of several gene-for-gene resistance interactions (e.g. barley::powdery mildew (*Mla*), tobacco::TMV (*N*), tomato::nematode (*Mi*), wheat::leaf rust (*Lr21*), *Arabidopsis*::*Pseudomonas syringae* resistance (*RPS2*) (for review see SHIRASU 2009). One would therefore expect that zearalenone production is a virulence factor of *Fusarium*. ZON biosynthesis in *F. graminearum* requires the polyketide synthetase genes *PKS4* and *PKS13*. Three different groups have previously reported that loss of ZON production due to gene inactivation does not alter virulence (GAFFOOR et al. 2005, KIM et al. 2005, LYSØE et al. 2006). In the light of our results this needs to be carefully reinvestigated. Microarray data indicate that the ZON biosynthesis cluster is not expressed at the high temperature used during barley head infection, so lack of an effect of gene disruption is not surprising. Many *Fusarium* strains produce ZON in meaningful amounts only under cool conditions or after a cold shock (JIMENEZ et al. 1996). We therefore hypothesize that ZON may have a detectable virulence function under environmental conditions favorable for ZON production in barley (cool weather during grain filling) or in maize (cool nights late in the season). Experiments are ongoing to test this hypothesis. Furthermore, living plants seem to have a high capacity to antagonize ZON by formation of the masked mycotoxin ZON-glucoside (BERTHILLER et al. 2009b). At present it is unknown whether genetic differences in *Fusarium* resistance and ZON accumulation in plants are correlated with this detoxification ability.

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