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# KP4 to control *Ustilago tritici* in wheat: Enhanced greenhouse resistance to loose smut and changes in transcript abundance of pathogen related genes in infected KP4 plants

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ABSTRACT

*Ustilago tritici* causes loose smut, which is a seed-borne fungal disease of wheat, and responsible for yield losses up to 40%. Loose smut is a threat to seed production in developing countries where small scale farmers use their own harvest as seed material. The killer protein 4 (KP4) is a virally encoded toxin from *Ustilago maydis* and inhibits growth of susceptible races of fungi from the Ustilaginales. Enhanced resistance in KP4 wheat to stinking smut, which is caused by *Tilletia caries,* had been reported earlier. We show that KP4 in genetically engineered wheat increased resistance to loose smut up to 60% compared to the non-KP4 control under greenhouse conditions. This enhanced resistance is dose and race dependent. The overexpression of the transgene *kp4* and its effect on fungal growth have indirect effects on the expression of endogenous pathogen defense genes.
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## 1. Introduction

Loose smut of wheat is favored by a cool and moist climate during anthesis. Therefore, losses can be significant in regions with such favorable climatic conditions, although losses have also been reported in dry and warm regions. The optimum time for infection is between early and mid anthesis, but successful infection can occur even after anthesis. The pathogen and remains dormant in the mature seed at the growing point. After seed germination, the mycelium grows in the crown node and later invades inflorescence tissues. This allows the fungus to be transported into the developing spike, where it sporulates, and disperses telio spores at spike emergence [1].

Although losses due to loose smut infestation are not devastating, it can cause moderate economic losses, resulting in profit reductions of 5–20% at an infection level of 1–2% [2]. Several methods are currently available to control loose smut; in many parts of the world, the use of resistant wheat cultivars, certified seed and fungicide seed treatments is highly effective in controlling loose smut of wheat. However, most of the resistant wheat cultivars detected to have a narrow resistance spectrum and seed treatment is either not used or can have limited efficacy [2]. Therefore, genetically modified (GM) wheat might be an environmentally safe and economically advantageous alternative to control loose smut infections.

Inter-strain inhibition in *Ustilago maydis* was first reported by Puhalla in 1968 [3]. The system was described as the production of an "extracellular substance" by certain races of *U. maydis* that inhibit other, sensitive, races of the same species. Moreover, the ability of producing or being sensitive to this inhibitor was heritable and under extranuclear control. Today, these inhibitors are known as killer proteins (KPs) and more have been reported, e.g. KP4 and KP6 [4].

The KPs are encoded by double strand RNA viruses. While KP1 and KP6 are processed by the protease Kex2 into one and two active products, KP4 activity is independent of post-transcriptional modifications [4–6]. Mature KP4 is a 105 amino acid polypeptide encoded by the *U. maydis* virus 4 (UMV4). It inhibits growth of sensitive *U. maydis* races by deregulating transmembrane calcium channels [7,8]. Using seed *in vitro* assays, Clausen et al. [9] showed

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that genetically modified (GM) wheat expressing *kp4* under the control of a maize ubiquitin promoter specifically inhibited the growth of sensitive *U. maydis* strains. Antifungal activity of the KP4 wheat lines was further confirmed both in the field and the greenhouse leading to increased resistance to *Tilletia caries*, a wheat pathogen of the Ustilaginales order that causes stinking smut [10]. In addition, KP4 wheat showed *in vitro* inhibited growth of *Ustilago tritici* another seed-borne wheat pathogen that is responsible for loose smut. These results were consistent with data reported by Koltin and Day [11] showing that KPs inhibit growth of the grass-infecting species in the Ustilaginales order, that cause smut and bunt diseases. This inhibition is highly specific since the growth *in vitro* of several bacterial and of fungal species was not affected by KP4 [10]. In this work we show that KP4 wheat lines have increased quantitative resistance to the loose smut pathogen, *Ustilago tritici* (Persoon) Rostrup. and that the strength of the KP4 effect depends on the *U. tritici* race used for inoculation. We also compare the expression profile of genes involved in pathogen defense between KP4 and non-KP4 control wheat.

## 2. Material and methods

### 2.1. Inoculation of plant material

Two GM lines of spring wheat varieties Golin and Greina, one line of each variety, already characterized in detail previously [9,10] were used in this study. Greenhouse grown seeds of non-KP4 controls, Greina null segregant (plants which lost their transgene by mendelian segregation) and non-transformed Golin, and of KP4 lines of the 6th generation after gene transfer (T6) of KP4, were grown by 3–5 seeds in pots of 12 cm of diameter in the greenhouse for fungal infection assays. Growth conditions were as follows: 19 °C/14 °C (day/night temperature), 60%/50% (day/night humidity); 16 h of light; minimum 3400 lx (cd sr $m^{-2}$) light intensity. Infection of plant material was performed as described by Wilcoxon and Saari [2] by injecting spore suspensions into flowering spikes. Dose effect was performed by applying different spore concentrations in the injection suspension. A natural spore field collection ("Furrer collection"), which contained different unknown races, was used for a first greenhouse assay. This collection was kindly provided by H.R. Furrer, Research Station Agroscope Reckenholz-Tänikon ART, Zurich, Switzerland (www.agroscope.admin.ch). In a second greenhouse experiment isolated races, *U. tritici* races T9, T10 and T39, were used for a race and dose experiment. The teliospores from these races were obtained from Agriculture and Agri-Food Canada (http://www.agr.gc.ca) by courtesy of J. Menzies. A second field spore collection ("Spiess collection"), also containing different unknown races, used in field trials 2010 were kindly provided by H. Spiess from Getreidezüchtungsforschung Dottenfelderhof, Dottenfelderhof, 61118 Bad Vilbel, Germany.

### 2.2. Greenhouse and field trials

Growth conditions used in the greenhouse were as described above. In the dose response assay with spore collection, per dose 50 inoculated seeds of Greina KP4 and its non- KP4 control (null segregant) and 70 inoculated seeds of Golin KP4 and non-KP4 control (non-transformed Golin) were sown in pots of 12 cm in diameter. In the dose response assay with different races, an average of 60 (±10) hand-inoculated greenhouse grown seeds were sown for each combination of genotype (Golin and Greina KP4 and both non-KP4 controls), dose and race used for inoculation. Scoring of disease symptoms was done in all assays at BBCH61-69 [12,13] as percentage of infected spikes. Infected spikes were identified by the presence of telio spores instead of developing seeds. Statistical analysis was done by Fischer Exact Test.

Field trials were carried out at AgroBioTechnikum Groß Lüsewitz, Groß Lüsewitz, Germany (http://www.biovativ.de) in two different field sites: Gross Lüsewitz and Üpplingen. Seeds from greenhouse grown, hand-inoculated plants were sown in March 2010. Microplots containing 100 seeds of each line were organized in randomized blocks in four independent replicates from the same inoculation. As described earlier, scoring for disease symptoms and collection of the material was performed at BBCH 61-69 (flowering). Agronomic treatment was done according to common local farmers practice, but without any fungicide treatment.

### 2.3. Plant material for expression profiles

In the field trial, three biological replicates of 15 fifth leaves (leaf 5) samples were collected separately from tillers with and without symptoms. In the dose response greenhouse experiment with different races, plants having tillers with and without symptoms were sampled, collecting separately leaf number 5 from each scoring result, dose and race used for inoculation. Samples were collected between BBCH61-69, frozen in liquid nitrogen immediately after collection and stored at −80 °C until RNA extraction.

RNA was extracted using TRIzol reagent (Invitrogen, Basel, Switzerland), according to the manufacturer's instructions. To avoid DNA contamination, RNA samples were treated with RQ1 RNase-free DNase kit (Promega).The quality of the isolated RNA was determined with a NanoDrop ND 1000 (NanoDrop Technologies, Delaware, USA) and gel electrophoresis. Total RNA samples (1.5 μg) were reverse-transcribed into double-stranded cDNA with RevertAidTM First Strand cDNA Synthesis Kit (Fermentas).

### 2.4. Dynamic arrays

Fluidigm® 48.48 Dynamic Array™IFCs (Integrated Fluidic Circuits) dynamic arrays were performed. Three technical replicates per sample and a non-template control were tested. The dynamic arrays were performed as described by Fluidigm® 48.48 Dynamic Array™IFCs (PN 100-1208 B) with minor modifications. Total reaction volumes were changed: "STA (specific target amplification) reaction volume" was 6 μl instead of 5 μl and "sample pre-mix volume" was 8 μl instead of 5 μl. The total volume per inlet for the "assay mix" chosen was 6 μl. Biomark™-System was used to run the chips and data was collected with Fluidigm™Real-Time PCR Analysis Software.

Supplementary Tables 1 and 2 show the lists of primers used in the experiment. Genes were selected either because they have been reported to be involved in plant defence response or they were differentially expressed in microarray experiments performed with KP4 and control lines (Fammartino et al. [16]). Three reference primers for RT-qPCR normalization in wheat were used: Ta542297, Ta2291 and Ta2776 [14]. Primers whose melting curves had more than one peak and, therefore, different melting temperatures ($T_m$) were eliminated from further analysis. *Ct* values were calculated by Fluidigm™Real-Time PCR Analysis Software, linear derivative and automatic detectors methods were set for baseline correction. PCR efficiencies were calculated with LinRegPCR (12.x) software and $\Delta Ct$ calculated according to Karlen et al. [15]. The expression stability of the reference genes was checked using the geNorm technology within qbase$^{PLUS}$ software which recommended to use only Ta542297 and Ta2291 as reference targets. Graphs and statitiscal analysis were performed in SigmaPlot for Windows Version 12.0, Copywright©2011, Systat

Software, Inc. Principal component analysis was performed using R language (http://www.R-project.org).

## 3. Results

### 3.1. KP4 wheat shows enhanced resistance to loose smut

From earlier experiments that investigated the antifungal specificity of the *kp4* transgene [9] we expected that KP4 could also inhibit the development of loose smut (caused by *Ustilago tritici*). KP4 wheat was tested in a greenhouse trial for quantitative loose smut resistance using different doses of *U. tritici* spores (Furrer collection). Fig. 1 shows the number of plants with and the number of plants without symptoms for the varieties Greina and Golin both KP4 lines and non-KP4 controls for four different *U. tritici* spore doses. The scoring results are shown in Supplementary Table 3. The presence of the transgene had a significant effect (Fischer Exact Test) on the number of plants with and without symptoms for the doses 1, 5 and 10 g/L in both varieties. For the 20 g/L dose, the difference between the number of plants with and without symptoms was not significant for the presence of the transgene. The highest increase in resistance was for the dose 5 g/L, 31% and 57% less plants with symptoms for Greina and Golin KP4 respectively. There was no significant correlation between dose and percentage of plants with symptoms for non-KP4 control lines for the three lower *U. tritici* doses. On the other hand, there was a significant correlation for dose (logarithm base 10) and percentage of plants with symptoms in the KP4 lines, $R^2$ 0.94440 and 0.9415 for Greina and Golin KP4 respectively (Pearson correlation coefficient, $P < 0.05$). This indicates that KP4 provides quantitative resistance to loose smut telio spore development in wheat plants expressing the killer protein 4 and

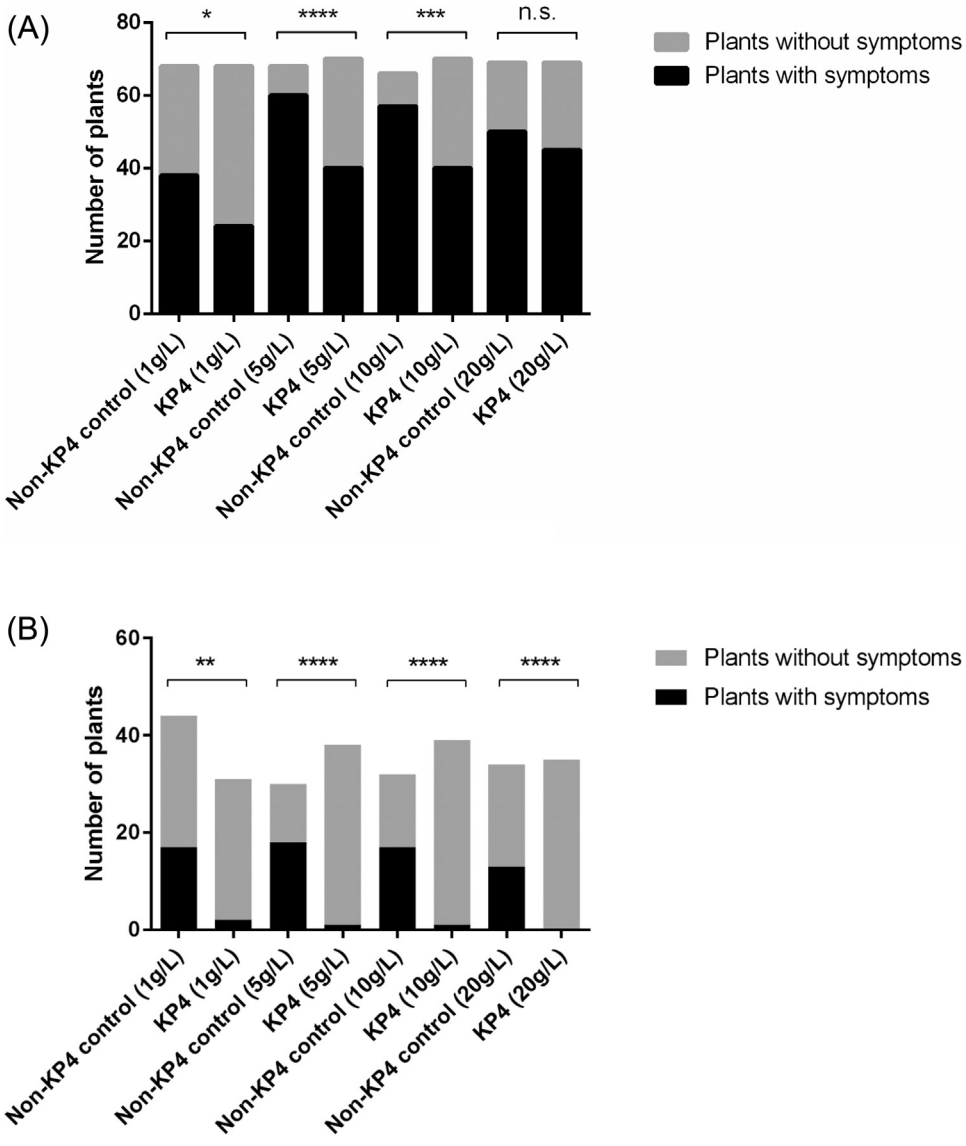


**Fig. 1.** Greenhouse scoring results for infection test of two wheat varieties, genetically modified by KP4 Golin and Greina, inoculated with different doses of loose smut spores. Plants were inoculated with different doses of loose smut spores, 1, 5, 10 and 20 g per liter of spores suspended in water. (A) The bars represent the number of plants with (black) and without (grey) symptoms for Golin KP4 and non-KP4 control. (B) The bars represent the number of plants with (black) and without (grey) symptoms for Greina KP4 and non-KP4 control. The asterisks represent the probability (Fischer Exact Test) that the difference observed in plants with symptoms is independent of the presence of the transgene. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$, n.s.= non significant = $P > 0.05$. In both varieties there was an increase in resistance in KP4 plants compared to their non-KP4 control.

that the relationship between dose and percentage of plants with symptoms is exponential.

The KP4 Greina line and its non-KP4 control were also tested in a field trial in 2010 in two locations in Germany: Üpplingen and Groß Lüsewitz. In order to ensure the viability of the spores used in the field trial, a fresh collection (Spiess collection) was used. The *U. tritici* spore concentration used for inoculation was 5 g/L because this dose had shown the highest difference between KP4 lines and non-KP4 control in the greenhouse experiment. The infected ears developed a mass of spores instead of the cereal grain and were easily detected during the flowering stage (BBCH 62-68) by their black color. The numbers for infected and non-infected ears were

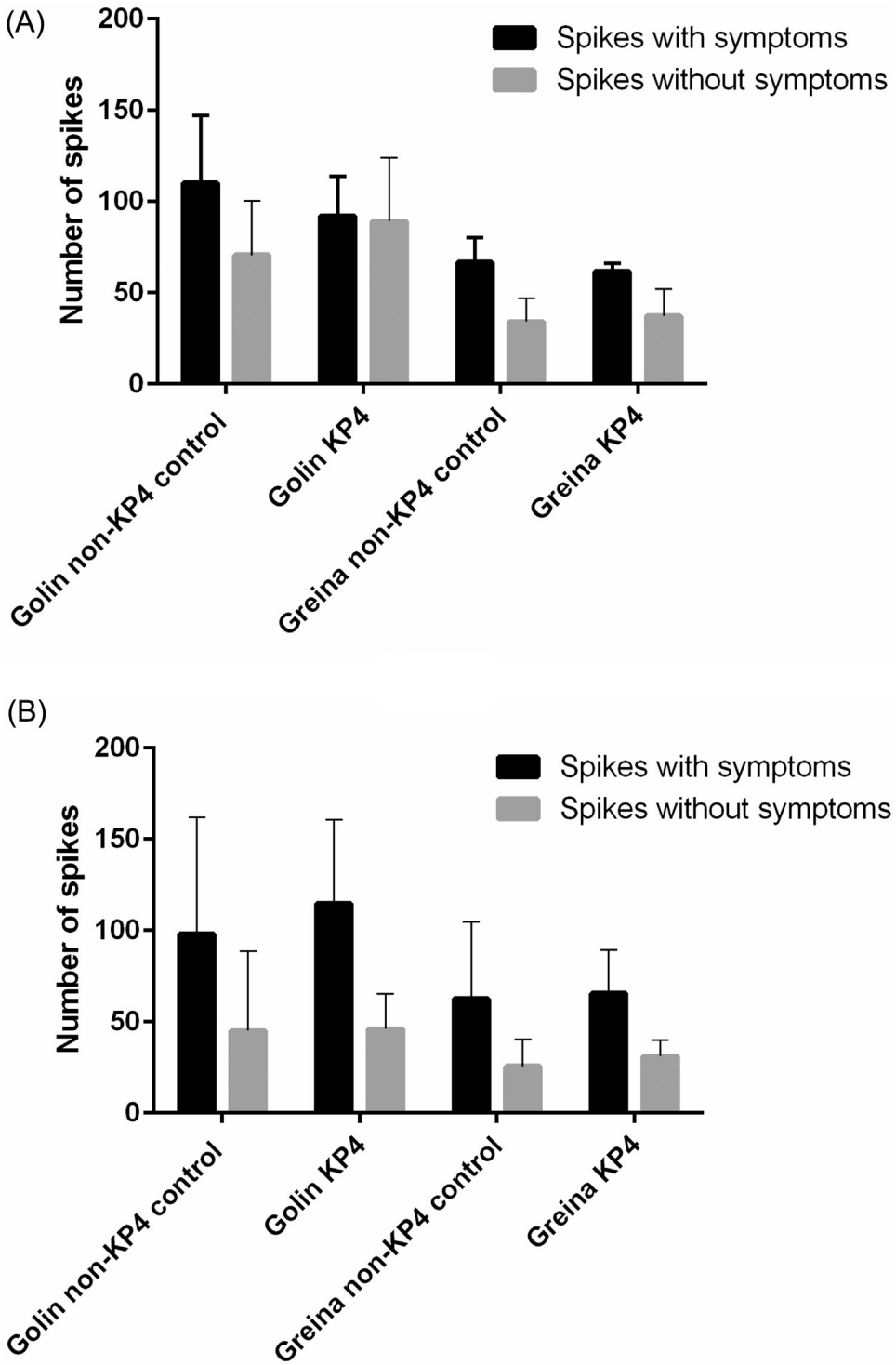


**Fig. 2.** Scoring results of field trial with KP4 and non-KP4 control plants inoculated with loose smut. The bars represent the number of plants with (black) and without (grey) symptoms for Golin and Greina, KP4 and non-KP4 control in: (A) field site Üpplingen (three biological replicates ±SEM) (B) field site Gross Lüsewitz (four biological replicates ±SEM). There is no significant difference between KP4 wheat and non-KP4 control.

determined and percentage of infected spikes was calculated. In this field trial, no significant difference was observed between KP4 and its non-KP4 control line in both locations (Fig. 2). The scoring results are shown in Supplementary Table 4.

### 3.2. KP4 enhanced resistance is race and dose dependent

The field spore collections used in the greenhouse (Furrer collection) and field (Spiess collection) experiments were different, thus their infection pressure might differ depending on their respective race composition. We therefore tested whether the enhanced resistance provided by KP4 varied, if different *U. tritici* races were used for inoculation. Three different representative isolated *U. tritici* races of defined virulence, T9, T10 and T39 were used for inoculation with the doses that showed a significant increase in resistance in the greenhouse trial: 1, 5 and 10 g/L. Fig. 3 shows the number of plants with and the number of plants without symptoms for the varieties Greina and Golin both KP4 lines and non-KP4 controls for the different doses. The scoring results are shown in Supplementary Table 5.

In case of the dose 5 g/L, both for Golin and Greina inoculated with T10 and T39, the number of plants with or without symptoms is not independent of the presence of the transgene, but no dependence was detectable when the plants are inoculated with T9. Therefore, the resistance provided by KP4 depends on the *U. tritici* race used for inoculation. The scoring results for all three doses are shown in Supplementary Table 5. The results for doses 1 and 10 g/L were summarized in Supplementary Table 6 and confirmed that the resistance provided by KP4 depends both on the *U. tritici* race used for inoculation and the dose.

### 3.3. Effect of different races on expression of genes related to plant pathogen defense

In order to find possible explanations for the difference between races (Fig. 3), we studied the expression profiles of genes

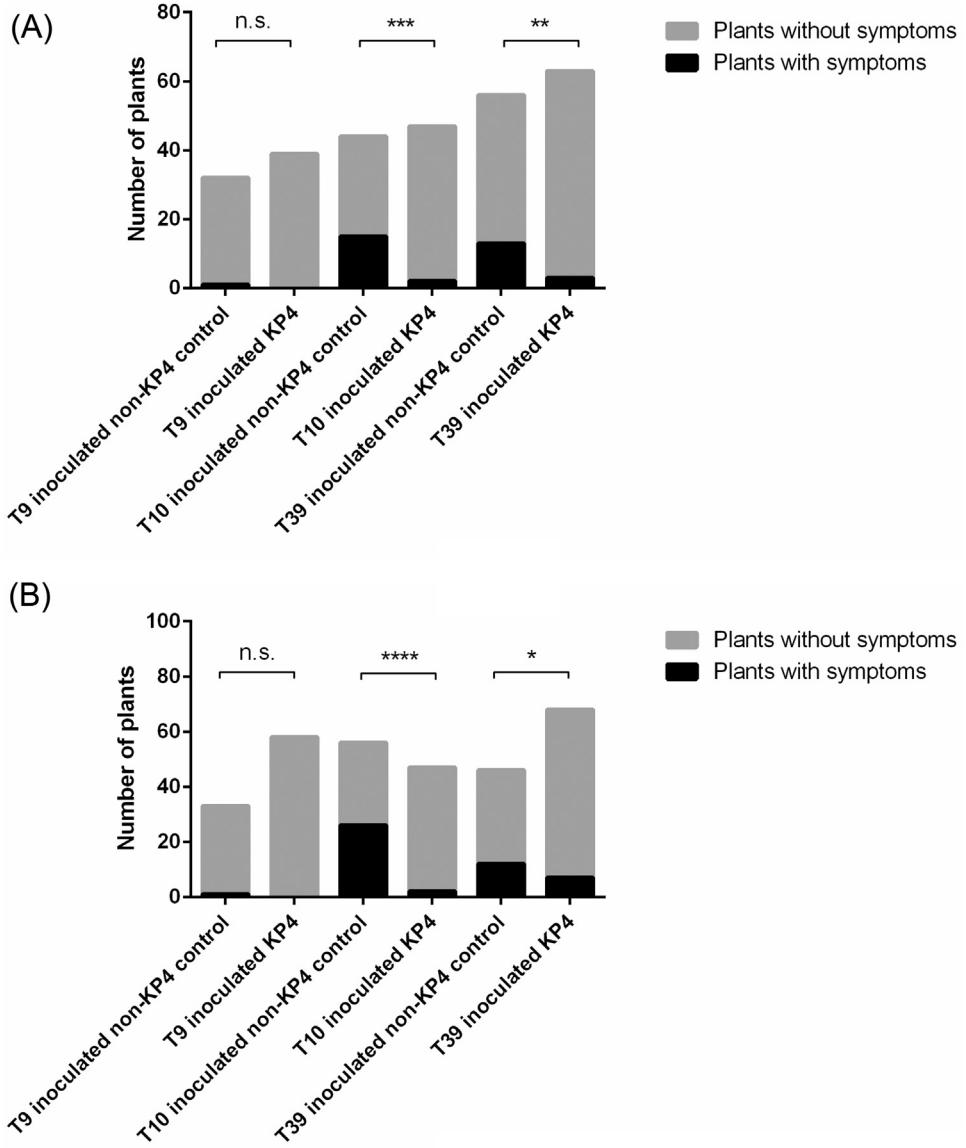


**Fig. 3.** Variation of resistance in KP4 plants according to different races used for inoculation for a 5 g/L spore suspension dose. (A) The bars represent the number of plants with (black) and without (grey) symptoms for Golin KP4 and non-KP4 control. (B) The bars represent the number of plants with (black) and without (grey) symptoms for Greina KP4 and non-KP4 control. Increase of resistance in KP4 plants is race dependent. The asterisks represent the probability (Fischer Exact Test) that the difference observed in plants with symptoms is independent of the presence of the transgene. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$, n.s.= non significant = $P > 0.05$.

related to plant pathogen defense in leaves (pool of leaf number 5) from plants inoculated with the different races at the same spore concentration. Twentytwo genes were selected either because they were found to be differentially regulated in microarray analysis previously performed using a KP4 line inoculated with *Tilletia caries* [16] or because they are known to be related to pathogen-defense. The absence of loose smut symptoms in the spikes might be due to unsuccessful inoculation or unsuccessful pathogen infection, as a result of plant defense mechanisms or KP4 inhibiting effects. We observed plants that had spikes both with and without symptoms. Therefore, in order to confirm that the spikes without symptoms came from successfully inoculated plants, we used leaves from tillers with spikes with symptoms and leaves from tillers with spikes without symptoms coming from the same plant. Given that the only dose that had tillers with and without symptoms on the same plant in both KP4 and non-KP4 control line for the three races was 10 g/l and only in Golin lines, the samples for the expression profile correspond to this variety and dose. Fig. 4 shows a Principal Component Analysis (PCA) which visualizes the relationship between the mentioned samples regarding the transcript abundance of 15 genes (Supplementary Table 1) measured with Fluidigm Dynamic Array™IFCs. Principal component 1 (PC1) accounted for 77% of the variance and PC2 accounted for 13%. The genes that contribute most to differences among samples along PC1 are: polyubiquitin, Pto kinase interactor 1 and an ef-hand calcium-binding protein. Those that contribute most to differences among samples along PC2 are: xylanase inhibitor, Pto kinase interactor 1 and an alternative oxidase. Samples do not cluster separately according to the race they have been inoculated with. Interestingly, while samples from plants inoculated with T39 are grouped together according to the absence or presence of symptoms, samples from plants inoculated with T9 are grouped together according to their genotype, Golin KP4 or Golin non-KP4 control.

### *3.4. Pathogen defense related genes are down-regulated in KP4 wheat and others correlate with* kp4 *expression*

Transcript abundance of 22 genes (Supplementary Table 2) was measured by Fluidigm Dynamic Array™IFCs in leaves samples (pool of 15 leaves, leaf number 5) from tillers with and without symptoms collected in the field trial at Groß Lüsewitz in 2010. The results were used to perform the PCA in Fig. 5 that visualizes the relationship between the mentioned samples regarding the transcript abundance of the 22 genes (Supplementary Table 2). PC1 accounted for 55% and PC2 for 32% of the variation. The gene that contributes the most to variation along PC1 is chalcone synthase and along PC2 is pathogen-related (PR) 1.1 protein. Interestingly, there is no clear separation by genotype or biological replicate. However, PC2 differentiates between samples coming from tillers with and without symptoms except for one sample.

In order to find possible explanations for the differentiation of two groups, with and without symptoms, in the field trial analyzed with PCA (Fig. 5), we analyzed the transcript abundance of each of the 22 genes (Supplementary Table 2) individually. We found five genes that were significantly up-regulated in samples from tillers with symptoms compared to those without symptoms: PR 1.1 protein ($P = 0.022$), thaumatin-like protein ($P = 0.025$), chalcone synthase ($P = 0.025$), PR10 protein ($P = 0.028$) and a class I chitinase transcripts abundance ($P < 0.001$) (Fig. 6A). The down-regulation of these genes in samples without symptoms indicates that these genes might not be involved in loose smut defense. Moreover, we observed that thaumatin-like protein and a class I chitinase mRNAs abundance was significantly lower ($P = 0.045$ and $P = 0.023$, respectively) in KP4 compared with non- KP4 control line (not shown). The down-regulation of these defense genes in the

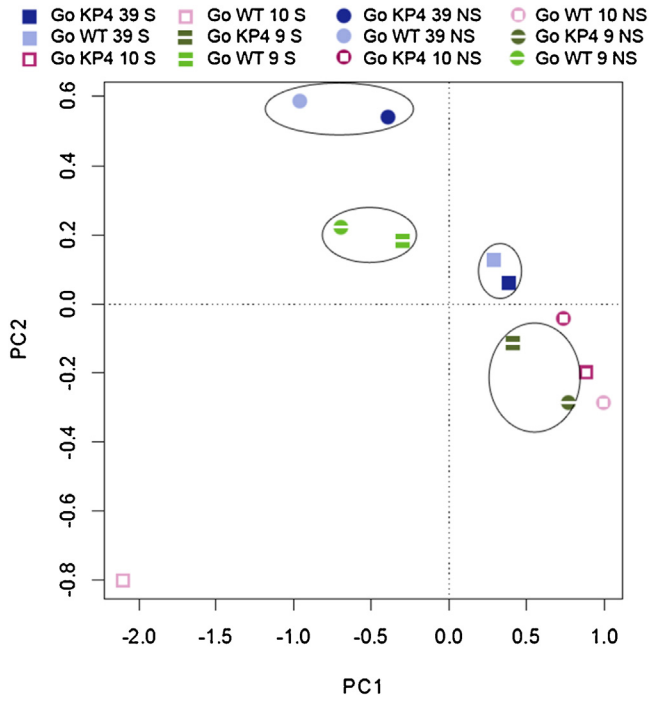


**Fig. 4.** PCA of transcript abundance using samples from greenhouse experiment with different races. Samples inoculated with race T39 (circled) are grouped together while samples inoculated with races T9 and T10 do not show a grouping pattern. S: symptoms. NS: no symptoms.

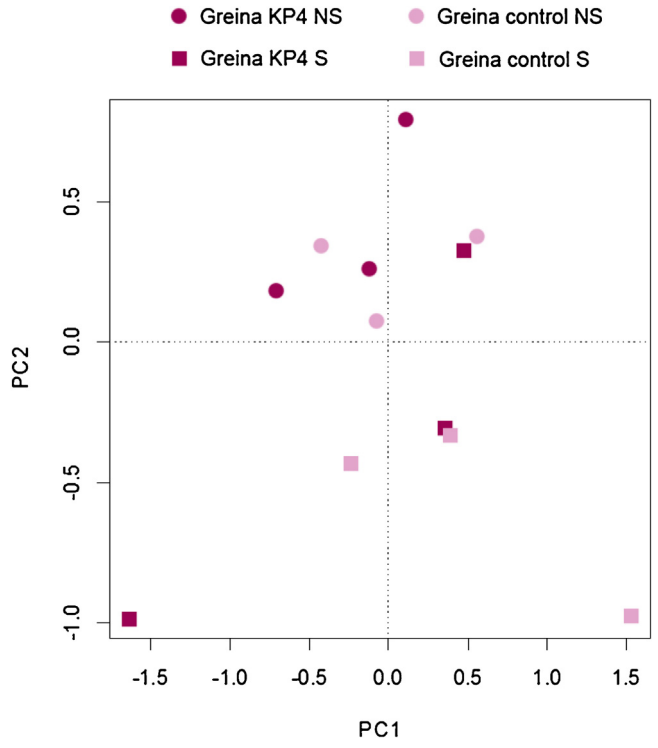


**Fig. 5.** PCA of transcript abundance with samples from field trial at Gross Lüsewitz. Symptomatic and asymptomatic samples are grouped separately from each other but there is no clear separation by genotype or replicate. S: symptoms. NS: no symptoms.

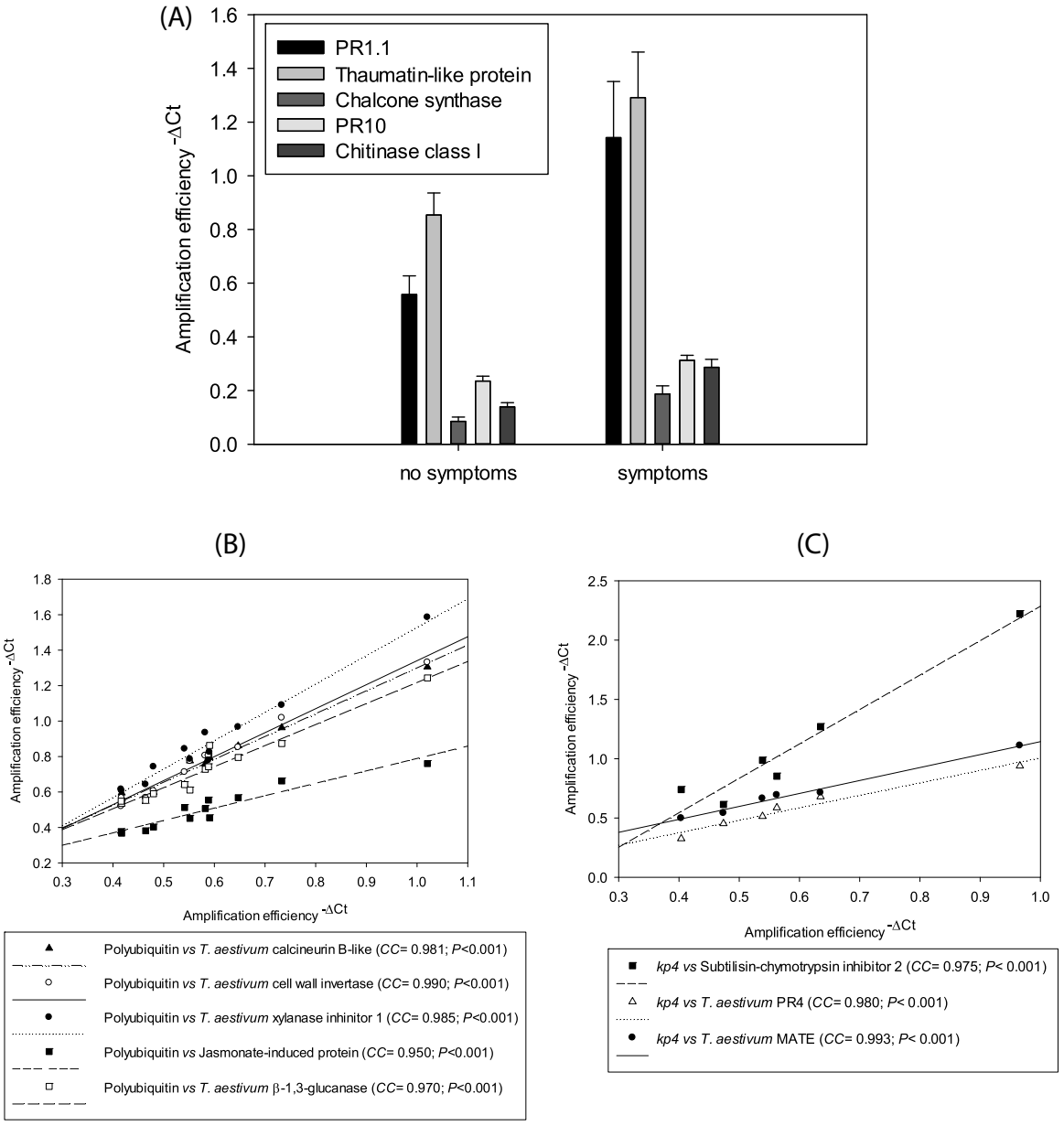


**Fig. 6.** Significant differences in transcript abundance and correlations with polyubitin and *kp4*. (A) Genes differentially expressed in tillers with and without symptoms. Data are normalized to the reference genes transcript abundance mean of three replicates collected at the field trial (Gross Lüsewitz) ±SEM. Significance was calculated using ANOVA. *T. aestivum* PR1.1, thaumatin- like protein, chalcone synthase, *T. aestivum* PR 10 and *T. aestivum* chitinase chitinase class I are significantly up-regulated in samples that showed symptoms. (B) and (C) Correlation of tested genes with polyubiquitin and KP4 respectively. Data are the transcript abundance of three biological replicates of Greina KP4 and non-KP4 control from tillers with and without symptoms. *CC* = Pearson's correlation coefficient.

KP4 line might be due to the fact that plants expressing *kp4* do not require the expression of these genes to maintain the same percentage of infected spikes as plants that do not express *kp4*.

Previous results in our group have shown that a race of *Tilletia caries* is able to down-regulate polyubiquitin as a possible way of overcoming plant defenses [16]. Thus, we measured the mRNA abundance of polyubiquitin in our samples. No significant difference was observed between KP4 and its non-KP4 control nor between samples with and without symptoms. However, we found that some of the pathogen defense genes transcript abundances correlated with polyubiquitin mRNA abundance both in Greina KP4 and non-KP4 control: calcineurin B-like protein (CBL) interaction protein kinase 5; cell wall invertase; xylanase inhibitor; a jasmonate-induced protein; β-1,3-glucanase (Glc2) (Fig. 6B). Interestingly, for some of the defense genes, the polyubiquitin expression profile correlated only in Greina KP4 plants, and not in the non-KP4 control, indicating the transgene has a pleiotropic effect in the expression of the following pathogen defense genes: a multi antimicrobial extrusion (MATE) protein; subtilisin-chymotrypsin inhibitor 2; PR4 protein (Fig. 6C).

## 4. Discussion

### *4.1. KP4 plants are more resistant under greenhouse conditions to* Ustilago tritici *than their non-KP4 control*

Previous work had shown that KP4 was able to enhance wheat resistance to *Tilletia caries* in green house and field conditions [9,10]. The objective of this work was to test this increased

resistance with another important fungus of the same order: *Ustilago tritici* causing loose smut. The results in the greenhouse showed that KP4 wheat plants are up to 60% more resistant to *U. tritici*. Moreover, the significant correlation between the logarithm of the dose and the percentage of infected plants showed this resistance is quantitative. In addition, we observed that the resistance provided by the transgene is also dependent on the race used for inoculation, possibly due to differences in virulence among races [17,18]. Furthermore, in the PCA using transcript abundance of known pathogen-defense related genes, the lines grouped according either to their genotype or to the symptoms depending whether the race used for inoculation was T9 or T39. This indicates that each race might have a different effect on the expression profiles of the lines, at least for the studied genes. Therefore, we conclude that the resistance provided by KP4 will be different for each collection of wild spores depending on its race composition and dose. Consequently, in order to be able to test KP4 effects quantitatively, a dose response experiment would be necessary for each new spore collection.

### *4.2. Indirect effects of KP4 transgene in endogenous gene expression*

In samples coming from tillers of infected plants with no symptoms in the field, significantly lower transcript abundance was observed for PR1.1 protein, thaumatin-like protein, chalcone synthase, PR10 protein and class I chitinase. These genes are all reported to be involved in plant pathogen defenses [19–23]. Therefore, it is conceivable that the corresponding transcripts are less abundant in tillers with no symptoms because they are not involved in defense against loose smut. Remarkably, two of these genes are down-regulated in KP4 plants compared to the non-KP4 control: thaumatin-like protein and class I chitinase. It is unlikely that KP4 participates in the regulation of endogenous genes, but it might indirectly affect the expression of these genes. We hypothesize that even when the observed symptoms showed no evidence of KP4 enhanced resistance under these dose and spore collection conditions, KP4 plants are able to maintain the same symptom status (i.e. amount of infected spikes) as non-KP4 control without the expression of some pathogen defense genes that non-KP4 control plants need for protection.

In agreement with previous work (Fammartino et al.), we found that wheat polyubiquitin gene expression correlates positively with the expression of *kp4*. This correlation is not surprising, since *kp4* is under the regulation of a maize ubiquitin promoter [9]. In addition, we found other genes whose transcript abundance correlated with the polyubiquitin transcript abundance: calcineurin B-like protein (CBL) interaction protein kinase 5, cell wall invertase, xylanase inhibitor, a jasmonate–induced protein and β-1,3-glucanase (Glc2). The positive correlation of the transcript abundance of these genes with polyubiquitin expression is conceivable since they are related to pathogen defense [24–28], and, thus, possibly regulated by the same pathways. Interestingly, other genes involved in pathogen defense, i.e. subtilisin-chymotripsin inhibitor 2 [29], PR4 protein 4 [20], and a multi antimicrobial extrusion (MATE) protein [30] correlated positively only in KP4 plants (with the high mRNA abundance of the polyubiquitin gene) and not in non-KP4 control plants. Apparently, in KP4 plants, the expression of certain pathogen defense related genes is induced with increasing amounts of *kp4*.

These results show that either expression of KP4 under the control of a maize ubiquitin promoter and/or the effects of the toxin may have pleiotropic effects on the expression of pathogen-related genes, either increasing or decreasing their transcript abundance depending of the gene. However, it is worth mentioning that no undesired phenotypic effects were observed in these KP4 plants [9,31].

## Conflict of interest

We did not receive financial or any support other than from ETH Zurich or Univesity of Rostock and are committed to scientific research only.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.btre.2016.08.002.

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