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Masked mycotoxins: does breeding for enhanced *Fusarium* head blight resistance result in more deoxynivalenol-3-glucoside in new wheat varieties?

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Abstract

From economic and environmental points of view, enhancing resistance to *Fusarium* head blight (FHB) in wheat is regarded as the best option to reduce fungal colonisation and the concomitant mycotoxin contamination. This review focuses on the effect of FHB resistance on deoxynivalenol (DON) and the masked metabolite deoxynivalenol-3-glucoside (DON-3-glucoside) in wheat. Based on published information complemented with our own results we draw the following conclusions: (1) All investigated wheat cultivars can convert DON to DON-3-glucoside. Hence, detoxification of DON to DON-3-glucoside is not a new trait introduced by recent resistance breeding against FHB. (2) The amount of DON-3-glucoside relative to DON contamination can be substantial (up to 35%) and is among other things dependent on genetic and environmental factors. (3) Correlation analyses showed a highly significant relationship between the amount of FHB symptoms and DON contamination: breeding for FHB resistance reduces DON contamination. (4) DON contamination data are highly correlated with DON-3-glucoside concentration data: in other words, reduction of DON content through resistance breeding results in a concomitant reduction in DON-3-glucoside content. (5) The DON-3-glucoside/DON ratio increases with decreasing DON contamination: the most resistant lines with the lowest DON contamination show the highest relative level of DON-3-glucoside to DON. In summary, introgressing FHB resistance reduces both DON and DON-3-glucoside levels in the grain, but the reduction is lower for the masked toxin. DON-3-glucoside can represent a possible hazard to human and animal health, especially in wheat samples contaminated with DON close to permitted limits.

Keywords: cereals, trichothecene, *Gibberella*, ratio masked toxin

1. Introduction

The genus *Fusarium* belongs to the order of the *Hypocreales*, the class *Ascomycetes* and phylum *Ascomycota*. The perfect stages of *Fusarium* spp. are mainly classified in the genus *Gibberella* (Liddell, 2003). *Fusarium* fungi occur worldwide and can cause many plant diseases, such as *Fusarium* head blight (FHB) on the ears of small grain cereals including wheat. At least 17 *Fusarium* species have been associated with FHB in cereals (Parry *et al.*, 1995). The most abundant pathogens on wheat are *Fusarium graminearum* (Schwabe) [teleomorph: *Gibberella zeae* (Schw.) Petch]

followed by *Fusarium culmorum* (Wm. G. Smith) Sacc. Other species, including *Fusarium avenaceum* (Corda ex Fr.) Sacc. [teleomorph *Gibberella avenacea* (Cook)], *Fusarium poae* (Peck) Wolenw. and *Fusarium langsethiae* (Torp & Nirenberg) play a minor role, but depending on conditions can be locally the dominant species (Bottalico, 1998; Bottalico and Perrone, 2002; Parry *et al.*, 1995; Stepien and Chelkowski, 2010). On wheat *F. graminearum* and *F. culmorum* are the most aggressive species in terms of symptom severity (Wong *et al.*, 1992).

Fusarium head blight symptoms

The fungus usually first colonises the anthers and subsequently infects the caryopsis, floral bracts and rachis (Bai and Shaner, 1994; Sutton, 1982). The first symptoms are brown, water-soaked spots on the glumes. The initially infected spikelet soon dies and the green colour changes into the typical straw-like colour of ripe ears. From the inoculated spikelet symptoms can spread through the rachis to neighbouring spikelets. A pink, orange to red discoloration may be observed on the glumes or at the base of the spikelet. Premature death (wilting) of entire upper parts of the ear above the initial point of infection is another common symptom. Severe FHB can result in disease symptoms on grains. *Fusarium* colonised kernels are characteristically smaller than normal, shrivelled in appearance and white to pale pink in colour (Abramson *et al.,* 1987). If ears are invaded at a very early stage, kernels may fail to develop entirely.

Mycotoxins

Colonisation of the ear can result in yield and quality loss of the grain, but of main concern is the mycotoxin contamination of the grains. Many *Fusarium* species have the potential to produce mycotoxins contaminating the grain. The most prevalent mycotoxins produced by *Fusarium* spp. are trichothecenes, (e.g. deoxynivalenol (DON), nivalenol, T-2 toxin, HT-2 toxin, diacetoxyscirpenol) and zearalenone. Other important mycotoxins are beauvericin and enniatins (produced by pathogens, such as *F. poae* and *F. avenaceum*) and moniliformin (synthesised by e.g. *F. avenaceum*) (Bottalico, 1998; Bottalico and Perrone, 2002). Trichothecenes are inhibitors of protein synthesis and they affect the immune system (Da Rocha *et al.*, 2014; Marin *et al.*, 2013). Worldwide, *F. graminearum* and *F. culmorum* are the most important DON producers and this mycotoxin is the main toxin detected in wheat grains.

Masked mycotoxins

Gareis *et al.* (1990) were the first to mention 'masked mycotoxins'. Masked mycotoxins were defined as plant generated conjugated mycotoxins which are not routinely analysed, but nevertheless present a possible health hazard because the toxin moiety could be released during digestion (for a more recent treatise on the definition of masked toxins the reader is referred to Rychlik *et al.*, 2014). Metabolisation of mycotoxins *in planta* can lead to the generation of masked toxins, such as with the conversion of DON to DON-3-glucoside (Poppenberger *et al.*, 2003) or to DON-sulphates (Warth *et al.*, 2015). In the meantime, various conjugated forms of DON, zearalenone and fumonisins, as well as T-2 and HT-2 toxins have been identified (Berthiller *et al.*, 2013; De Saeger and Van Egmond, 2012). These have not been the focus of routine mycotoxin analyses so far, but

recent information indicates that they pose a possible health hazard. The metabolism of DON-3-glucoside was studied in rats (Nagl *et al.* 2012). Urine and faeces were analysed by a validated LC-MS/MS biomarker method. DON-3-glucoside was readily hydrolysed to DON during digestion, but most of the DON-3-glucoside was metabolised by gut microbiota and recovered in faeces. It was therefore concluded that DON-3-glucoside was of considerably lower toxicological significance than DON, at least in rats. The metabolism of DON-3-glucoside was also investigated in pigs (Nagl *et al.* 2014). Orally administered DON-3-glucoside was nearly completely hydrolysed in the intestinal tract of pigs, but only partially absorbed. Compared to DON, the oral bioavailability of DON-3-glucoside and its metabolites seems to be reduced by a factor of up to 2.

The European Commission has set maximum levels for DON in foodstuffs (EFSA, 2007), but levels for masked DON have not been taken into account. More recently the EFSA considered it appropriate to assess human exposure to modified forms of the various toxins in addition to the parent compounds, because many modified forms are hydrolysed into the parent compounds or released from the matrix during digestion (EFSA, 2014). Hence, DON-3-glucoside, if undetected in food and feed, could pose a potential risk to food and feed safety particularly in batches contaminated with DON near the maximum allowed limit.

2. Control of *Fusarium* head blight in wheat

Control of FHB is challenging. It is generally agreed that an integrated approach including crop rotation, proper land preparation, the use of fungicides and antagonists and last but not least growing resistant wheat cultivars would be the best approach to control FHB. From economic and environmental points of view, introgressing and combining resistance factors for FHB in productive wheat cultivars is regarded as the best way to reduce fungal colonisation and the risk of toxin contamination.

Resistance breeding to *Fusarium* head blight

FHB resistance breeding aspects in general and more specifically in relation to toxin contamination in wheat have recently been reviewed by Buerstmayr and Lemmens (2015). Breeding for improved FHB resistance is an important element in many cereal breeding programs. Essential tools for a successful resistance breeding program are (1) the availability of resistant sources and (2) proper tools to select the resistant lines in the offspring.

1. Extensive genetic variation for FHB resistance exists in hexaploid wheat. Quantitative variation for resistance to FHB was reported in numerous studies when genebank collections, introduced lines, breeding lines or cultivars were evaluated. Snijders (1990) grouped FHB resistance sources into three gene pools: winter wheat germplasm

from Eastern Europe, spring wheat from China/Japan and spring wheat from Brazil. Other studies have shown that genotypes with a moderate to good level of FHB resistance are available also in other gene pools, like germplasm from different parts of Europe (Buerstmayr *et al.*, 1996; Mesterhazy, 1983, 1995; Saur, 1991) and North America (McKendry, 2008; Rudd *et al.*, 2001; Sneller *et al.*, 2010; Zhang *et al.*, 2008).

Durum wheat cultivars are generally considered to be susceptible to FHB. Indeed, no high level in resistance to FHB has been found within *T. durum* lines. This fact may be due to several factors: a narrow genetic base compared to hexaploid wheat might be linked to the fact that durum wheat is tretraploid and that limited breeding efforts have been undertaken on this crop. Attempts to transfer resistance from hexaploid into tetraploid wheat have been met with limited success (Cirlini *et al.*, 2014). DNA-based markers are a relatively recent tool that can be applied to support breeding, especially for traits that are difficult or costly to select using conventional methods, such as FHB. Numerous studies have shown that inheritance of resistance in wheat is quantitative, governed by multiple genes and modulated by the environment. More than 100 quantitative trait loci (QTL) for FHB resistance have been reported. In total 22 different QTL regions located on 16 different wheat chromosomes have been described (see Buerstmayr and Lemmens, 2015; Buerstmayr *et al.*, 2009 and references therein). Examples of well validated QTL with positive effects on FHB resistance are those on chromosome-arms 3BS (*Fhb1*), 5AS (*Qfhs.ifa-5A*) and 6BS (*Fhb2*). No systematic negative effects of the spring wheat-derived QTL on grain yield, thousand grain weight, hectolitre weight and protein content was found (Salameh *et al.*, 2011). However, small significant negative effects on grain yield were detected in some, but not all $BC_3F_{2:5}$ populations (Von der Ohe *et al.*, 2010). Selection of lines with improved resistance level and similar high yield level like the recurrent parent was feasible. All other differences in agronomic and quality traits were in all cases small although often significant (Von der Ohe *et al.*, 2010).

2. Most FHB resistance breeding programs use artificial inoculation to assure *Fusarium* infections (see Dill-Macky, 2003). The ultimate goal of FHB resistance breeding is of course the reduction of mycotoxin contamination in the grain. But mycotoxin content in the grain is usually not chosen as the main selection parameter because the analyses are very costly. Breeders are handling yearly thousands of wheat lines in their breeding programs. The percentage of diseased ears/spikelets and the percentage of visually damaged kernels are disease parameters which can be visually assessed rapidly and above all cheaply. Toxin analyses are usually restricted to a reduced set of advanced lines to check and to confirm the effect of progress in

FHB resistance on the toxin content. DON is the most prevalent mycotoxin in cereals and is therefore also regarded as the so called 'lead toxin'. In other words if 'toxin' content is checked, then this usually means DON content only because of cost reasons. Therefore information on e.g. zearalenone or other trichothecene contamination (e.g. 15-acetyldeoxynivalenopl) is scarce, not to mention other types of toxins produced by *Fusarium* spp. playing a smaller role in FHB in wheat (e.g. *Fusarium sporotrichioides* with T-2 and HT-2 toxin). Buerstmayr and Lemmens (2015) reviewed the effect of FHB resistance breeding on mycotoxin content in the grain. Their conclusions were: (1) in general FHB symptoms induced by either *F. graminearum* or *F. culmorum* are related to DON content. Increasing FHB resistance reduces DON content; (2) at the same time the contamination with other mycotoxins produced by the same *Fusarium* species are reduced; (3) FHB resistance is not *Fusarium* species-specific: in other words, FHB resistance factors are effective against all *Fusarium* spp. tested (Mesterhazy *et al.*, 1999; Snijders and Van Eeuwijk, 1991; Van Eeuwijk *et al.*, 1995). Improving FHB resistance simultaneously increases resistance to all investigated *Fusarium* species causing FHB and results in a coincident reduction of the respective mycotoxin contamination. For example, increasing FHB resistance against *F. graminearum* in a specific wheat cultivar indirectly results in an improved resistance towards *F. avenaceum* in the same cultivar. In more resistant wheat lines not only the DON content is reduced after infection with *F. graminearum,* but also e.g. the moniliformin contamination after inoculation of the same wheat line with *F. avenaceum.*

3. Deoxynivalenol-3-glucoside in wheat

Occurrence of deoxynivalenol-3-glucoside in wheat and wheat products

Several reports have been published on surveys of DON and DON-3-glucoside content in wheat and wheat products. DON-3-glucoside is the masked toxin most often detected in contaminated wheat and wheat products. In Austria, Berthiller *et al.* (2009) found DON-3-glucoside in 23 naturally contaminated wheat samples in concentrations ranging from 76 to 1,070 µg/kg. In 2012, De Boevre *et al.* reported up to 425 µg/kg DON-3-glucoside (mean: 34 µg/kg) in bran enriched bread in Belgium. In the same country DON-3-glucoside was detected in 54 out of 93 investigated wheat samples (mean DON-3-glucoside content 250 µg/kg) and in 15 out of 25 bread samples (mean: 490 µg/kg) (Vanheule *et al.*, 2014). In a Chinese contribution (Li *et al.*, 2012) DON-3-glucoside was detected at concentrations of 4-238 µg/kg (mean 52 µg/kg) in wheat kernels and 3-39 µg/kg (mean 11 µg/kg) in wheat flour in 2008; and 3-235 µg/kg (mean 22 µg/kg), 3-53 µg/kg

(mean 14 μg/kg) and 3-87 μg/kg (mean 19 μg/kg) in wheat products in 2009, 2010 and 2011, respectively. In 68% of 22 flour samples from the Czech Republic DON-3-glucoside was found with a maximum of 72 μg/kg DON-3-glucoside (mean: 15 μg/kg) (Malachova *et al.*, 2011). In Italy 128 out of 150 durum wheat samples (85%) contained DON-3-glucoside at concentrations varying between 46 and 842 μg/kg (Dall'Asta *et al.*, 2013). In a survey in Serbia 54 winter wheat samples were investigated and the DON-3-glucoside content ranged from 17 to 83 μg/kg and for DON from 41 to 309 μg/kg (Skrbic *et al.*, 2011).

Deoxynivalenol-3-glucoside/deoxynivalenol ratio in wheat or wheat products

The ratio of DON-3-glucoside to DON has been shown to vary between years and among wheat genotypes (Berthiller *et al.*, 2009). The ratio of DON-3-glucoside to DON has been found to be as high as 29% in wheat samples (Berthiller *et al.*, 2009), and a ratio of 70% was detected in a sample of bran enriched bread (De Boevre *et al.,* 2012). Berthiller *et al.* (2009) detected ratios (in mol%) of 7 to 29% in a set of 22 wheat grain samples. The DON-3-glucoside to DON ratio varied from 5 to 20% in a set of 21 winter wheat grain samples and from 18 to 23% in spring wheat (4 varieties) following artificial inoculation with *F. graminearum* and *F. culmorum* in Denmark, and from 8 to 19% in naturally infected winter wheat (6 samples) (Rasmussen *et al.*, 2012). Similarly, in durum wheat grain DON-3-glucoside/DON ratios of up to 30% were determined in many samples (Dall'Asta *et al.*, 2013). The average relative ratio of DON-3-glucoside to DON in samples of wheat grown in China was 33% in wheat kernels and 10% in wheat flour in 2008 and 22%, 9% and 14% in wheat products in 2009, 2010 and 2011, respectively (Li *et al.*, 2012). In general the DON-3-glucoside/DON ratios reported to date in wheat typically range from 5% to about 35% with occasional exceptions showing higher ratios (up to 2,119%; Li *et al.*, 2012).

Correlation studies between data for DON and DON-3-glucoside content in the wheat grain are rare. In a Chinese study the natural occurrence of DON-3-glucoside was positively correlated with that of DON in all wheat and wheat-based samples over the period 2008-2011 with correlation coefficients of 0.75 in wheat kernels and 0.83 in wheat flour in 2008, and 0.48, 0.64 and 0.74 in wheat-based products in 2009, 2010 and 2011, respectively (Li *et al.*, 2012). They pointed out that the positive correlation between DON and DON-3-glucoside concentrations shows that the ratio of DON-3-glucoside to DON was relatively stable, about 21±4% in the samples over the four-year period (Li *et al.*, 2012). Also Dall'Asta *et al.* (2013) found a positive correlation coefficient (r=0.78) between DON and DON-3-glucoside content in a durum wheat breeding nursery (see below). In general, an increase of DON content is accompanied by an increase in DON-3-glucoside content

or to put it from the breeders' perspective: the lower the DON content, the lower the DON-3-glucoside content.

4. Effect of *Fusarium* head blight resistance in wheat on deoxynivalenol-3-glucoside/ deoxynivalenol ratio

Information on the fate of masked mycotoxins in FHB resistance breeding programs is very rare: of course financial issues concerning mycotoxin analyses play a role, but also the fact that most masked mycotoxins have been discovered only recently and/or only recent toxicological studies have revealed their potential role in food and feed toxicology.

In a first report on the effect of FHB resistance on the DON-3-glucoside/DON ratio twenty-five advanced breeding lines selected from different crosses developed within the durum wheat breeding program of an Italian seed company were investigated (Dall'Asta *et al.*, 2013). The lines were tested under natural infection conditions in 2 replications and 3 environments. A positive correlation between DON and DON-3-glucoside contamination data was reported $r_{spearman}$=0.78** (with ** = significantly different from 1 at *P*<0.01). Durum lines with a high DON content also showed a high DON-3-glucoside level. On the other hand the DON-3-glucoside/DON ratio was negatively related to the DON content ($r_{spearman}$ is -0.37**). Their results indicated that lines with low DON content had a relatively higher DON-3-glucoside/DON ratio. They also calculated the correlation between DON-3-glucoside/DON content with the FHB resistance data ($r_{spearman}$ = -0.27**): a low disease incidence (= high FHB resistance) was associated with a higher DON-3-glucoside/DON ratio. They concluded that DON-3-glucoside formation may be a possible detoxification mechanism exerted by the more FHB tolerant/resistant durum wheat lines as a response to *Fusarium* infection (Dall'Asta *et al.*, 2013).

Ovando-Martínez *et al.* (2013) analysed DON and DON-3-glucoside content in 556 wheat samples. They performed inoculated field trials with hard red spring wheat breeding lines or cultivars during three seasons at two locations in Minnesota, USA. The correlation between DON and DON-3-glucoside was investigated. A second order equation model was fitted over all samples, resulting in a high coefficient of determination (R^2 = 0.872, significantly different from 1 at *P*<0.001). The DON-3-glucoside content rose as the DON content increased in samples with DON content between 0 and 30 mg/kg. However, at higher DON concentrations, a slight decrease in the DON-3-glucoside content was observed. The authors stated: 'the results obtained in this study lead us to think that the samples which presented lower FHB susceptibility (lower DON), will produce high levels of DON-3-glucoside; whilst the samples with higher FHB susceptibility will have lower levels of this 'masked' mycotoxin. This means the DON

and DON-3-glucoside formation exerted by the less FHB susceptible wheat lines is a response towards *Fusarium* infection'. Ovando-Martínez *et al.* (2013) demonstrated that the line and location have a greater effect on variation of DON and DON-3-glucoside than do their interaction among years. The most important factor affecting DON and DON-3-glucoside was the growing location. They concluded that the year, environmental conditions and location have an effect on the DON-3-glucoside/DON ratio in response to *Fusarium* infection.

Audenaert *et al.* (2013) studied FHB resistance, DON and DON-3-glucoside content in 11 commercial cultivars grown in Belgium. After artificial inoculation of single ears with *F. graminearum*, they found a clear association between DON content, DON-3-glucoside content and the masked fraction. High DON contamination was associated with a high DON-3-glucoside content but with the lowest DON-3-glucoside/DON ratio. *Vice versa*, the most resistant variety 'Sahara' showed the lowest DON contamination, a low disease index and low DON-3-glucoside content, but the highest DON-3-glucoside/total DON ratio. Disease index was inversely related to the DON-3-glucoside/total DON content (R^2=0.8432).

Schweiger *et al.* (2013) generated and investigated near isogenic lines (NILs) descending from a cross of the susceptible spring wheat cv. 'Remus' (recipient) and the FHB resistant line 'CM-82036' (donor) carrying all four possible combinations of the two QTL *Fhb1* and *Qfhs.ifa-5A*. 10 μl of a *F. graminearum* suspension, with a concentration of 10^4 conidia/ml, was pipetted between the palea and lemma of the two basal florets of the central spikelet of the ear. Spread of the disease was assessed at several time points. The more resistant NIL containing both QTL exhibited the lowest DON content (on average 9.45 mg/kg) and the highest DON-3-glucoside/DON ratio (17%). The NILs containing one QTL only showed an intermediate resistance level and contained, on average, 20.25 and 18.52 mg/kg DON and ratios of 12 and 14% DON-3-glucoside/DON, respectively. The susceptible line (no QTL) showed the highest DON content (41.35 mg/kg) and the lowest DON-3-glucoside/DON ratio (8%). Also in this report the wheat lines with the highest FHB resistance level showed the lowest DON contamination, but the highest DON-3-glucoside to DON ratio.

These data from the literature on the effect of FHB resistance on DON and its masked toxin DON-3-glucoside indicate that the DON content is positively related to the DON-3-glucoside content, but the DON-3-glucoside/DON ratio depends on the DON content and increases as the DON content decreases.

Supporting experiments on the effect of FHB resistance on the DON-3-glucoside/DON ratio

In the second part of this review we supplement the scarce information on the DON and DON-3-glucoside issue in the published literature with our own unpublished results of five independent experiments performed during the past years at our institute. The investigated wheat lines represent wide genetic variation and differed in their FHB resistance factors (QTL). Our prime aim is to present novel evidence to validate the published findings, discussed above. We present data of four disease/toxin parameters: (1) FHB symptom severity: in all experiments visual evaluation of symptoms (typically the percentage of visually diseased spikelets 26 days after inoculation) was chosen as a measure for severity of FHB symptoms; (2) DON content in the harvested grains, (3) DON-3-glucoside content in the grains and (4) the ratio of DON-3-glucoside to DON content (DON-3-glucoside/DON), expressed in % of DON-3-glucoside relative to DON based on their molar amounts (mol%).

Statistical analyses were done with SAS Version 9.4 (SAS Institute Inc., Cary, NC, USA). If required, data were transformed to fulfil the conditions for valid ANOVA analyses (PROC GLM). Rank correlation coefficients were calculated with PROC CORR. The reader is referred to the supplementary data for box plots, scatter plots and a summary of the ANOVA analyses of the experiments presented below.

First experiment: the effect of introgressing Fhb1 and Qfhs.ifa-5A in near isogenic lines

In a first experiment NILs differing in the two well-known resistance QTLs, *Fhb1* and *Qfhs.ifa-5A* (Buerstmayr *et al.,* 2002, 2003) have been developed from a cross of the highly resistant 'CM-82036', carrying both QTLs, and the susceptible European spring wheat cultivar 'Remus' using 'Remus' as the recurrent parent (see Schweiger *et al.*, 2013). In the back-cross$_5$F$_2$ generation lines were selected for the presence of the resistant alleles at both QTLs ('NIL1', 'NIL32', 'NIL5'), harbouring only the *Fhb1* ('NIL2', 'NIL6', 'NIL20') or only the *Qfhs.ifa-5A* ('NIL3', 'NIL17', 'NIL22') resistance allele, or carrying the susceptible alleles at both QTLs ('NIL4') (Figure 1).

In 2006 the NILs were phenotyped for FHB resistance in the field using spray inoculations. The ten NILs differing in the possession of *Fhb1* and *Qfhs.ifa-5A* and the parental lines 'CM-82036' and 'Remus' were planted in three replications in 0.45 m^2 plots. The field testing method was the same as in Salameh *et al.* (2011). Briefly, at anthesis the heads were spray-inoculated with 100 ml conidial suspension of the *F. graminearum* single spore isolate IFA86 (5×10^4 conidia/ml) using a motor driven backpack sprayer. Two days later the spray inoculation was repeated. An automated
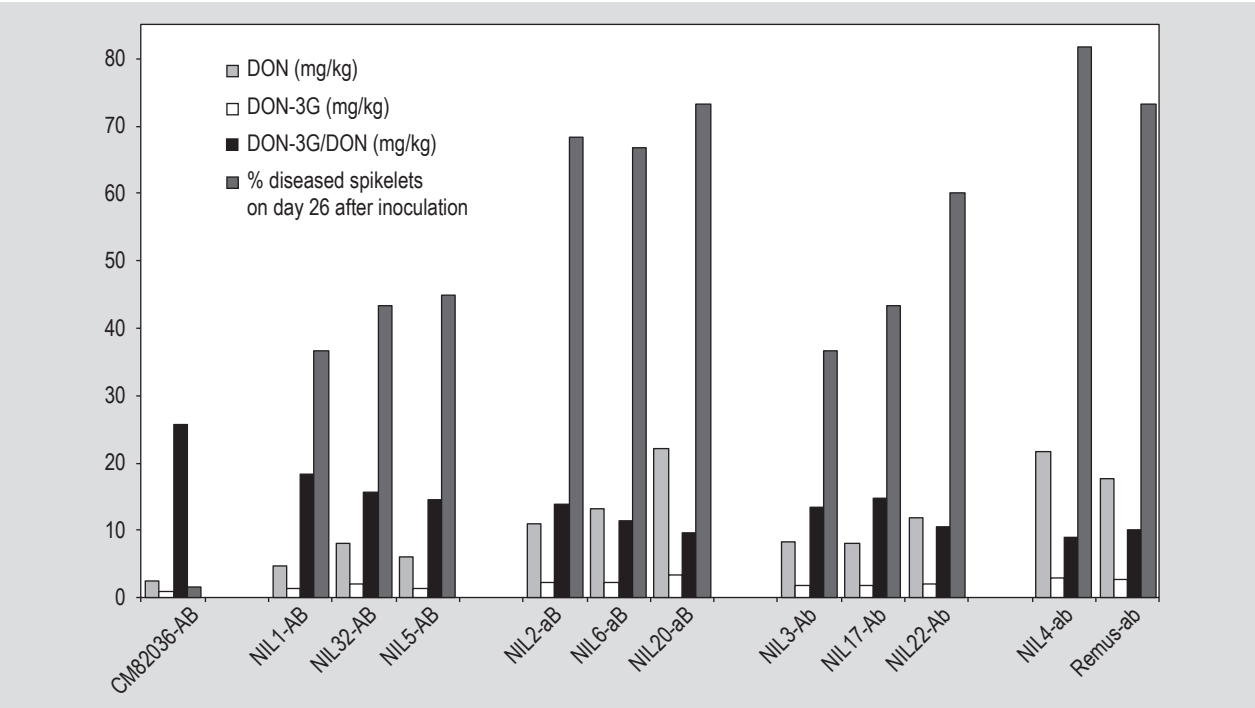
Figure 1. Results of the first experiment. Summary of deoxynivalenol (DON) and DON-3-glucoside (DON-3G) content in the seeds, the ratio DON-3-glucoside/DON and *Fusarium* head blight resistance of the near isolgenic lines (NILs) after spray inoculation with *Fusarium graminearum*. Data are means over 3 replications. The names of the lines include the respective introgressed QTL: AB, both *Fhb1* and *Qfhs.ifa-5A*; Ab, *Qfhs.ifa-5A* only; aB, *Fhb1* only; ab, none.

mist-irrigation system maintained high humidity for 20 h after inoculation. Disease symptom severity (average % infected spikelets) was recorded on day 26 after the first spray inoculation. At the end of the season the seeds were harvested using a plot combine harvester (Wintersteiger, Ried, Austria) set to low wind speed. Harvested samples were cleaned, manually ground, and subjected to toxin quantification according to Sulyok *et al.* (2006).

The results of the first experiment are illustrated in Figure 1 and the statistics summarised in Table 1 (Experiment number 1). Additional data can be found in Supplementary Figure S1 and S2. Variance analyses revealed highly significant differences in FHB infection (% diseased spikelets on day 26 after inoculation), DON and DON-3-glucoside content in the seeds as well as in the ratio DON-3-glucoside/DON between the investigated lines. Provoking FHB artificially with a highly aggressive *Fusarium* isolate led to high toxin concentrations, higher than typically found under natural conditions. A second important remark is, that wheat lines in general (and in this case the variety 'Remus') which were not specifically bred nor selected for FHB resistance, can metabolise DON to DON-3-glucoside. Hence, detoxification via this pathway resulting in masked toxins is not a 'new' trait introduced by specific resistance breeding against FHB. DON content in the tested lines varied from 2.530 to 22.143 mg/kg, DON-3-glucoside contamination from 1.003 to 3.327 mg/kg (Table 1). In

each line the absolute DON-3-glucoside concentration is lower than the DON contamination (Figure 1). Comparing the susceptible variety 'Remus' with the highly resistant variety 'CM82036' reveals a remarkable reduction in FHB symptoms as well as in DON and DON-3-glucoside contamination, accompanied with a significant increase in the DON-3-glucoside/DON ratio (10.1 and 25.6% for 'Remus' and 'CM82036', respectively). NILs carrying one QTL for FHB resistance show an intermediate behaviour. The effect of *Fhb1* only is not very pronounced in this susceptible genetic background but the combination of both QTLs shows an additive effect as compared to the effect of the individual QTL resulting in higher FHB resistance, lower DON content and a tendency to a higher ratio of the masked toxin. It is also clear that 'CM82036' is superior: apart from *Fhb1* and *Qfhs.ifa-5A* this line carries additional (unknown) resistance factors resulting in a very low disease level. Correlation analyses of the complete set of 12 lines showed a highly significant positive relation between DON contamination and the amount of FHB symptoms (r=0.89***) and between DON content and DON-3-glucoside concentration data (r=0.92***). The DON-3-glucoside/DON ratio increases with decreasing DON contamination (r=-0.96***) (Table 1). Progress due to resistance breeding by introducing both QTLs can be estimated by comparing the NILs which are genetically identical apart from the QTL for FHB resistance. 'NIL4' without QTL was contaminated with 21.663 mg DON/kg

Table 1. Summary of the statistical data of the 5 *Fusarium* head blight resistance – deoxynivalenol/DON-3-glucoside breeding experiments.[1,2]

Experiment	1	2	3	4	5
Number of lines	12	21	23	27	45
DON_{min} (mg/kg)	2.530	5.466	4.990	1.640	1.110
DON_{max} (mg/kg)	22.143	24.497	96.910	28.790	9.896
DON_{mean} (mg/kg)	11.292	15.050	27.354	9.571	4.477
DON_{median} (mg/kg)	9.700	15.254	19.785	6.875	4.193
DON-3-glucoside$_{min}$ (mg/kg)	1.003	0.987	1.458	0.510	0.195
DON-3-glucoside$_{max}$ (mg/kg)	3.327	2.284	7.300	3.125	1.052
DON-3-glucoside$_{mean}$ (mg/kg)	2.083	1.596	3.148	1.665	0.594
DON-3-glucoside$_{median}$ (mg/kg)	1.940	1.525	2.778	1.475	0.599
DON-3-glucoside/DON$_{min}$ (mol%)	9.0	5.3	3.4	7.0	5.9
DON-3-glucoside/DON$_{max}$ (mol%)	25.6	14.2	22.1	20.1	15.7
DON-3-glucoside/DON$_{mean}$ (mol%)	13.9	7.3	9.5	13.0	8.9
DON-3-glucoside/DON$_{median}$ (mol%)	13.6	6.5	8.5	12.7	8.3
$r_{spearman}$					
DON versus FHBseverity	0.89***	0.53***	0.93***	0.88***	0.82***
DON versus DON-3-glucoside	0.92***	0.82***	0.89***	0.95***	0.84***
DON versus DON-3-glucoside/DON	-0.96***	-0.73***	-0.80***	-0.82***	-0.45***

[1] The data (means for the individual lines in each experiment) are described by maximum (max), minimum (min), mean and median mycotoxin content in the seeds. At the bottom of the table the results of rank correlation analyses (Spearman) for the experiments are summarised.

[2] DON = dexoynivalenol; DON-3G = deoxynivalenol-3-glucoside; FHB = *Fusarium* head blight.

seeds, 'NIL1' carrying both QTL with 4.660 mg/kg DON. So DON content was reduced by 78%. Similarly, DON-3-glucoside was 3.020 and 1.327 mg/kg for 'NIL4' and 'NIL1', respectively. For DON-3-glucoside we calculate a reduction of 56%. We conclude that both DON and DON-3-glucoside content was reduced after introgressing two QTLs, but the reduction of DON-3-glucoside was less effective resulting in a relative increase of the DON-3-glucoside/DON ratio from 9.0% ('NIL4') to 18.4% ('NIL1').

Second experiment: the effect of pyramiding various Fusarium *head blight resistance QTLs in wheat lines*

A second experiment presented is a trial in cooperation with the John Innes Centre (JIC, UK) conducted in 2013. A set of 21 wheat QTL combination lines including the parents (Figure 2) was tested for FHB resistance in four replications in Austria using a highly aggressive *F. graminearum* isolate (IFA66). The lines contain several combinations of QTL on chromosomes 1B (associated with the 1BL-1RS wheat-rye translocation), 3B (*Fhb1*), 4A (*QFhs.jic-4AS*) and 5A (*Qfhs.ifa-5A*). During anthesis plots were repeatedly sprayed with 100 ml/m^{-2} of a 1×10^4 conidia/ml macroconidial suspension using a backpack sprayer. The crop canopy was kept wet with a computer controlled mist irrigation system to promote infection. For more details on the lines and the inoculation technique the reader is referred to Burt

et al. (2015). The percentage of visually infected spikelets was assessed on day 26 after inoculation. At the end of the growing season the seeds were harvested and analysed for DON and DON-3-glucoside content according to Sulyok *et al.* (2006).

A summary of the data is illustrated in Figure 2; additional data can be found in Supplementary Figure S3 and S4. The lines were ranked according to increasing DON content in the grain. ANOVA analyses confirmed highly significant differences in FHB infection, in the DON-3-glucoside/DON ratio as well as DON and DON-3-glucoside concentration among the lines. The lines combining 3 or 4 QTLs (at the left in Figure 2) showed the additive effect of pyramiding several QTL resulting in the best FHB resistance level (the lowest amount of diseased spikelets), the lowest DON contamination level but the highest DON-3-glucoside/DON ratio. The ratio DON-3-glucoside/DON varied from 5.3 to 14.2% for the '5A-parent' line and the most resistant line ('1B3B5A'), respectively (Table 1). Correlation analyses showed a highly significant positive relationship between DON contamination and the amount of FHB symptoms (r=0.53***): increasing FHB resistance reduces DON contamination. DON content data are highly correlated with DON-3-glucoside concentration data (r=0.82***): in other words, reduction of DON contamination results in a concomitant reduction in DON-3-glucoside content.
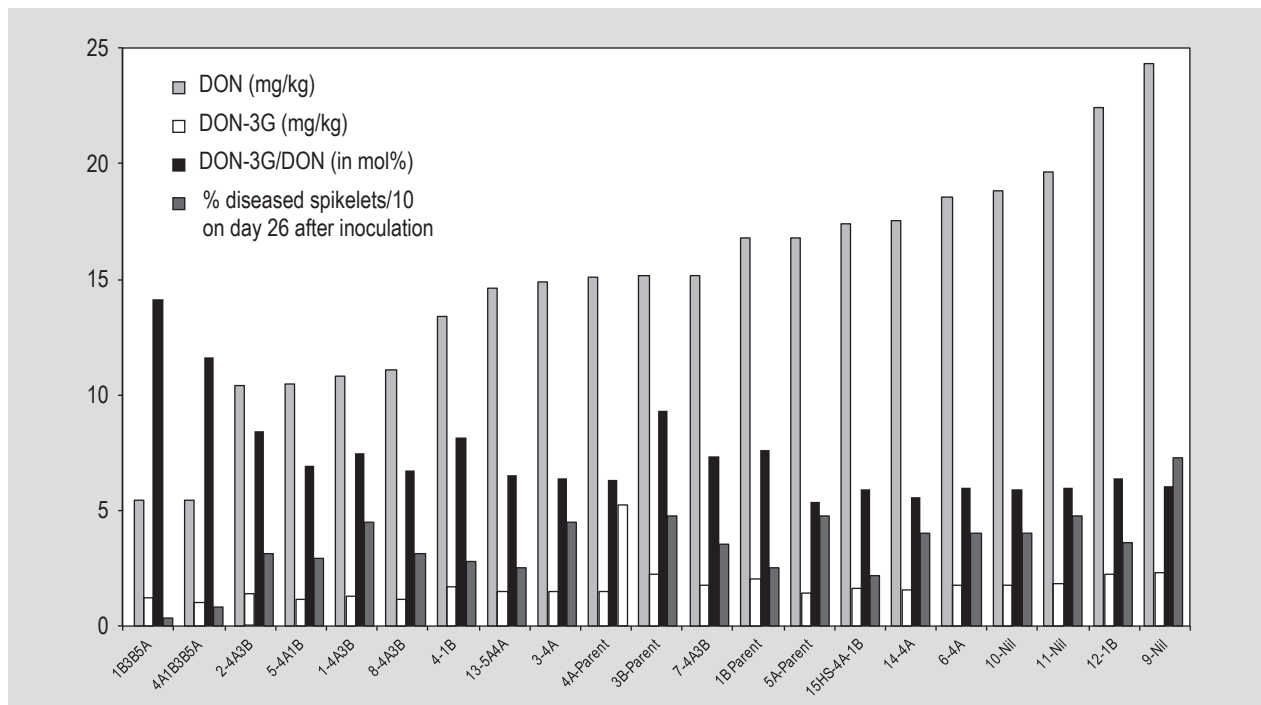
Figure 2. Results of the second experiment. The results for deoxynivalenol (DON) and DON-3-glucoside (DON-3G) content in the seeds, the ratio DON-3-glucoside/DON and *Fusarium* head blight resistance of the near isogenic lines (NILs) after inoculation with *Fusarium graminearum* are summarised. Data are means over 4 replications. The names of the lines include the respective introgressed QTL (1B, 3B, 4A and 5A). The lines were ranked according to increasing DON content.

However, the DON-3-glucoside/DON ratio increases with decreasing DON contamination (r=-0.73***): hence the most resistant lines with the lowest DON contamination show the highest relative level of DON-3-glucoside to DON. In this specific example, the DON concentration was reduced from 24.497 mg/kg for the most susceptible line ('9-Nil') to 5.466 mg/kg for the most resistant line '1B3B5A' (a reduction of 78%). In the same lines DON-3-glucoside content was reduced from 2.282 to 1.199 mg/kg which represents a reduction of 45%. Hence, also in this experiment, introgressing FHB resistance reduced both DON and DON-3-glucoside levels in the grains, but the reduction was less pronounced for the masked toxin.

Third experiment: investigation of various wheat resistance sources for masked deoxynivalenol

The results of a third experiment are illustrated in Figure 3; additional data can be found in Supplementary Figure S5 and S6. In a two year's experiment conducted in the growing seasons 2005 and 2006, a series of winter wheat cultivars or breeding lines were evaluated in two replications per experiment. This experiment includes winter wheat lines or cultivars representing a broad range of FHB response: from highly resistant to highly susceptible. Breeding lines number '20818', '20816', '20817-3', '20812-2' and '20828-3' descend from the cross between the Austrian winter wheat cultivar 'Capo' with the highly FHB resistant Chinese

cultivar 'Sumai-3' and possess the QTLs *Fhb1* and *Qfhs.ifa-5A* (Buerstmayr *et al.,* 2002, 2003). BVAL-numbers are old Austrian landraces, 'A39-9-2-1', 'G16-92' and 'Ar_Fo-338' are experimental lines from Austria, Germany and Switzerland, respectively. '*T. macha*' is a spelt wheat from the Caucasus, and the other entries are cultivars from different European countries. The field testing method was the same as already described for Experiment 1 with a slight modification: samples from two replications within years were pooled in equal amounts prior to extraction for toxin measurement.

ANOVA showed again highly significant differences between the lines for the four parameters investigated. It is obvious that the tested cultivars are generally highly susceptible (e.g. 'Riband', 'Furore') or show a medium resistance at best (e.g. 'Capo', 'Arina') (Figure 3). Note that they can convert DON into DON-3-glucoside. The results nicely illustrate the variability in FHB resistance that is available in the hexaploid wheat pool and exploitable for resistance breeding: 26 days after inoculation almost 100% spikelets were bleached in the susceptible variety 'Furore' but only 2 to 3% in the most resistant experimental lines. As can be seen in Table 1, the data for DON and DON-3-glucoside content in the lines are closely correlated (r=0.89***). The DON-3-glucoside to DON ratio varied from 3.4 to 22.1% for 'Furore' and '20818', respectively. DON content in the seeds increased in the opposite direction from the most resistant line '20818' with 4.99 mg/kg up
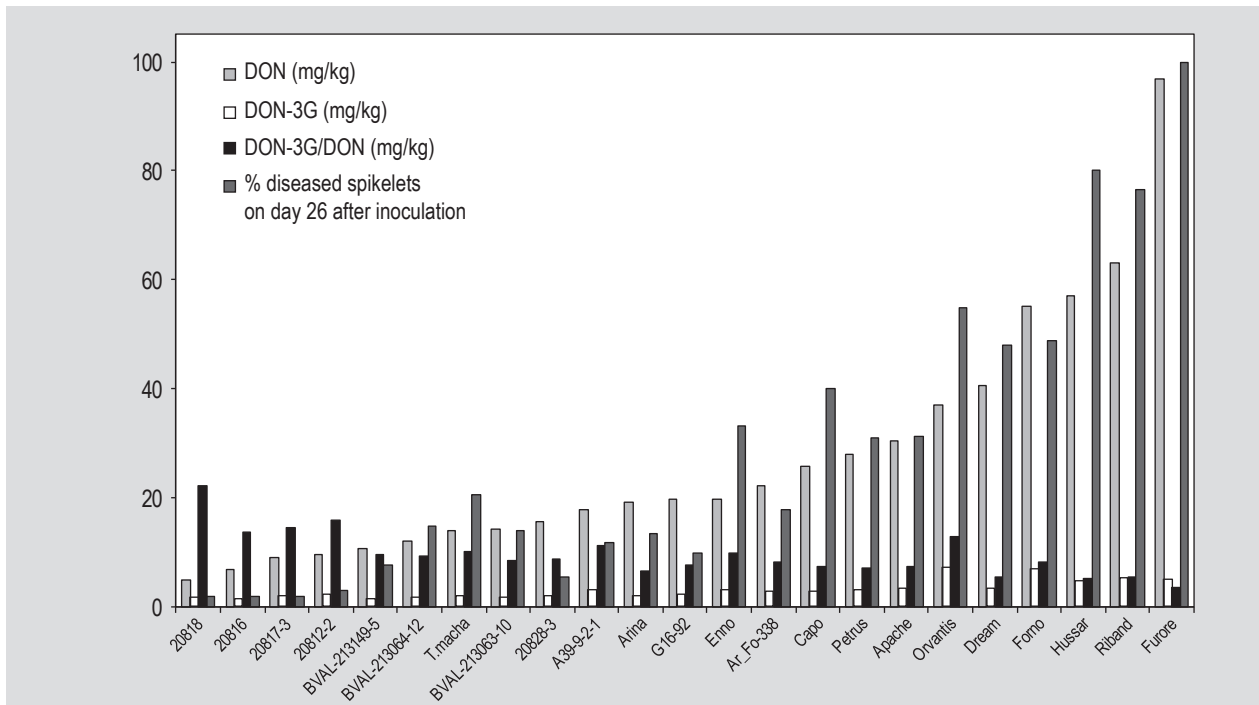
Figure 3. Results of the third experiment. The results for deoxynivalenol (DON) and DON-3-glucoside (DON-3G) content in the seeds, the ratio DON-3-glucoside/DON and FHB resistance of a nursery containing breeding lines, *Fusarium* head blight resistance sources and commercial varieties (see text). The genotypes were inoculated with *Fusarium graminearum*. The data are means over 2 years and 2 replications per year. The lines were ranked according to increasing DON content.

to 96.91 mg/kg for the most susceptible line 'Furore'. The Spearman correlation coefficient calculated between both datasets was -0.80*** (Table 1, Experiment 3). Introgressing both QTLs *Fhb1* and *Qfhs.ifa-5A* from 'Sumai-3' in the Austrian variety 'Capo' increased the DON-3-glucoside/DON ratio from 7.4 to 22.1% for 'Capo' and '20818', respectively. In conclusion: the most resistant lines in this wheat nursery have the lowest amount of FHB symptoms, the lowest absolute DON and DON-3-glucoside level, but again the highest DON-3-glucoside to DON ratio.

Fourth experiment: effect of introgressing Fhb1 and Qfhs.ifa-5A in commercial varieties

Two further independent experiments done at the IFA (Experiment 4 and 5) provide additional support for the findings described above. In season 2006, a set of sister lines with or without FHB-resistance QTL in back-cross derived populations was evaluated for toxin content. '20816-4' is a highly FHB resistant breeding line. 'Arina', 'Petrus', 'Apache', 'Ludwig', 'Augustus' and 'Orvantis' are winter wheat cultivars (Figure 4). All other lines are experimental lines which descend from back-cross-two plants of the highly resistant spring wheat line 'CM-82036' crossed with the winter wheat cultivars 'Apache' (AP), 'Petrus' (PE), 'Augustus' (AU), 'Ludwig' (LU), or 'Orvantis' (OR), respectively. The end-digits code for the allelic state at two previously known FHB resistance QTLs: *Fhb1* and

Qfhs.ifa-5A, respectively descending from the resistant parent 'CM-82036' (Buerstmayr *et al.*, 2002, 2003; Salameh *et al.*, 2011). The field testing method was again the same as described for Experiment 1 and in Salameh *et al.* (2011). Quantification of DON and DON-3-glucoside was done according to Sulyok *et al.* (2006).

The lines were grouped according to the adapted winter wheat parent they originate from and ranked according to the QTL-alleles present (Figure 4). ANOVA over all lines showed highly significant differences for all four investigated disease and toxin parameters. The differences in FHB severity of the parental lines is apparent: 'Orvantis' is very susceptible, 'Apache' moderately resistant. For example, the DON-3-glucoside content in the 'Orvantis' (sensitive) derived lines was higher than in the 'Apache' (more resistant). Correlation analyses over all lines revealed again a high and positive correlation coefficient between the DON data on one side and FHB severity data (r=0.88***) or DON-3-glucoside data (r=0.95***) on the other side (Table 1, Experiment 4; Supplementary Figure S7 and S8). A closer look to Figure 4 illustrates the effect of the two QTL on FHB symptoms, the DON and the DON-3-glucoside content. In general each individual QTL reduces FHB symptoms and DON content and the combination of both QTL has an additive effect on both parameters. The DON-3-glucoside/DON ratio was negatively related with the DON content (r=-0.82***, calculated over all lines).
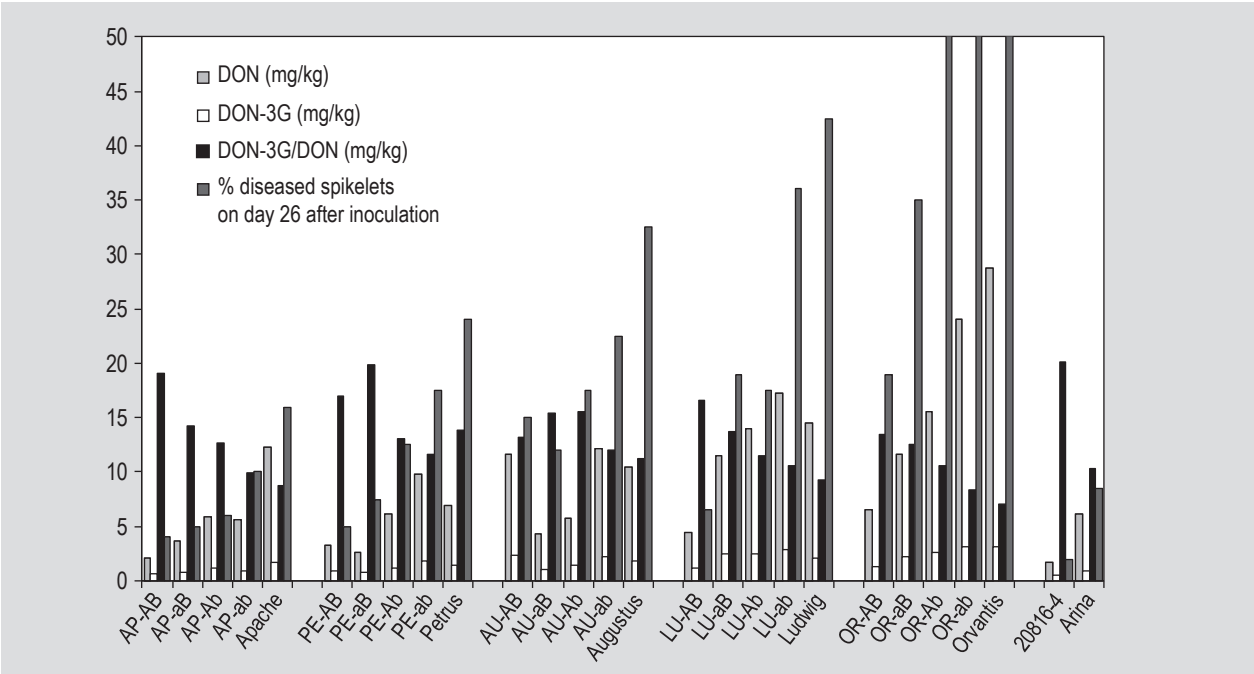
Figure 4. Results of the fourth experiment. Deoxynivalenol (DON) and DON-3-glucoside (DON-3G) content in the seeds, the ratio DON-3-glucoside/DON and *Fusarium* head blight resistance of wheat near isogenic lines after inoculation with *Fusarium graminearum*. Data are means over two seasons and two replications. The last two digits of the line names: AB = line with both quantitative trait loci (QTL) alleles (*Fhb1* and *Qfhs.ifa-5A*) from the highly resistant parent CM-82036, aB = resistant allele at *Fhb1* only, Ab = resistant allele at *Qfhs.ifa-5A* only, ab = both QTL alleles from the winter wheat parent. AP, 'Apache'; AU, 'Augustus'; LU, 'Ludwig'; OR, 'Orvantis'; PE, 'Petrus'.

For 'Apache' for example, DON was reduced from 12.245 to 2.085 mg/kg when both QTL were introgressed: this represents a reduction of 83%. DON-3-glucoside content was reduced from 1.650 to 0.615 mg/kg which is a reduction of 63%. For 'Orvantis' the introgression of the two QTL reduced DON by 77%, but reduced DON-3-glucoside by 57% only. Hence, also in this experiment introgressing FHB resistance QTL in wheat cultivars reduced average FHB severity as well as DON and DON-3-glucoside levels in the grain, but the reduction is smaller for the masked toxin. In general the increase in the DON-3-glucoside/DON ratio in more resistant wheat lines seems to be independent from the type of QTL which was introgressed: in other words, if the presence of a QTL reduces DON content, the DON-3-glucoside/DON ratio increases independently from the 'type' of QTL.

Fifth experiment: masked trichothecenes in Hungarian and Austrian wheat lines

Last but not least we mention an experiment (Experiment 5 in Table 1; Supplementary Figure S9 and S10) done in close cooperation with the Cereal Research Centre (CRC) in Szeged (Hungary) (data not shown). A wheat nursery of 43 wheat lines was selected containing 23 wheat breeding lines including varieties from Hungary (CRC) and 20 breeding lines/varieties from Austrian origin (Saatzucht Donau).

The Hungarian material contained highly resistant lines originating from crosses with 'Sumai-3' and 'Nobeokabozu'. The lines were tested over 3 years (2009, 2010 and 2012) for FHB resistance using three different inoculation techniques: (1) the kernel spawn method using *Gibberella zeae* colonised maize kernels spread on the soil surface; (2) spraying the *F. graminearum* inoculum on the flowering wheat ears under mist irrigation; and (3) spraying plus the use of a plastic bag to cover the inoculated ears in order to assure sufficient humidity for infection. Variance analyses over the 3 seasons showed highly significant differences between the lines in FHB resistance, DON and DON-3-glucoside content as well as the DON-3-glucoside/DON relation. The Genotype×Inoculation Method interaction was not significant for all parameters. The mol% DON-3-glucoside/DON varied from 5.9 to 15.7% (Table 1). DON contamination was closely related to DON-3-glucoside content and FHB resistance with r equalling 0.84*** and 0.82***, respectively. DON content was not so tightly related to DON-3-glucoside/DON compared to the other experiments (r=-0.45**), but the general picture and conclusions remain the same.

Conclusions on introgression of Fusarium *head blight in wheat*

We conclude that the effects of introgressing QTL for FHB resistance in wheat are: (1) a decrease in FHB severity; (2) a decrease in the absolute contamination of both

DON and the masked toxin DON-3-glucoside with (3) a concomitant increase in the relative fraction of the masked toxin compared to DON. The latter is due to the fact that upon increasing the FHB resistance the reduction in DON-3-glucoside content is less pronounced than the reduction in the DON contamination. As far as we can ascertain, every FHB resistance factor (QTL) or combination of different QTL that significantly reduces DON content shows this effect (increasing DON-3-glucoside/DON ratio) in the phenotype.

Introgression of any resistance factor that hampers *Fusarium* infection or spreading and therefore reduces *Fusarium* biomass in the plant will, if all other factors are kept constant, reduce fungal trichothecene production and hence contamination with DON (see review by Buerstmayr and Lemmens, 2015) and DON-3-glucoside, as shown in this review. This most likely explains the decrease of both DON and DON-3-glucoside contamination with increasing FHB resistance. But on the causes of the shift of the DON-3-glucoside/DON ratio we can only speculate. The following hypotheses are put forward to explain this phenomenon:

1. The simplest explanation for a higher DON-3-glucoside to DON ratio could be the fact that in resistant varieties the fraction of healthy, metabolically active plant tissue remains higher, thus contributing more and/or for a longer period of time to the detoxification of DON. The translocation of DON through the xylem and phloem has been reported, resulting in detectable amounts of toxin also in those kernels that did not show any fungal infection (Kang and Buchenauer, 1999; Pritsch *et al.*, 2001). We speculate that in active plant cells over time more DON can be metabolised to DON-3-glucoside resulting in a shift towards a higher DON-3-glucoside/DON ratio. Any type of resistance QTL or pyramiding of different QTL resulting in a reduction of FHB damage to the head tissue could contribute to this shift. On the other hand, local DON concentrations in susceptible lines could be so high that the detoxification enzymes (both glucosylation and glutathione pathway (see below)) gets saturated followed by fast dying of the plant tissue. In such tissues all plant conversions of DON will come to a halt and this could result in a comparatively lower DON-3-glucoside to DON ratio.

2. A second possibility is that in the scope of co-evolution between host plant (e.g. wheat) and pathogen, mechanisms developed *in planta* that detoxify DON. DON is a non-host specific toxin and it is a fungal virulence factor. Conjugation is known to be a detoxification process: DON-3-glucoside showed a strongly reduced ability to inhibit protein synthesis of wheat ribosomes compared to DON (Poppenberger *et al.*, 2003). The QTL on chromosome 3B (*Fhb1*) protects wheat against the phytotoxic effect of DON (Lemmens *et al.,* 2005). It was suggested that *Fhb1* is involved in a faster detoxification of DON. Data from Cirlini *et al.*

(2014) showed that 'Sumai-3' – a highly FHB resistant variety carrying *Fhb1* – detoxifies DON to DON-3-glucoside at a faster rate than susceptible durum lines: as soon as 24 h after inoculation 'Sumai-3' showed the highest conversion rate (25 versus 5-6% for the durum lines) and its detoxification ability was confirmed as very high at the time of harvest (84%). Kluger *et al.* (2015) reported that after application of DON in wheat ears, the mycotoxin can be detoxified via two major pathways: either glucosylation or via the glutathione (GSH) pathway. *Fhb1* enhances and intensifies the detoxification via the glucosylation pathway. Wheat cultivars carrying the resistance QTL *Fhb1* showed similar metabolism kinetics: formation of DON-3-glucoside was faster, while DON-GSH production was less efficient compared to cultivars which lacked *Fhb1*. Moreover, all wheat lines harbouring *Fhb1* showed significantly elevated DON-3-glucoside/DON abundance ratios (Kluger *et al.*, 2015).

3. A combination of 1 and 2.

5. General conclusions

A review of literature data supplemented with own experimental results on the effect of resistance breeding against *Fusarium* head blight in wheat on the masked toxin DON-3-glucoside leads us to the following conclusions:

1. All wheat lines that we investigated, even if not specifically bread for FHB resistance, can metabolise DON to DON-3-glucoside. Hence this plant detoxification pathway is not a new trait introduced via recent resistance breeding. Still, some specific QTL (e.g. *Fhb1*) possibly enhance the speed or rate of DON detoxification.

2. Combining several QTL for FHB resistance decreases FHB symptom severity.

3. DON-3-glucoside content is in general lower than the DON content in the grains (DON-3-glucoside/DON ratio usually <35%).

4. Increasing FHB resistance results in a decrease in the absolute contamination of both DON and the masked toxin DON-3-glucoside in the grain.

5. An increase in FHB resistance together with a decrease in DON content results in a concomitant increase in the relative fraction of the masked toxin DON-3-glucoside compared to DON.

6. This is due to the fact that upon increasing FHB resistance, the reduction of DON content is more efficient than the reduction of the DON-3-glucoside contamination.

7. As far as we can judge it, every FHB resistance factor (QTL) or combination of different QTL that significantly reduce DON content shows this effect (increasing DON-3-glucoside/DON ratio) in the phenotype.

8. It is not clear which biochemical/physiological mechanism is responsible for this increase in the DON-3-glucoside to DON ratio. Probably a relatively higher

proportion of DON is converted *in planta* to DON-3-glucoside in lines with an improved FHB resistance.

Resistance breeding against FHB is contributing – and will continue to do so – to a significant reduction of the risk for toxin contamination in wheat grain. The use of molecular markers will speed up the development of new cultivars combining several FHB resistance factors in commercial wheat cultivars. A higher level of FHB resistance in wheat cultivars results in a massive reduction of the total trichothecene content (masked and non-masked) as can be seen in the experiments presented in this review. Breeders are aware that their goal must be to keep the sum of both masked and non-masked DON as low as possible. Whether it is also possible to reduce the DON-3-glucoside/DON ratio in highly resistant cultivars remains to be investigated. Since the underlying mechanism is not known it might well be impossible to achieve this.

Supplementary material

Supplementary material can be found online at http://dx.doi.org/10.3920/WMJ2015.2019.

Figure S1. Box plots for the disease data of Experiment 1 described for the percentage visibly infected spikelets 26 days after inoculation (FHB26), deoxynivalenol (DON) content, DON-3-glucoside content and the ratio DON/DON-3-glucoside.

Figure S2. Scatter plots of DON data versus FHB26, DON-3-glucoside and DON/DON-3-glucoside ratio of Experiment 1.

Figure S3. Box plots for the disease data of Experiment 2 described for FHB26, DON content, DON-3-glucoside content and the ratio DON/DON-3-glucoside.

Figure S4. Scatter plots of DON data versus FHB26, DON-3-glucoside and DON/DON-3-glucoside ratio of Experiment 2.

Figure S5. Box plots for the disease data of Experiment 3 described for FHB26, DON content, DON-3-glucoside content and the ratio DON/DON-3-glucoside.

Figure S6. Scatter plots of DON data versus FHB26, DON-3-glucoside and DON/DON-3-glucoside ratio of Experiment 3.

Figure S7. Box plots for the disease data of Experiment 4 described for FHB26, DON content, DON-3-glucoside content and the ratio DON/DON-3-glucoside.

Figure S8. Scatter plots of DON data versus FHB26, DON-3-glucoside and DON/DON-3-glucoside ratio of Experiment 4.

Figure S9. Box plots for the disease data of Experiment 5 described for FHB26, DON content, DON-3-glucoside content and the ratio DON/DON-3-glucoside.

Figure S10. Scatter plots of DON data versus FHB26, DON-3-glucoside and DON/DON-3-glucoside ratio of Experiment 5.

Table S1. Summary of the ANOVA analyses for Experiment 1 to 5.

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