Transcriptomic analysis of quinoa reveals a group of germin-like proteins induced by *Trichoderma*

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Abstract

Symbiotic strains of fungi in the genus *Trichoderma* affect growth and pathogen resistance of many plant species, but the interaction is not known in molecular detail. Here we describe the transcriptomic response of two cultivars of the crop *Chenopodium quinoa* to axenic co-cultivation with *Trichoderma harzianum* BOL-12 and *Trichoderma afroharzianum* T22. The response of *C. quinoa* roots to BOL-12 and T22 in the early phases of interaction was studied by RNA sequencing and RT-qPCR verification. Interaction with the two fungal strains induced partially overlapping gene expression responses. Comparing the two plant genotypes, a broad spectrum of putative quinoa defense genes were found activated in the cultivar Kurmi but not in the Real cultivar. In cultivar Kurmi, relatively small effects were observed for classical pathogen response pathways but instead a *C. quinoa*-specific clade of germin-like genes were activated. Germin-like genes were found to be more rapidly induced in cultivar Kurmi as compared to Real. The same germin-like genes were found to also be upregulated systemically in the leaves. No strong correlation was observed between any of the known hormone-mediated defense response pathways and any of the quinoa-*Trichoderma* interactions. The differences in responses are relevant for the capabilities of applying *Trichoderma* agents for crop protection of different cultivars of *C. quinoa*.

Background

Trichoderma is a genus of ascomycete fungi widely studied for its versatile interactions with other organisms. *Trichoderma* can feed or parasitize on other fungi, bacteria, oomycetes and nematodes. Several species of *Trichoderma* are also symbionts with plants and can promote plant growth by several, yet so far only partially known mechanisms. Strains of several symbiotic species in the *Trichoderma harzianum* species complex (e.g. *Trichoderma afroharzianum* T22 (1), previously called *T. harzianum*) are used commercially because they can substantially improve yields of several species of crops. The strain T22 can enrich the soil nutrient availability to plants (2), and several species of *Trichoderma* have also been shown to enhance plant growth through volatile compound emission (3, 4) and stimulate plant systemic defense responses (5, 6). Nevertheless, plants do not always benefit from these interactions as described for some maize cultivar in field trials and lab experiments (7). Plant growth inhibition by *T. harzianum* has also been observed in axenic co-cultures with quinoa seedlings (8).

Quinoa (*Chenopodium quinoa* Willd.) is an emerging crop of great interest due to its nutritional values (9) and its resistance to hostile environmental conditions, especially salinity and drought (10, 11). Quinoa seeds are gluten-free, contain all essential amino acids and its composition (vitamins, antioxidants, fatty acids and minerals) is

highly suitable for human nutrition (12). Quinoa has a high genetic diversity, e.g. more than 4,000 accessions have been registered by the Food and Agriculture Organization (13). The high genetic diversity of cultivars is the result of many years of selection by the indigenous people of the Andean Altiplano, where quinoa may have been domesticated 7,000 years ago by pre-Columbian cultures (14).

Quinoa agricultural yields can be boosted by *Trichoderma* application, as previously described (15). However, the outcome of plant-*Trichoderma* interactions is not always beneficial. Plant genotype-specific growth inhibition by commercially available *Trichoderma* strains have been reported for lentils (16), tomato (17) and maize (7). Thus, the incompatibility of particular plants with particular biocontrol strains can lead to undesired agricultural losses. Therefore, there is a need to understand the genotype-specific interaction mechanisms that determine whether plant growth is promoted or inhibited by biocontrol agents like T22.

In this work, we have studied the molecular response mechanisms of two *C. quinoa* cultivars that experienced plant growth inhibition when treated with *T. harzianum* BOL-12 and *T. afroharzianum* T22 in axenic co-cultures. The response of quinoa to BOL-12 and T22 in the early phases of interaction was studied by transcriptomic analysis and RT-qPCR verification. Overall, we observed that upon interaction with the two fungal strains, a broad spectrum of putative quinoa defense genes were activated in Kurmi but not in the Real cultivar.

Methods

Biological materials

Seeds of quinoa (*Chenopodium quinoa* Willd.) cultivars Maniqueña Real (*Real*) and Kurmi were kindly supplied by PROINPA (Quipaquipani, Bolivia). *Trichoderma afroharzianum*, Rifai, T22, anamorph ATCC 20847 (1) was purchased from the American Type Culture Collection (Manassas, VA, USA). *Trichoderma harzianum* BOL-12QD (BOL-12) was isolated and provided by the Instituto de Investigaciones Farmaco-bioquímicas (IIFB-UMSA, La Paz, Bolivia).

Fungal growth

T22 and BOL-12 were maintained on potato dextrose agar (BD-Difco, Detroit, USA) at 25°C. To isolate conidiospore suspensions, one ml of sterile water was added to two-week-old *Trichoderma* cultures on potato dextrose agar and collected spores were filtered through a sterile piece of cotton wool. The spores were washed

twice with sterile ddH_2O and pelleted at 3700g for 5 min at 4 °C in an Allegra X-12R centrifuge (Beckman, Brea, CA, USA). Spores were resuspended in sterile ddH_2O and kept at 4°C until experiments.

Germination of T22 and BOL-12 spores for *C. quinoa* treatment was performed as described by Yedidia, Benhamou (18) using 15 ml tubes shaken at 200 rpm for 18 h. The germinated spore suspension was washed twice by centrifugation as described above and finally resuspended in sterile ddH_2O. The final spore concentration was adjusted to be 1 germinated spore/µl and verified by colony forming unit (CFU) counts in potato dextrose agar Petri dishes.

Sterilization of *C. quinoa* seedlings and germination

Seeds of *C. quinoa* were surface-sterilized by soaking in commercial bleach (NaClO; 27 g/kg) for 20 min., followed by 6 rinses in sterile ddH_2O. Immediately thereafter, the seeds were placed on sterile water agar (8 g/L) in Petri dishes and incubated in darkness at 24°C for 14 hours (8).

Co-culture of quinoa and *T. harzianum* in Petri dishes

Five germinated axenic seedlings of each cultivar Kurmi and Real with similar root length were aligned on a straight line on 12x12 cm square Petri dishes containing 0.1X Murashige and Skoog Basal Salts Mixture (MS; Duchefa, Haarlem, The Netherlands), supplemented with 8 g/L agar. The Petri dishes were then tilted 45° during growth with the agar/air interface facing upwards and seedlings having the roots pointing towards the bottom part of the Petri dish. The seedlings were incubated at 24°C for 4 hours before treatment with T22 or BOL-12.

C. quinoa seedlings were treated by adding 10 µl [1 CFU/µl] of either T22 or BOL-12 germinated spore suspension on the neck of the primary root. Ten µl of sterile ddH_2O was added to each seedling in the mock control group. After treatment, the seedlings were incubated at 24°C in a 16 h light /8 h dark photoperiod. Co-cultivation was done under fluorescent lights (Polylux XLr 30W, GE, Budapest, Hungary) at 50 µmol m^{-2} s^{-1} for 12 and 36 hours.

Seedling growth analysis

Hypocotyl length was analyzed from images taken with a Digital Camera Canon EOS Rebel T3. Measurements from the photographs were done with the segmented line tool of *ImageJ* 1.49 (19).

Sample collection and RNA extraction

For RNA extraction quinoa seedling were sampled 12 and 36 h after *Trichoderma* treatment as follows. The roots were excised at the root-hypocotyl interface with a scalpel. One root from each of five plates per treatment was

pooled into one replicate on pre-weighed aluminum foil envelopes. The envelopes were weighed on a precision balance and shock-frozen in liquid nitrogen. Frozen samples were either processed immediately or stored at -80°C until RNA extraction. Roots and shoots were pooled separately.

Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), with the following modification: Root tissue samples preserved in liquid nitrogen were placed in a precooled mortar containing liquid nitrogen followed by thoroughly grinding without letting the samples thaw. Then 450 µL of Buffer RLT (Qiagen, Valencia, CA, USA) supplemented with B-mercaptoethanol (1%) was added. Grinding continued until samples thawed and were transferred to a 1.5 ml microcentrifuge tube. The rest of the procedure was followed according to Qiagen instructions. Total RNA quantity and quality was determined with a NanoDrop spectrophotometer. DNase treatment was performed with the DNA-*free* kit (Ambion, Carlsbad, CA, USA), following the instructions of the manufacturer. The integrity and quality of the RNA was determined as follows: 500 ng. of DNase-treated RNA were dissolved in 8 µl of sterile water and split it in two aliquots, one placed on ice and the other placed at 37°, both for 20 minutes, immediately 2 µl of loading buffer was added to each sample and together were loaded to an agarose gel (2%) stained with ethidium bromide. The gel was run at 80V for 30 min and visualized in an UV-transilluminator. Samples with sharp 18S and 28S rRNA and showing no evidence of degradation were retained.

RNA-seq library construction and sequencing

Total RNA treated with DNase was sent to IGA technology services (IGA, Udine, Italy; http://www.igatechnology.com) for poly(A)$^+$ mRNA purification, strand-specific cDNA synthesis, library construction (Truseq stranded mRNA-seq) and sequencing using a HiSeq2500 (Illumina Inc., San Diego, CA, USA) in paired-end mode with a read length of 125 bp. Raw sequences have been deposited at the National Center of Biotechnology Information NCBI under the Sequence Read Archive (SRA): SUB9370528.

Transcriptomic analysis

RNA-seq reads were checked for quality by FastQC (v.0.9.0) and mapped on the quinoa genome "Kd" (Yasui et al., 2016) and to the QQ74 coastal genome (20) by Tophat2 (v.2.2.9). Transcript abundances were assessed with HTSeq (v.0.9.1) with "intersection-nonempty" mode. Genes that had a minimum of 1 read mapped in each of the samples considered for analysis were included. Gene expression levels were measured as counts per million (CPM) (21). Library size normalization was performed using the trimmed mean of *M*-values (TMM) within the R package

125 edgeR (v.3.14.0) (22, 23). Differential gene expression analysis (treated vs mock-treated) was performed using
126 edgeR with TMM normalized libraries (21) with a false discovery rate (FDR) of 5% ($q < 0.05$) (24).

127 **Functional annotation of differentially expressed genes**

128 Gene ontology (GO) term enrichment for sets of differentially expressed genes were estimated with Argot2 through
129 sequence function prediction (25). Singular enrichment analysis (SEA) for biological processes was performed
130 with AgriGO v2.0 (26). The statistical test for SEA was Fisher's exact test and for false discovery rate the Yekutieli
131 method was applied (27).

132 **cDNA synthesis and gene expression by qRT-PCR analysis**

133 Synthesis of cDNA was carried out with 500 ng of total RNA added to each 20 µl reaction of the RevertAid H
134 Minus Reverse Transcription Kit (Thermo Scientific). The cDNA samples were stored at -20°C for downstream
135 analysis. qRT-PCR of plant RNA was performed in a CFX384 Touch Real-Time PCR system (Bio-Rad, Hercules,
136 CA, United States) using Maxima SYBR Green qPCR Master Mix (Thermo Scientific) supplemented with 0.25
137 µM of each specific primer and 10 ng of cDNA as template in a total volume of 10 µl/reaction. The PCR program
138 had the following conditions: 1 cycle of: 95° C, 20 s; 30 cycles of: (95° C, 15 s; 60°C, 20s; 72 °C, 20s). The
139 specificity of each PCR amplification was determined by melting curve analysis and by analysis in 2% agarose
140 gels. The primer sequences can be found in Table S7. The relative transcript expression was calculated by the
141 Pfaffl algorithm using, *CqACT2A* and *CqMON1,* as reference genes. Ten-fold dilutions of cDNA template were
142 used to determine the amplification efficiency for each gene (28).

143 Primer pairs were designed using Perlprimer (29) so that one of the primers in each pair spanned an exon-exon
144 border, and the primer pairs were additionally checked using Netprimer (premierbiosoft.com) to avoid primer-
145 primer interactions.

146 **Protein evolution**

147 Protein alignments were made using Muscle (30). Aminoacid substitution models were evaluated by MEGA X
148 (31). The protein evolutionary tree was performed by maximum likelihood using LG model (32) with gamma
149 distribution (LG + G) and 95% limit for partial gaps. Total positions in the final dataset were 191. Bootstrap testing
150 was conducted with 1000 replicates.

Results

Trichoderma BOL-12 and T22 inhibit the growth of *C. quinoa* seedlings under certain axenic conditions

Previously we have observed that quinoa growth was inhibited by two *Trichoderma* strains under axenic conditions (8). Therefore, we decided to investigate the axenic co-culture of quinoa with T22 and BOL-12 by gene expression analysis to detect the molecular signaling possibly responsible of the *C. quinoa* growth inhibition. Briefly, quinoa seedlings of cultivars Kurmi and Real were grown for 18 h in square petri dishes on 0.1x MS and 0.8% agar and co-cultivated with T22 or BOL-12 for 12 and 36 hours. The studied *Trichoderma* strains did not have any measurable effect on the growth of *C. quinoa* seedlings during this short time of co-cultivation. Thus, the growth pattern was consistent with previously reported.

Transcriptome sequencing of *C. quinoa* in axenic co-culture with *Trichoderma*

RNA samples from quinoa roots treated with *Trichoderma* for 12 and 36 hours were collected. RNA samples at 12 hours post inoculation (hpi) were analysed through RNA-seq and RNA samples at 36 hpi were evaluated through posterior qRT-PCR. For the transcriptomic analysis three biological replicates of each treatment were sequenced in paired-end mode. The final number of reads that passed the quality control varied between 10.2 and 23.1 million paired-end reads of 125 bp per sample (Table 1). Reads were mapped to the draft quinoa genome of cultivar Kd as well as to the chromosome-level assembly of the quinoa genome cultivar QQ74 (Table 1). On average, the proportion of mapped reads was substantially increased when reads were mapped to the QQ74 quinoa genome (93.9 %), as compared to the draft Kd quinoa genome (71.8%). Therefore, all downstream analyses were performed with data mapped to the QQ74 genome.

Differential gene expression in quinoa in response to *Trichoderma* treatment

The differential gene expression analysis considered only reads that mapped to unique locations in the QQ74 genome. The average number of reads that were mapped to unique locations in the QQ74 genome was 89.8% (Table 1). The remaining reads (10.2%) producing multiple alignments were discarded. Further, only quinoa genes with at least one read in each of the samples analysed were considered (Table 2). Gene expression levels were measured as counts per million (CPM) . CPM were TMM-normalized in order to compensate for library size differences. Differential gene expression analysis comparing mock-treated samples with samples treated with *Trichoderma* was performed with edgeR.

Quinoa roots in general induced more genes than they repressed upon interaction with *Trichoderma*, with the exception of Kurmi interacting with T22 where more genes were repressed than induced (Table 2). Kurmi treated with T22 showed 16 times more differentially expressed genes than in the treatment with BOL-12. Similarly, quinoa cv. Real treated with T22, compared to the mock-controls, had 5.5 times more differentially expressed genes than Kurmi in the treatment with BOL-12 (Table 2).

Regarding communal effects by both *Trichoderma* strains, we observed more genes differentially expressed in cv. Real (141 genes) than in cv. Kurmi (75 genes) (Tables S1-3). Among the quinoa genes up- or downregulated under one or several conditions, only 19 were communally differentially expressed in all experimental combinations, and all were induced (Figure 1, Table 3). That is, they were significantly induced during the interaction of each quinoa cultivar with each *Trichoderma* strain. The group of 19 differentially expressed genes were not significantly associated with any functional GO term upon analysis by SEA. However, the GO analysis suggests that 13 of the 19 gene products are localized outside the cytoplasm. This indicates activity at the plasma membrane, the cell wall and the extracellular compartment, indicating functions relating to interactions with external stimuli (Table 3).

Quinoa genes differentially expressed unique to each cultivar

We decided to analyze genes that were induced by *Trichoderma* and were uniquely expressed in each cultivar. The Kurmi cultivar upon interaction with either BOL-12 or T22, expressed 75 genes that were communally differentially expressed (DE) but were not differentially expressed upon either *Trichoderma* interaction in cv. Real (Figure 1 and 2, Table S1). The expression profiles of these genes were clustered by Euclidean distance and are shown in Figure 2. From the 75 DE genes in cv. Kurmi by both strains of *Trichoderma*, 59 genes were induced (Table S1), whereas 16 DE genes were repressed (Table S2). The 75 DE genes expressed in cv. Kurmi are expressed in cv. Real but are not responsive to the treatment with either of the *Trichoderma* strains (Figure 2).

Analysis of the 59 significantly induced genes revealed that 17 genes (*CqGLPs*) are highly expressed and share a high protein sequence identity (90%). These genes encode proteins that belong to the germin-like protein family (GLPs) (Figure 2; Table S1). Further, several genes involved in flavonoid biosynthesis were specifically responsive in Kurmi. We identified 9 genes whose orthologs in *Arabidopsis thaliana* are described to be involved in the flavonoid biosynthesis pathway. These differentially expressed genes are orthologs to four out of five enzyme-coding genes necessary for production of flavonol glycosides from naringenin, also known as chalcone (33) (Figure 2, Table S1).

The Real cultivar had 141 genes differentially expressed common to both *Trichoderma* strains tested (Figure 1, Table S3). The cv. Real response to both *Trichoderma* strains showed mostly activation of transcription factors and enzymes without a significant match to a known pathway. Among the genes that were differentially expressed there are 4 ethylene-responsive transcription factors, 9 probable WRKY transcription factors and 3 chitinases (Table S3).

Genes differentially expressed related to biotic interactions were observed in mayor proportions in quinoa cv. Kurmi than Real. Therefore, the focus of this study was on the response of the Kurmi cultivar. **Functional annotation of differentially expressed genes**

To assess the function of the differentially expressed genes we annotated the differentially expressed genes of all combinations with Gene Ontology (GO) terms for biological processes. The quinoa genome has 44 776 annotated genes (20) but the annotation with Argot only assigned GO terms to 50.5 % of the genes (i.e. 22 650 genes annotated with GO terms). Despite the low percentage of GO terms assigned, GO annotation for the biological process category in Kurmi plants treated with BOL-12 revealed defense response (GO:0006952) and response to biotic stimulus (GO:0009607) as the main and only processes associated to *Trichoderma* BOL-12 treatment (Table S4). In contrast, the interaction between Kurmi and T22 did not show any significant GO term for biological processes.

Quinoa plants of the Real cultivar had more genes associated to GO terms than Kurmi. However, no specific association to a cluster of similar GO terms were observed (Table S4). Specifically, Real treated with T22 showed 38 genes that were annotated to response to stress (GO:0006950) and 6 genes that were annotated to chitin catabolic processes (GO:0006032) and associated redundant GO terms (Table S4). Further, Real treated with BOL-12 did not show any GO terms directly associated to defense response or response to stress, yet highest significance was observed to GO terms for cell wall related processes (Table S4). Nonetheless, in the interaction between Real and each strain of *Trichoderma* we observed several genes related to defense being commonly activated. Among them WRKY transcription factors (9 differentially expressed genes), ethylene-responsive genes (4 differentially expressed genes) and chitinases (3 differentially expressed genes) (Table S3).

Validation of RNA-seq with qRT-PCR

Quinoa root transcriptomes have not previously been analysed. We therefore validated the gene expression data obtained by RNA-seq by performing qRT-PCR for 10 selected genes, including induced, repressed and stably

234 expressed genes (Figure 3, Table S5). A log-log linear model analysis of the RNA-seq data and the qRT-PCR data
235 showed a strong correlation (R^2) of 0.848. The correlation was higher when the different quinoa-*Trichoderma*
236 interaction pairs were assessed independently (Table S5).

237 **Changes in root gene expression at 36 hpi**

238 Time changes in the expression of quinoa genes by *Trichoderma* treatment were assessed by qRT-PCR at 36 hpi.
239 We followed time-dependent changes in two highly induced genes (*CqGLP1* and *CqGLP10*) representing the GLP
240 family and one gene that was induced in all quinoa-*Trichoderma* interactions (*CqHSP83).* The gene expression of
241 *CqGLP1* and *CqGLP10* was reduced at 36 hpi compared to 12 hpi in the Kurmi cultivar but its expression was
242 still higher than the mock-treatment. In contrast, the gene expression of *CqGLP1* and *CqGLP10* in the Real cultivar
243 was higher at 36 hpi than at 12 hpi, being statistically significant in the Real - BOL-12 interaction (Figure 4). The
244 *CqHSP83* gene maintained its level of gene expression between 12 hpi and 36 hpi by application of T22 in both
245 cultivars. In contrast, the application of BOL-12 to both cultivars downregulated *CqHSP83* gene expression at 36
246 hpi as compared to 12 hpi. However, the downregulation was only significant in the Kurmi cultivar (Figure 4).
247 Overall, the results indicate that the induction of the analysed genes is slower in cv. Real than in cv. Kurmi.

248 **Shoot gene expression**

249 We investigated changes in the quinoa shoot gene expression 36 h after *Trichoderma* treatment at the root neck
250 (Figure 5). Ten out of 12 genes investigated were also expressed in the shoots, *CqPER39* and *CqPR1C* gene
251 expression was not detected at the shoots in any of the combinations studied (Table S6). *Trichoderma*-induced
252 gene expression changes at the shoots (Figure 5) showed a generally similar pattern of gene expression as observed
253 in the roots at 36 hpi (Figure 4). *CqGLP1* and *CqGLP10* are significantly expressed in both cultivars upon
254 interaction with BOL-12 but not with T22. Likewise, *CqHSP83* is significantly expressed in both cultivars when
255 interacting with T22 but not when interacting with BOL-12 (Figure 5). The other genes did not show a significant
256 correlation in the Shoot-root expression in any of the quinoa-*Trichoderma* interactions studied (Table S6).

257 **Evolutionary analysis of the germin-like proteins**

258 Plant germins were first investigated and have been characterised in most functional detail in cereals (34). To
259 investigate the coincidental induction of germin-like proteins in cv. Kurmi (Table S1), we carried out BLAST
260 searches to identify all germin and germin-like homologues in *C. quinoa*, *Beta vulgaris*, *A. thaliana* and *Hordeum*
261 *vulgare* and performed alignments and evolutionary analyses. We found that 16 of the17 quinoa GLPs induced by

Trichoderma (highlighted in green) in cv. Kurmi belong to a single (98% bootstrap) *C. quinoa*-specific clade of 29 homologues (Figure 6 and Table S8). The remaining GLP induced by *Trichoderma* (*CqGLP20*) groups in an unresolved putative clade, which contains four quinoa GLPs and one sugarbeet GLP (Figure 6). The relation of the quinoa-specific clade to homologues in other species, including the closest relative *B. vulgaris*, was not resolved, whereas other groups of quinoa germin-like proteins were significantly associated with specific homologues in *B. vulgaris.* Species-specific gene groups were also observed for *B. vulgaris* and *A. thaliana*. The result suggests that recent expansions of gene groups have occurred independently in the amaranth family species *C. quinoa* and *B. vulgaris*.

Discussion

The outcome of plant-*Trichoderma* interactions with respect to both growth and physiological changes has been shown to be genotype-specific regarding both the plant and the *Trichoderma* biomaterial (5, 17). Here, we have observed a small set of quinoa genes being responsive in all combinations of *Trichoderma* strains and quinoa cultivars studied. However, we have found many more genes that are differentially expressed by a specific quinoa cultivar in response to either or both of two *Trichoderma* strains (Figure 1). Nonetheless, the outcome of the interaction of *Trichoderma* on plant growth in axenic co-cultures is negative for plants, similarly for both quinoa cultivars and consistent with previous observations (8).

The set of 19 genes that showed significant responses in all cultivar-strain combinations mainly include genes connected to biotic stress response and cell wall modification (Table 3). Orthologs of these genes are known to be involved in defense response. For example, the polygalacturonase inhibitor protein *AtPGIP1* (AT5G06860) in *A. thaliana* is thought to inhibit cell wall pectin degrading enzymes, commonly produced by fungal pathogens (35). Xyloglucan endotransglucosylase/hydrolases are cell wall repairing enzymes, many of which are induced by fungal infection (36). Further, two highly similar (protein sequence identity of 94,3%) heat-shock proteins (*CqHSP83A* and *CqHSP83B*) annotated to be involved in general stress responses (GO:0006950) were upregulated in all quinoa-*Trichoderma* interactions. Large heat-shock proteins (70 – 90 kDa) are known to be involved in plant defense response through the stabilization of protective plant proteins (37-39). The heat-shock proteins expressed by quinoa might have been induced to contribute to the stabilization of defense proteins that would prevent or counteract damages induced by *Trichoderma* (8).

289 Several differences in the defense response patterns between the quinoa cultivars were observed. Especially, the
290 Kurmi cultivar that displays specific activation of several homologs to biotic stress-associated plant genes (Table
291 S1-2). In contrast, the responsive genes in cv. Real were mostly involved in general cellular processes (Table S3),
292 and to a lesser extent involving defense response genes. The defense response gene set induced in cv. Real was
293 also completely different from the one activated in Kurmi (Figure 1, Table 2 and Table S1-3). Surprisingly, in
294 neither case an obvious association to known major pathogen response pathways like jasmonic acid, salicylic acid
295 or ethylene pathways (40-42) could be observed in the GO analysis at 12 hpi. The low association of the quinoa
296 differentially expressed genes with these known pathways could, however, be caused by the relatively low level
297 of GO annotation observed for the quinoa genome.

298 The quinoa genes specifically induced in the Kurmi cultivar upon interaction with *Trichoderma* (Table S1)
299 resemble a set of defense response genes observed in plant-pathogenic interactions (43). Several of these induced
300 genes (chalcone synthase, chalcone isomerase, flavonol synthase, UDP glycosyl transferase and *cis*-zeatin O-
301 glucosyltransferase) belong to the flavonoid biosynthetic pathway (33). Flavonoids have an important role in plant
302 defense (43, 44). For example, some *A. thaliana* mutants lacking the UDP glycosyl transferase gene (*AtUGT74F1*)
303 are more susceptible to *Pseudomonas syringae* infection than the wild-type (45). Further, a QTL analysis for
304 pathogen resistance in soybean identified two UDP glycosyl transferase genes as the candidate genes responsible
305 for resistance to *Fusarium* (46). Thus, the Kurmi cultivar might be producing flavonol glycosides in order to
306 prevent damage from *Trichoderma* overgrowth.

307 The Kurmi cultivar specifically induced several plant defensins that belongs to the germin and GLP family (Figure
308 6 and Table S1). The *Trichoderma*-responsive GLPs form a majority (16 genes out of 29) of a recently expanded
309 quinoa-specific clade (Figure 6, Table S8), which are thus strongly connected to *Trichoderma* interaction. Two of
310 the GLP genes were further tested, and were found to be also induced in leaves (Figure 5). The timing of the
311 induction further indicated that GLPs are induced in both Kurmi and Real, albeit more slowly in Real (Figure 4).
312 Especially studied in grains like barley, GLPs are plant proteins involved in defense response and characterized
313 by various enzymatic activities including oxalate oxidase (OXO), superoxide dismutase (SOD), ADP-glucose
314 pyrophosphatase/ phosphodiesterase (AGPPase) and polyphenol oxidase (PPO) (47, 48). A potential function of
315 germin-like proteins (GLPs) is found in its OXO and SOD activities, which may play a key role in production of
316 hydrogen peroxide (H_2O_2) during plant defense (34). Because of a potential major importance of GLP in protecting
317 cells from superoxide toxicity produced under pathogen attacks, germin or GLP genes (i.e. *HvOXO1*) have been

318 inserted into dicot plants like rapeseed or peanut to enhance its pathogen resistance (48-50). Given that several
319 quinoa GLPs were expressed upon interaction with *Trichoderma*, it is very likely that these GLP defensins have
320 an important role in the plant immune response of quinoa. Resistance to microbe attacks has been previously
321 connected to the speed of response in different cultivars (51, 52). Thus, the rapid induction of a cluster of GLPs in
322 both roots and leaves of Kurmi compared to Real (Table S2-3, Figure 4) makes these genes potential candidates
323 for breeding to increase the tolerance to microbial attacks in quinoa plants.

324 The set of defense-related genes (peroxidases, chitinases, ERF and WRKY transcription factors) induced in quinoa
325 cv. Real upon interaction with *Trichoderma* (Table S3) has been observed in *L. japonicus* roots upon 1 hour of
326 incubation with chitin oligosaccharides (53). However, the levels of such defense-related genes returned to normal
327 after 7 hours in *L. japonicus* whereas in quinoa remained induced after 12 hours, possibly due to the persistance
328 of interaction with the living *Trichoderma* agent as compared to the transient nature of the elicitor. Similar to the
329 *L. japonicus system*, the 24 h interaction of *Trichoderma* with *A. thaliana* resulted in the induction of the same
330 defense-related genes as seen here in quinoa (54). This set of genes could thus be a basal gene response of plants
331 after recognition of beneficial fungi like *Trichoderma* through chitin. In contrast, the Kurmi cultivar might have a
332 different set of receptors that helps the plant to perceive a possible negative effect from *Trichoderma* and thus
333 rapidly activate a different set of defense-related genes.

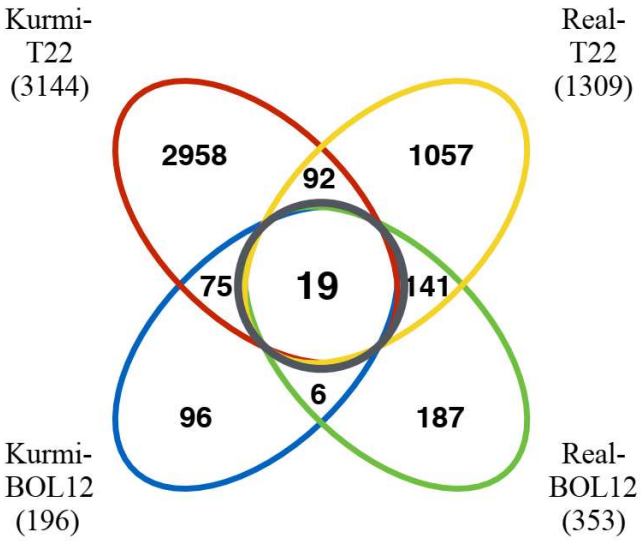
334 The plant root response to *Trichoderma* at transcriptomic level has been poorly studied compared to aerial parts
335 (55). Nevertheless, it has been observed that in tomato roots the recognition of *Trichoderma* at 24 hpi activates
336 ROS signaling, SA responses, cell wall modifications (56), JA responses and induction of plant defenses (54, 57).
337 In our study, we have observed a similar pattern for ROS signaling, cell wall modifications and induction of plant
338 defenses (Table 3, Table S1-3), confirming that the first response of root plants to beneficial fungi like
339 *Trichoderma* is to activate defenses. Further, our study reveals that the defense response against beneficial fungi
340 is variable between cultivars (Figure 2). The variable molecular response between cultivars to *Trichoderma*, could
341 help to create molecular markers of compatibility between certain plant cultivars and certain strains of
342 *Trichoderma*.

343 In conclusion, our study suggests that *Trichoderma* triggers a defense response in quinoa plants. Comparing the
344 defense response of two quinoa cultivars we can observe that the Kurmi cultivar mainly induced a set of genes
345 involved in plant defense. In contrast, the Real cultivar did not have a clear response because most of the changes

346 mediated by *Trichoderma* were related to general stress and regulation of biological processes. The Kurmi cultivar
347 might have higher tolerance to microbe attacks due to the expression of genes involved in the biosynthesis of
348 flavonol glycosides and a clade of GLP-defensins unique to quinoa. These genes are thus candidates for selection
349 of quinoa cultivars with higher resistance to microbe attacks.

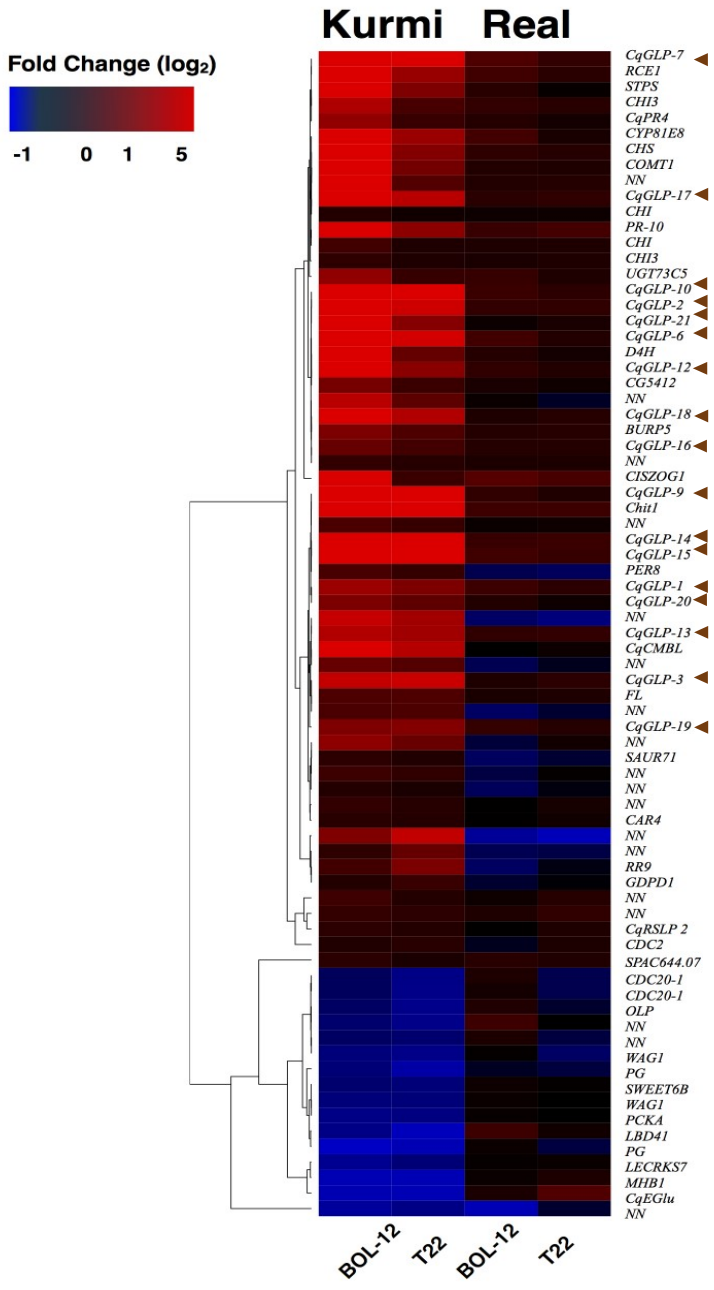
350

351 **Figures**



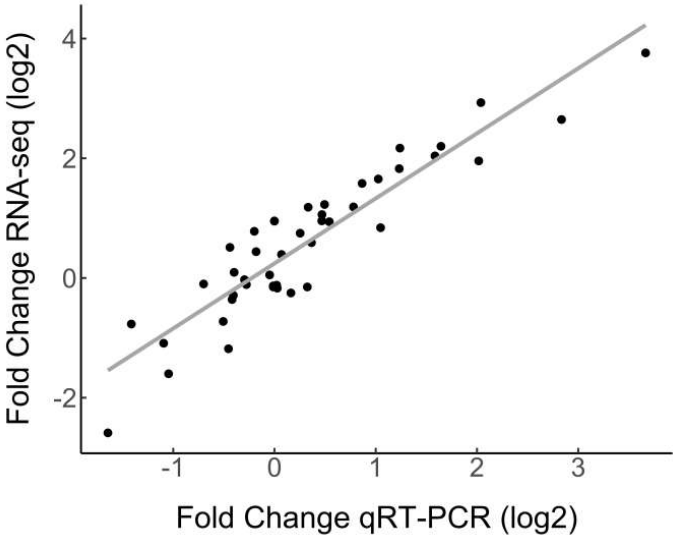
352

353 **Figure 1 Venn diagram of quinoa genes differentially expressed in response to *Trichoderma.*** Quinoa genes
354 differentially expressed were grouped according to the cultivars and *Trichoderma* strains studied. The black circle
355 indicates genes differentially expressed in both quinoa cultivars by each of the *Trichoderma* strains tested. The
356 numbers in parenthesis indicate the number of genes differentially expressed in each of the quinoa-*Trichoderma*
357 interactions studied.



358

359 **Figure 2 Heatmap profile of genes responsive to BOL-12 and T22 in Kurmi but not in Real.** Genes
360 differentially expressed in cv. Kurmi in response to either *Trichoderma* strain, but not significantly responsive in
361 cv. Real, were analysed. Clustering by Euclidean distance shows the similarity in expressional change upon
362 *Trichoderma* treatment. Brown arrows indicate *CqGLPs*.



363

364 **Figure 3 Correlation of RNA-seq and qRT-PCR gene expression data.** Ten differentially expressed genes and
365 two reference genes from the RNA-seq dataset were evaluated by qRT-PCR. Gene expression by qRT-PCR was
366 normalized to the *CqAct2* reference gene. Fold change was measured by comparing samples treated with
367 *Trichoderma* against mock- treated. The selected genes were assessed in all quinoa-*Trichoderma* combinations as
368 averages of triple biological replicates. The Pearson correlation coefficient between the RNA-seq and qRT-PCR
369 data was 0.921 (For data see Table S5).

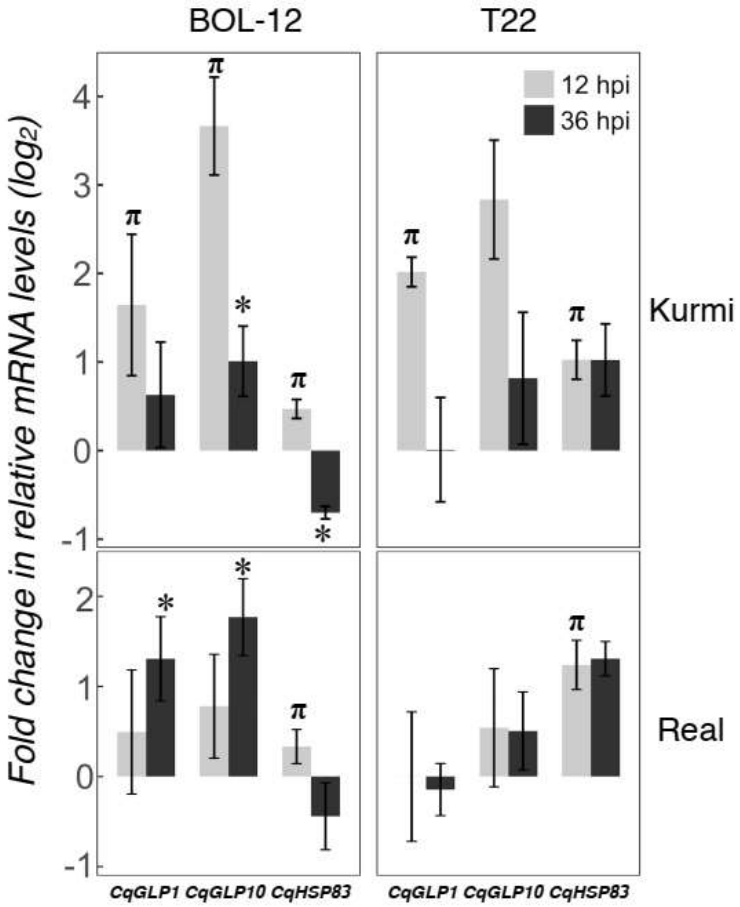


Figure 4 Gene expression changes at 12 and 36 hpi in representative *CqGLPs* and *CqHSP83*. Quinoa root samples treated with *Trichoderma* were assessed at 36 hpi by qRT-PCR. mRNA levels were normalized to the *CqAct2* reference gene. Fold changes (mean ± SE) were determined by comparing samples treated with *Trichoderma* against mock-treated ones. Asterisks denote significant changes in the gene expression as compared to the control treatment by qRT-PCR at 36 hpi ($p < 0.05$). Symbol π denotes significant changes in the gene expression as compared to the control treatment, using RNA-seq ($p < 0.05$) and confirmed by qRT-PCR ($p < 0.05$) at 12 hpi.

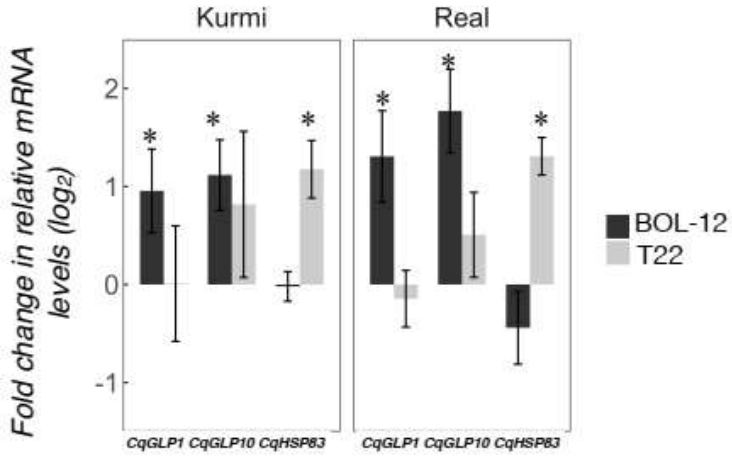
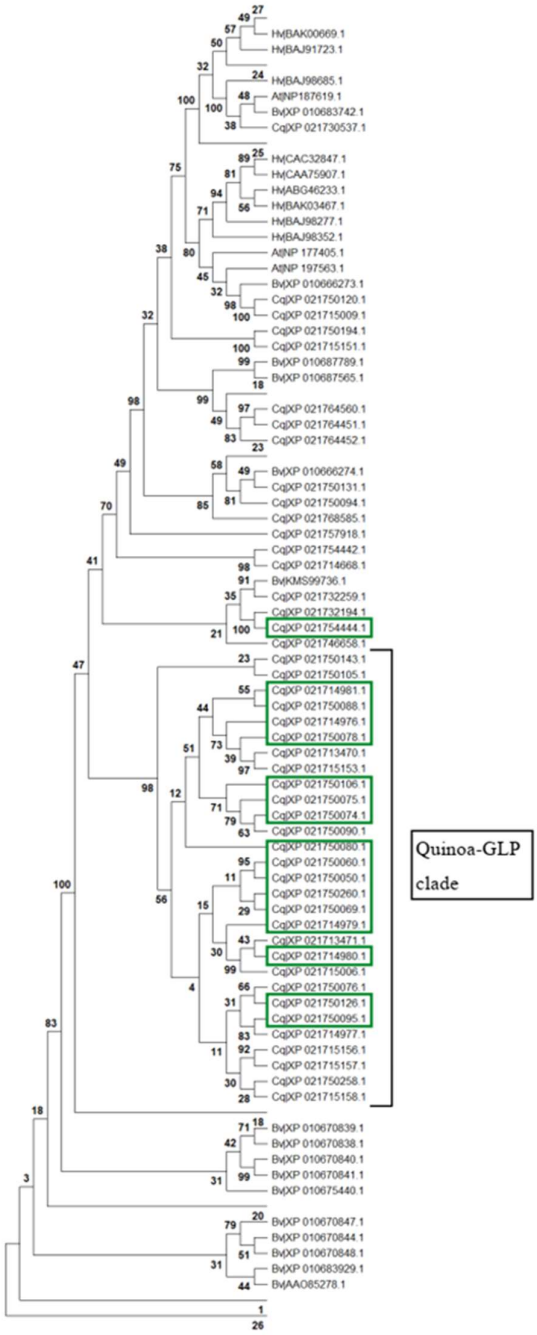


Figure 5 Gene expression changes in quinoa shoots after treatment of roots with *Trichoderma*. Quinoa shoot samples were assessed by qRT-PCR 36 h after treatment with *Trichoderma* to the roots. Gene expression was normalized to the *CqAct2* reference gene and is shown as mean ± SE. Asterisks denote significant changes in the gene expression as compared to the control treatment ($p < 0.05$). Two *CqGLP* genes that were induced in Kurmi roots at 12 hpi as well as the *CqHSP83* gene, which was induced in roots in all quinoa-*Trichoderma* interactions at 12 hpi are shown.



385

386 **Figure 6. Protein evolutionary tree of germin and germin-like proteins.** All identified germin-like proteins
387 found in *C. quinoa* (Cq), *B. vulgaris* (Bv), *A. thaliana* (At) and *H. vulgare* (Hv) homologues were aligned by
388 Muscle. The protein evolutionary tree was constructed by maximum likelihood using the LG + G model with 1000
389 iterations. Bootstrap values are given in percentage (%). Values below 30% are not shown except for in the
390 Trichoderma-responsive quinoa-specific GLP clade. The *Trichoderma*-induced homologues are marked in green.

Additional files

Additional file 1: Table S1. Quinoa genes significantly upregulated in the cultivar Kurmi but not in Real.
The table shows genes that were significantly upregulated in the Kurmi cultivar when treated with either BOL-12 or T22. The family of GLPs is highlighted in light green. The flavonoid biosynthetic pathway is highlighted in light purple. The numbers indicate averages of CPM values for each treatment (n = 3).

Additional file 2: Table S2. Quinoa genes significantly downregulated in the cultivar Kurmi but not in Real.
Genes observed to be significantly downregulated in the Kurmi cultivar when treated with either BOL-12 or T22 are included. The numbers indicate average of CPM values for every treatment (n = 3). CPM of reference genes are showed at the bottom for transparency.

Additional file 3: Table S3. Quinoa genes significantly up- and downregulated in the cultivar Real but not in Kurmi.
Genes shown were significantly and consistently upregulated or downregulated in the Real cultivar when treated with either BOL-12 or T22. Highlighted in green we can observe a family of chitinases. Light purple highlights WRKY genes and orange highlights ethylene-responsive genes.

Additional file 4: Table S4. Singular enrichment analysis of differentially expressed genes in quinoa roots treated with *Trichoderma.*

For each quinoa-*Trichoderma* interaction, quinoa genes differentially expressed (DE) were annotated for Gene Ontology with Argot2 and then analyzed for singular enrichment analysis with AgriGO2. Stress-related GO-term are highlighted in grey and cell wall-related terms in orange.

Additional file 5: Table S5. Gene expression assessed by RNA-seq and qRT-PCR.

RNA from quinoa roots treated with *Trichoderma* or mock treated (12 hpi) were analysed by RNA-seq and qRT-PCR in order to determine the correlation of expression levels. Fold change was determined by comparing samples treated with each *Trichoderma* strain against the mock-treated control.

Additional file 6: Table S6. Gene expression in quinoa shoot and root at 36 hpi with *Trichoderma*.

Quinoa shoot and root samples were assessed by qRT-PCR after 36 h treatment with *Trichoderma* added to the roots. Gene expression was normalized to the *CqAct2* reference gene. Fold change was determined by comparing samples treated with *Trichoderma* against mock-treated. Significant differences between treatment and control is highlighted in red. ND, not detected.

Additional file 7: Table S7. Primer sequences of quinoa genes analysed by qRT-PCR.

Forward and reverse primer sequences for qRT-PCR. Primer pairs were designed using Perlprimer aiming for exon-exon borders. *CqAct2* were used as reference genes for normalization of the mRNA abundances and were further verified by the *CqMon1* housekeeping gene.

Additional file 8: Table S8. Quinoa GLPs significantly upregulated upon treatment with *Trichoderma*.

Phytozome and NCBI codes for the germin-like proteins significantly upregulated by *Trichoderma* in the Kurmi cultivar. These GLPs belong to a quinoa-specific clade (Figure 6).

Additional file 9: Table S9. Differentially expressed quinoa genes upon treatment with *Trichoderma*.

Log2 fold changes, log2 CPM values, P-values and False Discovery Rates for the genes that were differentially expressed in any of the quinoa-*Trichoderma* interactions

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Table 1. RNA-seq summary of read numbers mapped to two quinoa reference genomes

Quinoa cv.	Treatment	Sample	Total reads[a]	Mapped reads[b]	%	Mapped reads[c]	%	Unique reads[d]	%	Multi-reads[e]	%
Kurmi	Mock	1	12609683	8902151	70.6	11717393	92.9	10428786	89.0	1288607	11.0
		2	13360579	9462168	70.8	12431446	93.0	11075758	89.1	1355688	10.9
		3	14186924	10144341	71.5	13301928	93.8	11987944	90.1	1313984	9.9
Kurmi	BOL-12	1	13017241	9321854	71.6	12231647	94.0	10952241	89.5	1279406	10.5
		2	11571126	8301337	71.7	10906882	94.3	9758090	89.5	1148792	10.5
		3	14321132	10211966	71.3	13449878	93.9	12018768	89.4	1431110	10.6
Kurmi	T-22	1	12371536	8500983	68.7	11225637	90.7	9860686	87.8	1364951	12.2
		2	11390953	8151138	71.6	10691577	93.9	9582419	89.6	1109158	10.4
		3	13144501	9423642	71.7	12345436	93.9	11154456	90.4	1190980	9.6
Real	Mock	1	14414857	10423990	72.3	13597954	94.3	12178545	89.6	1419409	10.4
		2	14949701	10780918	72.1	14143244	94.6	12694852	89.8	1448392	10.2
		3	13625079	9759644	71.6	12839479	94.2	11778409	91.7	1061070	8.3
Real	BOL-12	1	10245775	7405797	72.3	9599526	93.7	8553892	89.1	1045634	10.9
		2	11350821	8261409	72.8	10425272	91.8	9259149	88.8	1166123	11.2

		3	12542586	9091486	72.5	11767298	93.8	10725657	91.1	1041641	8.9
Real		1	23140059	17009224	73.5	21733959	93.9	19385551	89.2	2348408	10.8
	T-22	2	14568308	10646741	73.1	13743816	94.3	12645961	92.0	1097855	8.0
		3	11039194	8047239	72.9	10305738	93.4	9304963	90.3	1000775	9.7

[a] Total reads that passed the quality control per biological replicate in each treatment

[b] Average between right and left reads mapped with Tophat2 to the inbred Kd quinoa genome (58).

[c] Average between right and left reads mapped with Tophat2 to the QQ74 coastal quinoa genome (20).

[d] Unique reads mapped to the QQ74 coastal quinoa genome.

[e] Reads mapped to multiple positions

Table 2. Differentially expressed genes in quinoa roots treated with *Trichoderma*

Quinoa	Experiment	Induced	Repressed	Total	Ind/Repr	Genes evaluated[a]
Kurmi	BOL-12 vs. mock-treated	158	38	196	4.2	25 273
Kurmi	T22 vs. mock-treated	1417	1727	3144	**0.8**	25 379
Real	BOL-12 vs. mock-treated	277	76	353	3.6	30 108
Real	T22 vs. mock-treated	1170	139	1309	**8.4**	30 745

[a] Genes included had at least one read in each of the samples. The quinoa genome annotation contains 44 776 genes.

Table 3. Quinoa genes differentially expressed in both Kurmi and Real in response to both *Trichoderma* strains

Quinoa gene abbreviation	Quinoa gene code[a]	Gene name	Araport Code[b]	Quinoa protein length	Alignment length	Identity (%)	e-value	GO Cellular component
CqXTH6A	AUR62024859	Xyloglucan endotransglucosylase/hydrolase 6	AT4G25810.1	285	284	70,1	3E-154	apoplast
CqXTH6B	AUR62024861	Xyloglucan endotransglucosylase/hydrolase 6	AT4G25810.1	286	284	71,5	1E-151	apoplast
EXL4	AUR62018945	Protein Exordium-like 4	AT4G08950.1	311	298	67,5	4E-143	cell wall
CqPGIPA	AUR62012077	Polygalacturonase inhibitor protein	AT5G06860.1	311	311	48,6	6E-82	cell wall
CqPGIPB	AUR62024339	Polygalacturonase inhibitor protein	AT3G12145.1	333	329	44,4	3E-80	cell wall
CqChit1	AUR62021382	Glycosyl hydrolase with chitinase insertion domain-containing protein	AT4G19810.2	364	368	47,0	3E-113	extracellular region
CYP707A1	AUR62010485	Abscisic acid 8'-hydroxylase 1	AT4G19230.1	450	465	74,2	0E+00	plasmodesmata
PUB27	AUR62013534	U-box domain-containing protein 27	AT5G64660.1	392	424	40,3	4E-87	plasmodesmata
CqEP3.3	AUR62031316	Basic endochitinase C, homolog of carrot EP3-3	AT3G54420.1	242	232	62,9	1E-104	plasma membrane
NN	AUR62029900	Protein of unknown function	AT1G68390.1	286	216	60,2	1E-96	plasma membrane
AUR62005356	AUR62005356	Transmembrane protein	AT1G23830.1	253	138	39,1	3E-22	plasma membrane
SD25	AUR62006585	G-type lectin S-receptor-like serine/threonine-protein kinase SD2-5	AT4G32300.1	831	789	35,1	4E-124	plasma membrane
At1g35710	AUR62039001	Probable leucine-rich repeat receptor-like protein kinase	AT1G35710.1	478	451	37,3	5E-66	plasma membrane
CqHSP83a	AUR62031424	Heat-shock protein 83	AT5G52640.1	579	443	92,6	0E+00	cytoplasm

CqHSP83b	AUR62021118	Heat-shock protein 83-like	AT5G52640.1	703	700	93,1	0E+00	cytoplasm
CXE2	AUR62014711	Probable carboxylesterase 2	AT1G47480.1	304	301	50,5	7E-109	cytosol
AUR62011434	AUR62011434	Protein of unknown function	AT2G26530.1	269	184	33,7	2E-16	intracellular
CIGR1	AUR62001765	Chitin-inducible GRAS family transcription factor	AT2G29060.2	690	626	43,3	1E-165	nucleus
ERF071	AUR62025525	Ethylene-responsive transcription factor ERF071	AT2G47520.1	249	217	42,9	3E-44	nucleus
*CqWRKY33**	AUR62006298	WRKY33 transcription factor	AT2G38470.1	454	487	46,8	7E-116	nucleus

All genes commonly differentially expressed in all plant-*Trichoderma* combinations were induced.

[a] Genes annotated in the Quinoa QQ74 genome (Jarvis et al., 2017) curated with information from their closest ortholog in *A. thaliana*

[b] Gene codes from the *Arabidopsis* Information portal Araport.

* *CqWRKY33* was included in the list of genes because significant difference respective to the control was confirmed by qRT-PCR (Fig. 5)