

OPEN

# Protein phosphatases regulate growth, development, cellulases and secondary metabolism in *Trichoderma reesei*

Aroa Rodriguez-Iglesias &amp; Monika Schmoll

*Trichoderma reesei* represents one of the most prolific producers of plant cell wall degrading enzymes. Recent research showed broad regulation by phosphorylation in *T. reesei*, including important transcription factors involved in cellulase regulation. To evaluate factors crucial for changes in these phosphorylation events, we studied non-essential protein phosphatases (PPs) of *T. reesei*. Viable deletion strains were tested for growth on different carbon sources, osmotic and oxidative stress response, asexual and sexual development, cellulase and protease production as well as secondary metabolism. Six PPs were found to be positive or negative regulators for cellulase production. A correlation of the effects of PPs on protease activities and cellulase activities was not detected. Hierarchical clustering of regulation patterns and phenotypes of deletion indicated functional specialization within PP classes and common as well as variable effects. Our results confirmed the central role of catalytic and regulatory subunits of PP2A which regulates several aspects of cell growth and metabolism. Moreover we show that the additional homologue of PPH5 in *Trichoderma* spp., PPH5-2 assumes distinct functions in metabolism, development and stress response, different from PPH5. The influence of PPs on both cellulase gene expression and secondary metabolite production support an interrelationship in the underlying regulation mechanisms.

Adaptation to environmental conditions for survival and successful competition in nature requires an efficient machinery to detect signals as availability of nutrients, light or the presence of a potential mating partner or competitor. Perception, transmission and subsequent cellular responses are regulated by post-translational modifications such as phosphorylation and dephosphorylation<sup>1</sup>.

*Trichoderma reesei* is the major producer of cell wall degrading enzymes, an industrial source for the use of renewable lignocellulosic material towards production of biofuels and biorefineries<sup>2</sup>. Nevertheless, investigation of the impact of signal transduction pathways on biosynthesis of cell wall degrading enzymes has not been done in detail and it is still in its beginnings<sup>3</sup>.

Mechanisms involved in regulation of expression of cell wall degrading enzymes in *T. reesei* have been studied in detail over decades, in concern to carbon-source-dependent regulation pathways<sup>4,5</sup>. Posttranslational modifications like phosphorylation often regulate protein function, protein turnover, protein-protein interactions as well as intracellular signal transduction<sup>6,7</sup>. Concerning transcription factors, phosphorylation of CRE1 (carbon catabolite repressor 1) and ACE2 (activator of cellulases 2) are required for binding to their target sequences<sup>8,9</sup>. Moreover, analysis of the amino acid sequences of several transcription factors (XYR1 (xylanase regulator 1), ACE1 (activator of cellulases 1), ACE2, HAP2/3/5 (HAT associated proteins) and CRE1) have led to identification of phosphorylation sites for cAMP dependent protein kinase, casein kinase II and protein kinase C<sup>10</sup>. Putative XYR1 phosphorylation sites have been also identified *in silico*. Recently, a broad phosphoproteomic study<sup>11</sup> additionally confirmed phosphorylation of the regulators HAP2<sup>12</sup>, ACE1<sup>13</sup>, VEL1 (Velvet 1)<sup>14</sup>, VIB1 (vegetative incompatibility blocked)<sup>15,16</sup>, XPP1 (xylanase promotor binding protein 1)<sup>17</sup>. Accordingly, several of the mutations found in cellulase hypersecreting fungi created by random mutagenesis target genes involved in post-transcriptional processes<sup>18–20</sup>. However, neither the kinases nor the protein phosphatases counteracting the activity of these kinases have been identified so far. The relevance of phosphorylation in carbon source signaling

Austrian Institute of Technology GmbH, Health & Environment, Bioresources, Konrad-Lorenz-Straße 24, 3430, Tulln, Austria. Correspondence and requests for materials should be addressed to M.S. (email: [monika.schmoll@ait.ac.at](mailto:monika.schmoll@ait.ac.at))

is already known in filamentous fungi<sup>11,21</sup> and yeast<sup>22–25</sup>. For example, the transcription factor XlnR (xylanase regulator) in *Aspergillus oryzae*, which is a transcriptional activator in the induction of xylanolytic and cellulolytic enzymes, is regulated by reversible phosphorylation<sup>26</sup>. Cellulase and hemicellulase production in *A. nidulans* is modulated by seven PPs, resulting in reduced endocellulase activity when deleted<sup>27</sup>. Consequently, protein phosphatases crucially impact carbon-source signaling, metabolic processes and enzyme expression in fungi, which warrants detailed investigation.

Light represents an important environmental cue, which has a broad influence on metabolic pathways in fungi<sup>28</sup> and considerably influences regulation of plant cell wall degrading enzymes<sup>29</sup>. The circadian clock of fungi coordinates a daily rhythmicity of gene expression and phosphorylation tunes half-life, subcellular localization, transcriptional activity and conformation of clock components<sup>30,31</sup>. The Frequency (FRQ) protein is negative regulator of the heterodimeric photoreceptor complex formed by white collar-1 and white collar-2 in *N. crassa*. In *T. reesei* and in *N. crassa* the transcription factors forming this White Collar Complex (WCC) or the *T. reesei* equivalent, the Blue light regulator complex (BLRC), regulate cellulase gene expression<sup>32–34</sup>. FRQ is sequentially phosphorylated in the course of a circadian day in order to measure time and establish a circadian rhythm<sup>35,36</sup> and hence represents an interesting example for phosphorylation dependent regulation. Thereby, kinases and phosphatases act in balancing the phosphorylation status of FRQ and WCC. The actions of CK-1a (casein kinase 1a) and CKII (casein kinase II) over the WCC phosphorylation status are counteracted by PP2A and PP4 phosphatases in *N. crassa*. The half-life of FRQ increases as a result of dephosphorylation by PP1 and PP4. Interestingly, although PP2A can be deactivated by phosphorylation, it harbors auto-dephosphorylation activity and it can rapidly reactivate itself<sup>37</sup>. PP2A, besides its role in the maintenance of circadian rhythmicity, is also involved in rapamycin (TOR; target of rapamycin) signaling and may also be regulatorily connected to cAMP-PKA signaling<sup>25,38</sup>. PP1 phosphatases also play a role in a broad range of cellular functions, including glycogen metabolism, cell-cycle progression<sup>39–41</sup>.

Response to white light was shown to be linked to oxidative stress response in *T. reesei* via Cys96 of the photoreceptor ENV1. A mutation of this cysteine ENV1 interferes with the stress response in light<sup>42</sup>. Also in *T. atroviride*, crosstalk of carbon metabolism, light response and oxidative stress response was shown<sup>43,44</sup>.

Generally speaking, phosphorylation acts as the currency of signaling in transduction pathways. The significance of phosphorylation is reflected by the fact that kinases and phosphatases genes of *S. cerevisiae*, which represent only 6% of the genome of yeast, modify 30% of the proteome<sup>45</sup>. For genes encoding the responsible enzymes for (de)phosphorylation, protein kinases and protein phosphatases (PPs) in *T. reesei* a detailed manual annotation is available<sup>46</sup>. Briefly, PPs are classified based on substrate specificity, sequence homology and structural characteristics. This classification results in two major groups, serine/threonine protein phosphatases (PSP) and tyrosine protein phosphatases (PTP)<sup>47</sup>. The PSPs are classified into the protein phosphatase P (PPP) family, which includes multimeric proteins as PP1 (PPZ), PP2A, PP2B and PP5 groups, and the protein phosphatase M (PPM) family, which includes the PP2C, and the third group of aspartate-based phosphatases. The PTP group comprises the specific tyrosine protein phosphatases (PTPs), dual specificity protein phosphatases (DSPs), low-molecular-weight protein tyrosine phosphatases (LMW-PTPs) and the *cdc25* phosphatases (CDC25). The group of low-M<sub>r</sub> protein Tyr phosphatases (LMW-PTPs) belongs to an evolutionarily distinct category, whose members have converged on a similar catalytic mechanism<sup>48,49</sup>. Previously it was assumed that there are no tyrosine kinases in fungi<sup>50</sup>. Nevertheless, recently tyrosine residues were found to be phosphorylated in proteins from *T. reesei*<sup>11</sup>, indicating that besides the annotated protein tyrosine phosphatases also kinases phosphorylating tyrosine residues should be present in fungi. Besides catalytic subunits, there are protein phosphatase regulatory subunits that tune protein phosphatase specificity and function<sup>51</sup>. Furthermore, there are proteins regulating the activity of protein phosphatases, like phosphotyrosyl phosphatase activators (PTPA, also known as PP2A phosphatase activator) and phosphatase inhibitors.

After annotation of genes encoding PPs in the genome of *T. reesei*<sup>46</sup>, we obtained 15 viable phosphatase mutants and studied their phenotype in order to elucidate the function of the corresponding proteins. Phenotype assays revealed functions of PPs in growth, development, osmotic and oxidative stress response and secondary metabolite production. In many cases the effects caused by the deletions occurred in a light dependent manner, showing that protein phosphatases in part have a different relevance for fungal physiology in light than in darkness. Moreover, for some PPs functions in regulation of protease and cellulase production was observed. Additionally, we provide the first characterization of an additional homologue of PPH5 in *T. reesei*, which we designated PPH5-2.

## Results and Discussion

**Regulation patterns of phosphatase genes under different conditions.** To gain an overview on regulation of phosphatase genes at a transcriptional level, we analyzed the transcription patterns of protein phosphatase encoding genes in available transcriptome data. Genes specifically regulated under a certain condition are expected to play a regulatory role under this condition. Additionally, regulation of a certain gene in a mutant strain indicates an importance of this gene for the phenotype and the regulatory alterations in this mutant. Moreover, co-regulated genes are likely to have functions in the same pathway under the given conditions. This correlation can also be explained by the double-lock mechanism in which a major regulator impacts transcription factors as well as their target genes<sup>52</sup>, as for example with regulation of *xyl1* by CRE1<sup>53,54</sup>.

We analyzed protein phosphatase transcript patterns in photoreceptor mutants<sup>55</sup>, in mutants of the heterotrimeric G-protein pathway<sup>56</sup> reflecting nutrient sensing and upon growth on different inducing (cellulose, lactose, sophorose) and repressing/non inducing (glucose, glycerol) carbon sources [56]. The effects of components of the G-protein pathway on cellulase gene expression have already been studied in *T. reesei* and *N. crassa*<sup>56–59</sup>. Besides sensing of pheromones<sup>60–62</sup>, also different nutrients are sensed by fungi via G-protein coupled receptors

(GPCRs)<sup>63</sup>. For *T. reesei* of those nutrients only glucose was shown so far to be sensed by GPCRs<sup>64</sup>. Additionally, we compared expression patterns under conditions of sexual development and growth on cellulose<sup>65,66</sup>.

These analyses showed that depending on the conditions, transcript patterns or individual protein phosphatase genes changed forming clusters specific for the investigated condition (Fig. 1A–D), although quite some overlap in assignment to cluster 1 is obvious (Fig. 1E). The different clusters contained protein phosphatase genes of different groups, indicating functional specialization within every group. Co-regulation of different protein phosphatases suggests that groups of protein phosphatases act on their target pathways in different combinations depending on the environmental conditions as reflected by coregulation in signaling mutants (Fig. 1).

In particular, several genes showed statistically significant regulation by ENV1 (envoy 1), including a gene encoding a phosphatase inhibitor (*ppi1*/TR\_119724; 4.5fold up-regulated in  $\Delta env1$ ). The PTP encoding gene *pty2*/TR\_77689 and a gene encoding a LMW-PTP (*pty4*/TR\_23417) are up-regulated in  $\Delta env1$  as well (>2fold) and four PP genes are down regulated in this strain between 2 and 3fold (*pty10*/TR\_121175, *dsp2*/TR\_119697, *cdc14*/TR\_27406 and *pty8*/TR\_65499). Thereby, *pty8* is also downregulated in strains lacking *blr1* or *blr2* in light and TR\_79635 is down-regulated in  $\Delta blr1$  in light<sup>55</sup>.

Regulation by subunits of the heterotrimeric G-protein pathway occurs for *pty8*/TR\_65499 in light in strains lacking *phlp1*, *gnb1* or *gng1*. Additionally, the *pty9*/TR\_5737 gene encoding a PTP shows decreased transcript levels in light in  $\Delta phlp1$  and *ptpa2*/TR\_120498 under the same conditions in  $\Delta gnb1$ <sup>56</sup>.

Interestingly, no significant regulation of these genes has been detected between growth in light and in darkness in available transcriptome data. Regulation of protein phosphatase encoding genes by either photoreceptors or components of the heterotrimeric G-protein pathway only occurs in light. Consequently, a light dependent relevance of PPs for the physiology of *T. reesei*, that is mediated by photoreceptors and the G-protein pathway is likely. PP genes were not among the genes regulated in an induction specific manner<sup>64</sup>, indicating that their function in metabolic processes or carbon utilization depends on posttranslational regulation including (de) activation. Accordingly, phosphorylation of *dsp2*/TR\_119697, *rga1*/TR\_77135 and *sit4a*/TR\_35316 was confirmed for *T. reesei*<sup>11</sup>.

**Generation of knock-out mutants of protein phosphatases.** Our efforts to delete the genes annotated as protein phosphatases and related genes<sup>46</sup> resulted in viable knock out strains for 12 catalytic PP subunits and 3 regulatory PP subunits. In many cases when no viable mutants could be recovered, their homologs in *N. crassa* were essential as well, since the corresponding transformants in *N. crassa* could only be recovered as heterokaryons (Additional file 1; Supplementary Table S1). The respective genes are consequently assumed to be essential in *T. reesei* as well. For PP genes that are not essential in *N. crassa* transformation for gene deletion was repeated at least three times and it did not result in viable mutants in *T. reesei*.

The obtained strains were analyzed for phenotypes under diverse conditions in order to elucidate their functions and identify common pathways on which more than one PP acts.

**Protein phosphatases are important for normal growth.** For evaluation of the influence of protein phosphatase genes on growth on solid media, we analyzed colony diameters on minimal medium with carboxymethylcellulose (CMC) and on malt extract (MEX) in constant light and constant darkness. Upon growth on CMC, 8 deletion strains showed consistently reduced growth in both light and darkness (Fig. 2A; Additional file 2) and no strain showed a specific light dependent growth defect. Of those,  $\Delta pp2a$ /TR\_56872,  $\Delta sit4a$ /TR\_35316,  $\Delta pph9$ /TR\_58587 and  $\Delta dsp4$ /TR\_64938 showed a growth defect on malt extract as well in light and darkness (Fig. 2A), which indicates that these genes are of general relevance for growth in *T. reesei*. In darkness on malt extract,  $\Delta rgl1$ /TR\_120545 and also  $\Delta pzl1$ /TR\_79535 showed a statistically significant reduction in growth (Fig. 2A). Interestingly,  $\Delta rgl1$ /TR\_123502, and  $\Delta pph8$ /TR\_81164 exhibited reduced growth only on CMC, suggesting that these protein phosphatases are involved in carbon signaling and potentially cellulase regulation.

The mutant of the homolog of PPH8/TR\_81164 in *N. crassa* (NCU4600, PPH-8) also exhibited multiple defects in hyphal growth and asexual sporulation<sup>67</sup>. However, deletion of the homolog in *S. cerevisiae* (PTC2) only resulted in growth defect at high temperatures (37 °C), which is dependent on the HOG pathway<sup>68</sup>.

While deletion of several protein phosphatase genes resulted in decreased growth in light and darkness compared to the wildtype under the same conditions, none of the mutants we investigated showed faster growth than the wildtype under any condition. Moreover, no contrasting phenotypes in light and darkness (increased growth in light and decreased growth in darkness or *vice versa*) were observed.

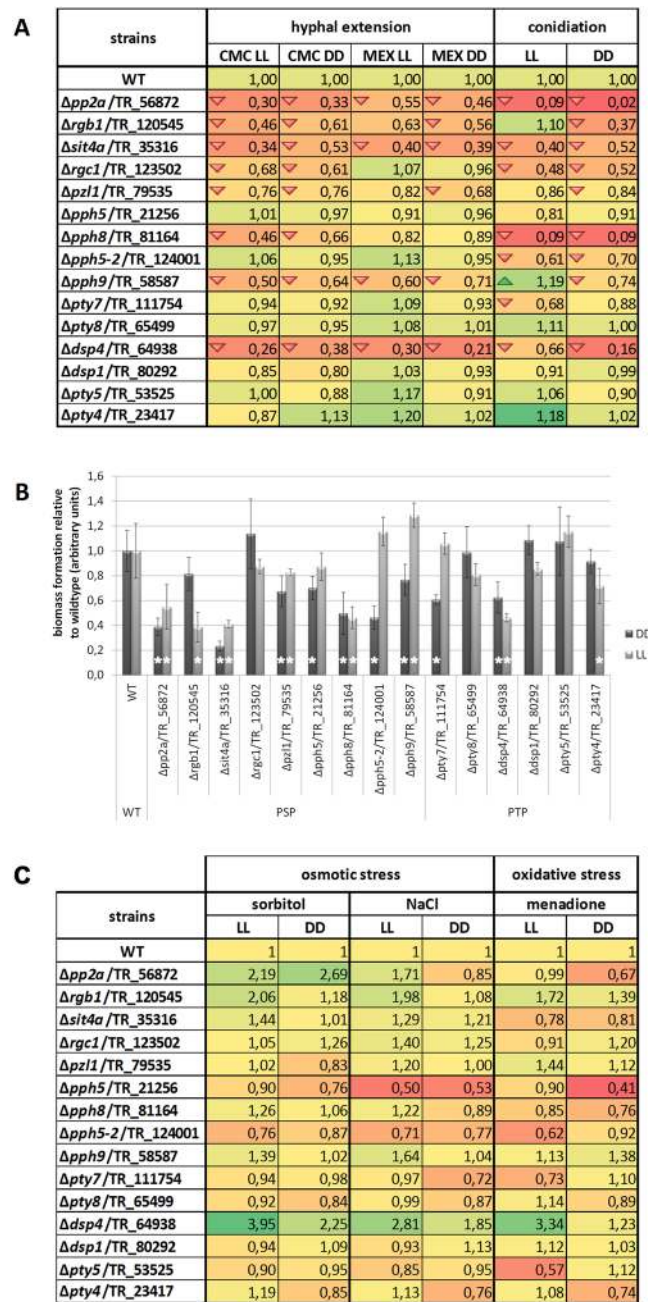
Investigation of biomass formation in shake flask cultures on minimal medium with cellulose as carbon source clearly confirmed the relevance of *pph8* for growth on cellulose, while *rgc1* was not required for normal growth under these conditions. Besides  $\Delta pph8$ /TR\_81164, also  $\Delta pp2a$ /TR\_56872 and  $\Delta sit4a$ /TR\_35316 showed a strong reduction in growth to about half the biomass formation of the wildtype and 8 more protein phosphatases were found to be relevant for growth on cellulose in liquid culture (Fig. 2B). In part the effect was light dependent. (i. e. for deletion of *pph5*, *pph5-2*, *pty7* and *pty4* significant changes were seen either in light or in darkness). We conclude that the role of these genes changes if the physiology of the fungus changes in response to light.

**Role of protein phosphatases in stress response.** Growth and development of microorganism can be affected by several chemicals mimicking environmental stresses. In order to determine whether protein phosphatases are involved in stress response, we performed osmotic and oxidative stress assays with phosphatase mutant strains. To induce osmotic stress we used NaCl and sorbitol which impose ionic stress (NaCl) or, non-ionic stress (sorbitol), respectively<sup>69,70</sup>. Menadione was used as oxidative stress inducing agent.

In general, all protein phosphatase deletion mutants showed statistically significant growth defects under one or more stress conditions in light and/or darkness (Additional file 1; Supplementary Fig. S1). Since several of the deletion strains showed growth defects already without adding stressors, we corrected the growth data (colony







**Figure 2.** Phenotypic characterization of strains lacking non essential protein phosphatases. **(A)** Analysis of colony diameter in constant light (LL) or constant darkness (DD) upon growth on minimal medium with carboxymethylcellulose (CMC) as carbon source or on malt extract. Conidiation/asexual development was analyzed upon growth on malt extract medium in constant light and constant darkness. Values are shown relative to wildtype. Statistically significant alterations of growth in this panel are marked with a triangle. **(B)** Biomass formation upon growth on minimal medium with cellulose as carbon source in constant light or constant darkness for 72 hours. Values with statistically significant difference from wildtype under the same condition are marked with an asterisk. Error bars show standard deviations of three biological replicates. **(C)** Evaluation of responses to osmotic and oxidative stress in constant light and constant darkness. Values were calculated relative to growth of the same strain without the stressor and then the difference was normalized to wildtype. The red background and arrows indicate a decrease and green background and arrows an increase. Details on analysis of statistical significance and p-values are provided in Additional file 2.

diameters) under stress conditions for the growth defects compared to wildtype. We found that particularly *pp2a* and *dsp4* had a negative effect on osmotic stress response because the mutants in these strains showed increased growth in the presence of sorbitol and NaCl, except for  $\Delta pp2a$  in darkness on NaCl where a slightly positive effect on growth was observed (Fig. 2C; Additional file 2). Deletion of *rgb1* or *pph9* resulted in better growth under

conditions of osmotic stress particularly in light (Fig. 2C; Additional file 2) although they have a growth defect without stress (Fig. 2A). Lack of either *pph5* or *pph5-2* causes no significant alteration in growth compared to the wildtype (Fig. 2A), but defects in response to osmotic stress in light and darkness, particularly in the presence of NaCl (Fig. 2C; Additional File 1).

In agreement with our data, the homolog of PP2A in *N. crassa* (NCU06563, PP2A) was suggested to be involved in osmotic stress response. The deletion mutant  $\Delta pp2a/TR\_81164$  showed somewhat altered sensitivity to both osmotic agents both in light and dark conditions. The *S. cerevisiae* homologs of both PP2A/TR\_81164 and PPH8/TR\_81164 are PPG1 and PTC2, respectively. The proteins PPG1 and PTC2 are required for glycogen accumulation and dephosphorylation of Hog1p, respectively<sup>68,71</sup>. The Hog1p homologue of *T. reesei*, TMK3 (*Trichoderma* MAPkinase 3), was shown to be relevant for cellulase regulation<sup>72</sup>.

The role of the homologs in *N. crassa* (NCU06563, PP2A and NCU04600, PPH-8) and *S. cerevisiae* (PPG1 and PTC2) was suggested to be in regulation of OS-2 dephosphorylation and have important roles in the MAPK signalling cascade<sup>67</sup>. More specifically, Ptc2p was also shown to dephosphorylate Cdc28p in *S. cerevisiae*, likewise functioning with proteins such as RAD53 to regulate DNA damage checkpoint pathways<sup>68,73,74</sup>. Therefore, the role of these protein phosphatases in *T. reesei* and *N. crassa* might be similar.

The regulatory subunit RGB1 is homologous to the gene product of NCU09377 in *N. crassa* (RGB-1), which was shown to be responsible for dephosphorylation by PP2A of the WCC, leading to activation of WC-1<sup>67,75</sup>. Ghosh *et al.* (2014) hypothesized that both the catalytic subunit and the regulatory (RGB-1) subunit of PP2A are able to control expression of the phospho-OS-2 MAPK via the WCC, thus providing additional layers of regulation of phospho-OS-2 MAPK expression<sup>67</sup>. Assuming that the same molecular mechanism occurs in *T. reesei*, in the absence of RGB1/TR\_120545, the WCC (BLR-1 and BLR-2) complex would be constantly inactive or at least less active. Therefore, the circadian clock would not function normally, and this is consistent with our results for stress response and conidiation of the mutant  $\Delta rgb1/TR\_120545$ , displaying light dependent functions. Compared to wildtype for osmotic stress, the growth of the mutant is only reduced in darkness. Accordingly, a link between light response via BLR1 and BLR2 and stress response was shown previously in *Trichoderma* spp.<sup>43,44</sup>. More generally, the connection between light response and stress response is supported by findings with the photoreceptor ENV1, which impacts activity of the photoreceptor complex. The evolutionarily conserved structure of ENV1 in *Hypocreales* integrates light response with stress response<sup>42</sup>.

The homolog of PZL1 in *S. cerevisiae*, PPZ1, has been characterized as an important regulator of salt and pH homeostasis<sup>76,77</sup>, suggesting a similar role in *T. reesei*. Deletion of the homolog in *N. crassa* (NCU07489, PZL-1) showed resistance to both sorbitol and sodium chloride, confirming the role of this protein in osmotic stress response<sup>67</sup>. In *T. reesei*, a light dependent effect on sorbitol and NaCl was found for *pzl1*, albeit it is less pronounced as that of the aforementioned protein phosphatases.

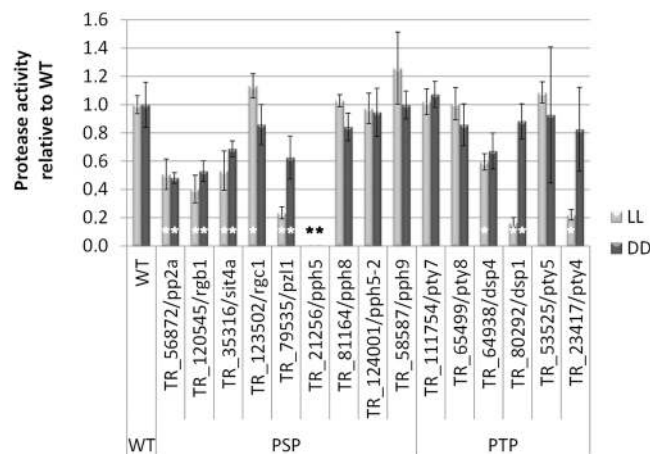
Upon growth under conditions of oxidative stress, we found different responses in constant light and constant darkness in many cases (Fig. 2C). The strongest effect was observed for *dsp4* in light, for which the severe growth defect on CMC alone was almost alleviated. *Rgb1* was found to have a negative effect on tolerance of oxidative stress in light and darkness. *Pph5* showed a growth defect under oxidative stress in darkness, while *pph5-2* showed a defect in light.

In *N. crassa*, deletion of the homologs for PP2A/TR\_56872 (NCU06563, PP2A) and PZL1/TR\_79535 (NCU07489, PZL-1) also showed sensitivity to menadione<sup>67</sup>. The homolog of PZL1/TR\_79535 in *A. fumigatus* (PHZA) is also involved in oxidative stress tolerance<sup>78</sup>. *T. reesei* PP2A/TR\_56872 (and its regulatory subunits) has a positive effect on oxidative stress response in darkness and PZL1/TR\_79535 has a negative effect on growth under oxidative stress conditions, particularly in light (Fig. 2C). Consequently, their function in oxidative stress response is conserved in *T. reesei*.

**Protein phosphatases have positive and negative functions in sexual development.** We examined sexual development in our protein phosphatase mutants and found that all mutants were able to undergo sexual mating with a fully-fertile strain (no defects in male or female fertility). Hence, the protein phosphatases we tested are not essential for sexual development. Five protein phosphatase mutants showed a delay in fruiting body formation compared to the wildtype ( $\Delta pp2a/TR\_56872$ ,  $\Delta dsp4/TR\_64938$ ,  $\Delta pzl1/TR\_79535$ ,  $\Delta pph8/TR\_81164$  and  $\Delta sit4a/TR\_35316$ ), while two showed an earlier time-point for fruiting body formation ( $\Delta pty4/TR\_23417$  and  $\Delta pph5-2/TR\_124001$ ). Of five mutants showing delayed fruiting body formation, three displayed defects in growth ( $\Delta pp2a/TR\_56872$ ,  $\Delta dsp4/TR\_64938$  and  $\Delta sit4a/TR\_35316$ ), which implies a somewhat longer time to encounter the mating partner and, therefore, undergo sexual development. Ascospores were discharged for all crosses with mutant strains, although five strains showed a delay in ascospore discharge ( $\Delta rgb1/TR\_120545$ ,  $\Delta pp2a/TR\_56872$ ,  $\Delta dsp4/TR\_64938$ ,  $\Delta pzl1/TR\_79535$ ,  $\Delta pph5/TR\_21256$  and  $\Delta sit4a/TR\_35316$ ).

The homolog of PP2A/TR\_56872 in *Sordaria macrospora* (SmPP2Ac) was shown to be involved in regulating cell-cell fusion and sexual development as an integral component of the STRIPAK complex<sup>67,79</sup>. Deletion of the homologous gene in *N. crassa* (NCU06563, *pp2a*) resulted in female-sterility<sup>67</sup>. However, since our mutants are in the background strain QM6a, which is female-sterile<sup>80</sup>, we cannot evaluate how the deletion of *pp2a* affects female-fertility in *T. reesei*. Absence of the homolog of DSP4/TR\_64938 in *N. crassa* (NCU08158, DSP-4) also exhibited abnormalities during sexual development. Deletion of the homolog of PZL1/TR\_79535 in *N. crassa* (NCU07489, PZL-1) only showed an abnormal phenotype during protoperithecia formation (Ghosh *et al.*, 2014). Interestingly, deletion of the homolog of PPH8/TR\_81164 in *N. crassa* (NCU04600, PPH-8) resulted in unregulated protoperithecia development. PPH-8 was suggested to affect nitrogen sensing and the sexual development pathway. It was hypothesized that PPH-8 regulates protoperithecial development via modulation of the OS-2 MAPK pathway<sup>67</sup>. Concerning the mutant strains that showed an earlier fruiting body formation, the deletion of the homolog of PTY4/TR\_23417 in *N. crassa* (NCU09841, PTY-4) exhibited decreased production of protoperithecia. This suggests that PTY-4 may regulate sexual development via OS-2 phosphorylation in *N. crassa*<sup>67</sup>. In





**Figure 3.** Analysis of protease production. Strains were grown in constant light (LL) or constant darkness (DD). Genes deleted in the respective strains are shown on the x-axis. Asterisks indicate statistically significant different protease production compared to wildtype under the same conditions. Strains were grown on TSA media amended with 10% (w/v) powdered milk at 28 °C after 2 days. Calculations are based on the ratio halo/mycelium and normalized to wildtype in light and darkness correspondingly. Errorbars show standard deviations of three biological replicates. Details on analysis of statistical significance and p-values are provided in Additional file 2.

*T. reesei*, the absence of PPH5-2/TR\_124001 resulted in faster formation of fruiting bodies after crossing. The gene encoding PPH5-2/TR\_124001 might be the result of a duplication event in the genome, arising from the gene *pph5*/TR\_21256 in *T. reesei*, due to the fact that direct homologs for PPH5-2/TR\_124001 are not found in other fungi. The mutant  $\Delta pph5$ /TR\_21256 showed a delay in ascospore discharge, which could suggest a necessary cooperation of both proteins (PPH5 and PPH5-2) for a normal sexual development. Also a subfunctionalization of the two paralogs cannot be excluded. However, in *N. crassa* the mutant of the homolog of PPH5/TR\_21256 (NCU01767, PPH-5) did not display an abnormal phenotype in sexual development<sup>67</sup>. Finally, the mutant strains of two regulatory subunits ( $\Delta rgb1$ /TR\_120545 and  $\Delta sit4a$ /TR\_35316) of PP2A proteins showed altered phenotypes during sexual development (delayed discharge of ascospores for both mutants and delayed fruiting body formation for the second). Considering that the catalytic subunits of PP2A are involved in sexual development, the defect observed in the mutants of regulatory subunits is consistent.

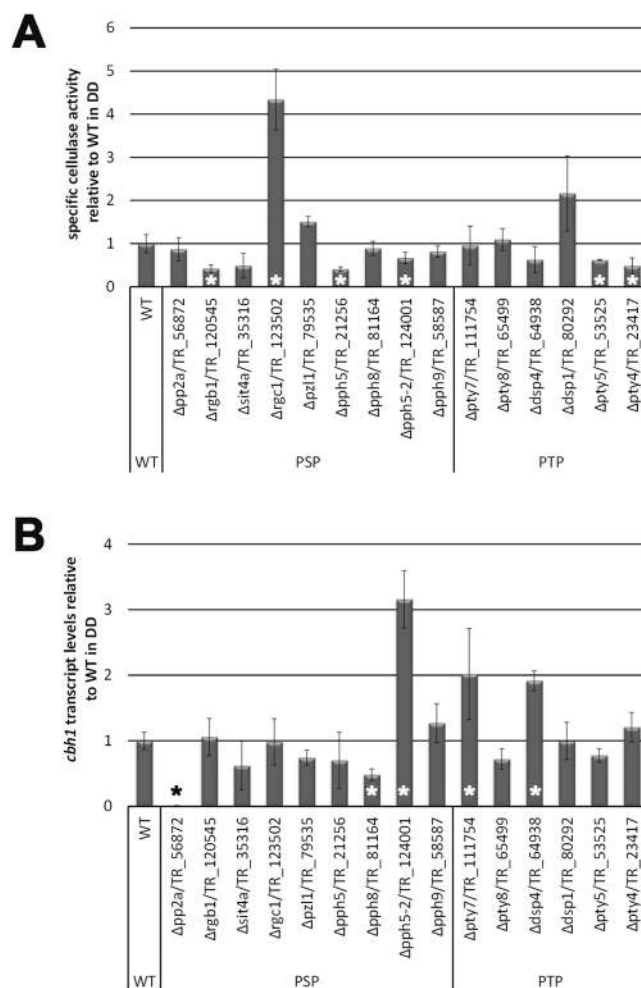
**Impacts on conidiation by protein phosphatases.** Assessment of asexual development showed that in the deletion strains  $\Delta pp2a$ /TR\_56872 and  $\Delta pph8$ /TR\_81164 conidiation was almost abolished. An increase in sporulation was only observed in  $\Delta pph9$ /TR\_58587 in light (Fig. 2A; Additional file 2). Interestingly,  $\Delta pph5$ /TR\_124001 showed decreased sporulation in light and darkness. This strain shows earlier fruiting body formation, but a delay in ascospore discharge. Therefore a potential shift in preference between asexual and sexual development by PPH5-2 warrants further investigations.

The mutant of the homolog to PPH8/TR\_81164 in *N. crassa* (NCU04600, PPH-8) also showed defects in conidiation<sup>67</sup>. Similarly, a mutant of the homologs to PP2A/TR\_56872 (NCU06563, PP2A) also showed a defect during asexual development, which is in line with our findings. Accordingly, also the PP2A regulatory subunit mutants ( $\Delta rgb1$ /TR\_120545 and  $\Delta sit4a$ /TR\_35316) show conidiation defects. However, deletion of the homolog to DSP4/TR\_64938, which affects conidiation, did not cause any adverse effects on conidiation in *N. crassa* but deletion of the homologs in *S. cerevisiae* (YVH1), and *M. oryzae* (MoYvh1) resulted in reduced growth and sporulation (yeast) and lower conidia production (*M. oryzae*)<sup>81</sup>.

There are also PPs involved in asexual development in *N. crassa* that show no significant effect in *T. reesei* (PPH5/TR\_21256-NCU01767 (PPH-5), DSP1/TR\_80292-NCU03426 (DSP-1) and PTY5/TR\_53525-NCU01010 (PTY-5)) revealing a different function of those PPs in both fungi<sup>67</sup>. Importantly, three mutant strains ( $\Delta rgb1$ /TR\_120545,  $\Delta dsp4$ /TR\_64938 and  $\Delta pph9$ /TR\_58587) exhibited reduced conidiation in constant darkness but not in constant light compared to the wildtype (Fig. 2A). These genes are regulated during different stages of conidiation<sup>82</sup>. Thus, *rgb1*, *dsp4* and *pph9* are not generally involved in regulation of conidiation. Instead their importance for conidiation depends on whether the fungus grows in light or in darkness.

**Pty4, Pzl1 and Dsp1 regulate protease production in a light dependent manner.** Fungal proteases are a major bottleneck in industry for efficient production of proteins of interest<sup>83,84</sup>. Although in microbial production systems the genes encoding proteases could be deleted<sup>85</sup>, the large number of genes encoding proteases in fungi makes it impossible to remove them all. A strain harboring the deletions of seven proteases was reported to support stable expression levels of therapeutic proteins<sup>85</sup>. Nevertheless, there is limited knowledge about the molecular regulation by which proteases are produced.

We investigated the proteolytic activity secreted by our deletion strains under constant light or dark conditions (Fig. 3).  $\Delta pp2a$ /TR\_56872,  $\Delta rgb1$ /TR\_120545,  $\Delta sit4a$ /TR\_35316,  $\Delta pzl1$ /TR\_79535,  $\Delta pph5$ /TR\_21256



**Figure 4.** Analysis of the influence of PPs on cellulase regulation. **(A)** Specific cellulase activity, i. e. cellulase activity in the culture filtrate relative to produced biomass. Strains were grown on Mandels Andreotti minimal medium with cellulose as carbon source in constant darkness. Errorbars show standard deviations of three biological replicates. Details on analysis of statistical significance and p-values for specific cellulase activity are provided in supplementary file 2. **(B)** Transcript abundance for the major cellulase gene *cbh1* analyzed in strains grown as described for **(A)**. For statistical significance, a p-value threshold of 0.05 was applied and three biological and three technical replicates were considered.

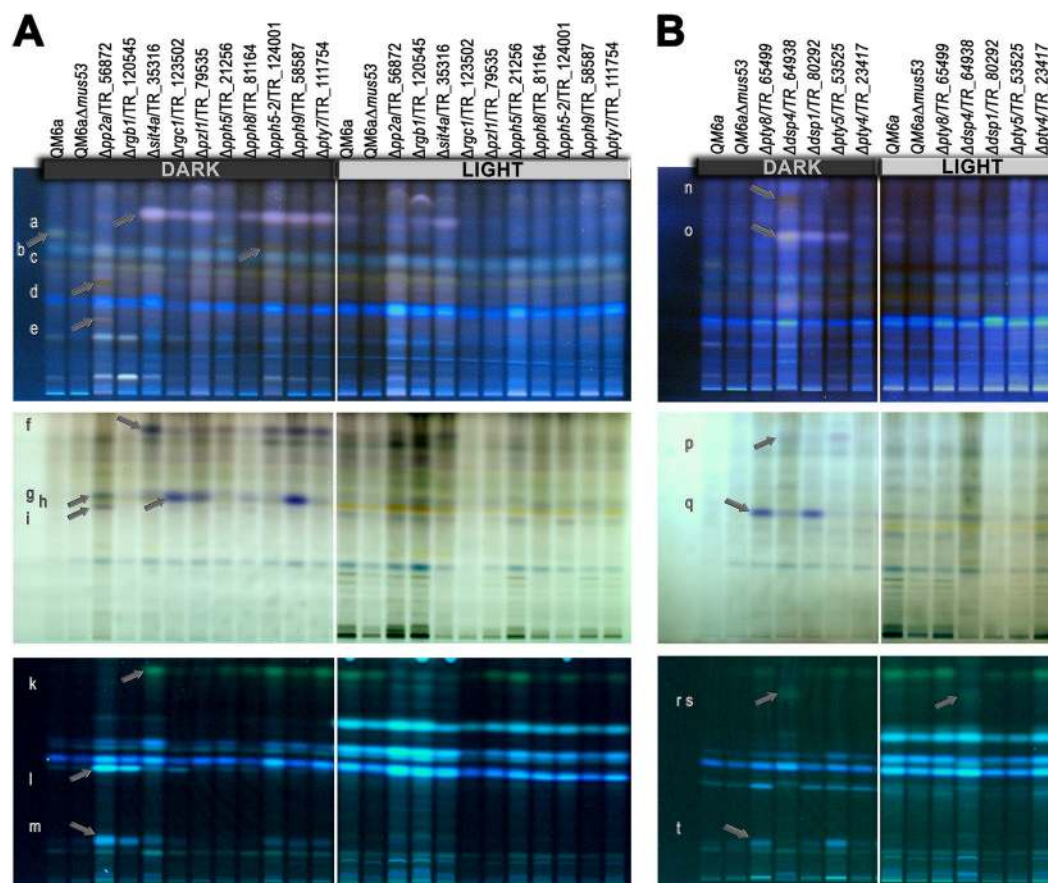
and  $\Delta dsp4/TR\_64938$  showed decreased protease activity in light and darkness.  $\Delta pty4/TR\_23417$  and  $\Delta dsp1/TR\_80292$  showed strongly decreased protease activity specifically in light.

Three mutant strains ( $\Delta pty4/TR\_23417$ ,  $\Delta pzl1/TR\_79535$  and  $\Delta dsp1/TR\_80292$ ) showed a more pronounced decrease of proteases under constant light compared to darkness. Although there is a difference in protease production in the wildtype between light and darkness (slightly decreased in darkness), the observed result for the deletion strains exceeds these differences and is therefore specific for the deleted genes.

**A relevance for protein phosphatases in cellulase regulation.** We studied the specific cellulase activity upon growth with cellulose as carbon source in constant darkness reflecting conditions in industrial fermenters. We found clearly increased specific cellulase activity compared to wildtype for  $\Delta rgc1/TR\_123502$  and a positive trend for  $\Delta dsp1/TR\_80292$  (Fig. 4A).  $\Delta rgc1/TR\_123502$  exhibited a defect in hyphal growth (as reflected in achieved colony diameter) in light and darkness only when growing on cellulose, but not on malt extract (Fig. 2), indicating more efficient cellulase production at the expense of biomass formation. Therefore, RGC1 might be involved in negative regulation on cellulase production. In contrast,  $\Delta rgb1/TR\_120545$ ,  $\Delta pp5/TR\_21256$ ,  $\Delta pp5-2/TR\_124001$ ,  $\Delta pty4/TR\_23417$  and  $\Delta pty5/TR\_53525$  showed decreased specific cellulase activity and in  $\Delta sit4a/TR\_35316$  we found a negative trend (Fig. 4A). This reduction suggests that these PPs positively regulate production of plant cell wall degrading enzymes.

Analysis of transcript levels of the major cellulase gene *cbh1* showed that the increased specific cellulase activity of  $\Delta rgc1/TR\_123502$  and the positive trend in  $\Delta dsp1/TR\_80292$  (Fig. 4B) do not correspond with an increased *cbh1* transcription level or altered biomass formation on cellulose (Fig. 2B). Hence, posttranscriptional regulation as shown recently<sup>64</sup> potentially contributes to this regulation. Alternatively, other enzymes regulated





**Figure 5.** Analysis of secreted secondary metabolite patterns. (A) Strains lacking PSPs or (B) strains lacking PTPs were grown on minimal medium with cellulose as carbon source in constant light or constant darkness. Amounts of supernatants applied for analysis were normalized to equal amounts of biomass produced by the respective strain. Letters at the edge of the figure refer to an arrow indicated an altered band to the right of the letter. Visualizations shown are derivatized/remission at 366 nm (upper panel), derivatized/transmission (middle panel) and developed, 366 nm (lower panel).

by these PPs could be responsible for this effect. *cbh1* transcript levels were decreased compared to wildtype in the deletion strains  $\Delta pp2a/TR_{56872}$  and  $\Delta pp8/TR_{81164}$ . In  $\Delta pp2a/TR_{56872}$  biomass production on cellulose was reduced (Fig. 2B), which is in agreement with lower *cbh1* transcript levels. However, the residual growth and activity indicate that cellulase degradation was not totally abolished.  $\Delta dsp4/TR_{64938}$ ,  $\Delta pty7/TR_{111754}$  and  $\Delta pp5-2/TR_{124001}$  showed higher transcript levels of *cbh1* compared to the wildtype, although this result does again not correlate with the specific cellulase activity levels. Interestingly, *dsp4/TR\_{64938}* is located within a genomic cluster of genes regulated in an induction specific manner (cluster 20<sup>64</sup>). We conclude that protein phosphatases play important roles in the posttranscriptional section of cellulase regulation, which was shown recently<sup>64</sup> and/or coordinated regulation of CAZymes for efficient cellulose degradation. This regulation likely involves (de) activation and modulation of protein stability of regulatory proteins, which is often dependent on the phosphorylation status.

**Protein phosphatases impact secondary metabolism.** *Trichoderma* spp. produce a great diversity of secondary metabolites<sup>86</sup>. In *T. reesei*, recent studies indicate a regulatory interconnection between carbon and secondary metabolism with the carbon catabolite repressor CRE1<sup>87</sup> and the transcription factor XPP1<sup>88</sup> as most important dual regulators. To study if protein phosphatases are involved in this regulation, secretion patterns of secondary metabolites in the deletion strains were analyzed. For that purpose, semi-quantitative high performance thin layer chromatography (HPTLC) was applied.

Altered secondary metabolite patterns were observed for several deletion strains (Fig. 5). Over all, we observed striking differences in secondary metabolite patterns between light and darkness. The differences between strains were more pronounced in darkness than in light.  $\Delta pp2a/TR_{56872}$  as well as the deletion strain for the putative regulatory subunit for PP2A,  $\Delta rgb1/TR_{120545}$  showed an increase in secondary metabolite production in light and darkness. These two strains also showed additional bands compared to the wildtype (Fig. 5A bands l and m), some of which were only visible in  $\Delta pp2a$  (Fig. 5A, bands d, e, g and i), but not  $\Delta rgb1$ . Hence, the secondary metabolite patterns of the two strains are not entirely similar on the different visualizations supporting that RGB1 acts as a regulatory subunit to PP2a, but has other functions as well. In the remaining strains except for  $\Delta pp5/$

TR\_21256, an additional band appeared (Fig. 5A band a).  $\Delta ppb5$ -2/TR\_124001 did produce this additional band and moreover an additional band that is not present in the other strains (Fig. 5A band c), reflecting functional differences between PPH5 and PPH5-2.

Bands f and k, which also appeared in these strains, were hardly detectable in  $\Delta rgc1$ /TR\_123502, but are present in  $\Delta ppb5$ /TR\_21256 (Fig. 5A). A band present in the wildtype samples was not detected in most mutant samples except for a weak appearance in  $\Delta ppb5$ /TR\_21256 (Fig. 5A band b). Band h is enhanced in  $\Delta rgc1$ /TR\_123502,  $\Delta pzl1$ /TR\_79535 and  $\Delta ppb9$ /TR\_58587 (Fig. 5A). The lower signal strength for  $\Delta rgc1$ /TR\_123502 in light indicates less secondary metabolites to be produced (Fig. 5A). Interestingly, the homologue of *pzl1* in *A. fumigatus* (*ppzA*) is involved in regulation of secondary metabolism as well, particularly in connection to iron starvation and siderophore production, with deletion of *ppzA* leading to loss of pathogenicity of *A. fumigatus*<sup>89</sup>.

$\Delta dsp4$ /TR\_64938 showed increased amounts of secreted secondary metabolites with additional bands (Fig. 5B bands n, o, p, r and s) not only in darkness but also in light. Band o is also additionally present in  $\Delta dsp1$ /TR\_80292 and  $\Delta pty5$ /TR\_53525 (Fig. 5B).  $\Delta pty8$ /TR\_65499 and  $\Delta pty5$ /TR\_53525 showed a further enhanced band in darkness compared to wildtype (Fig. 5B, band t).

In summary, our screening for secondary metabolite patterns showed a clear influence of many protein phosphatases on secondary metabolite production. In some cases individual bands (which could represent more than one compound) are concerned, but we also found an overall effect on the amount of secondary metabolites produced for some strains. The effects on individual compounds were mostly limited to darkness. Consequently, protein phosphatases contribute to the physiological effects of light on *T. reesei* as compared to growth in darkness, which are mediated by signaling pathways also by regulation of secondary metabolism.

**Functional clustering of protein phosphatases.** Based on the phenotypic traits we investigated, we performed hierarchical clustering of effects related to wildtype in our deletion strains. This analysis on the one hand provided us with information on common functions of individual protein phosphatases. On the other hand the clustering revealed phenotypic traits affected in the same way by several deletions, which indicates that the underlying regulatory pathways are connected. Assessing all traits, we found clustering of growth and osmotic stress together, but separate from development and oxidative stress (Fig. 6A). The results described above showed phenotypic similarities for some strains, but alterations of such similarities depended on the conditions, indicating the individual functions in the regulatory network of phosphorylation for all investigated genes.

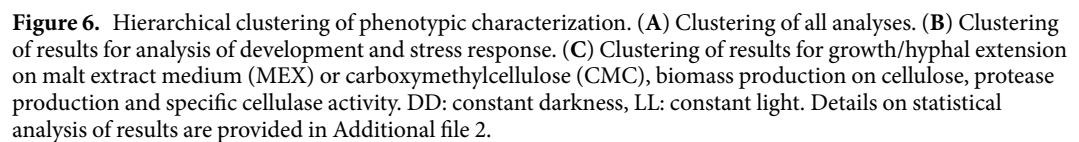
Separate clustering of phenotypes in stress response and development (Fig. 6B) as well as of growth, enzyme production and protease secretion (Fig. 6C) showed that deletion of *pp2a* or its putative regulatory subunit *rgb1* causes phenotypes that are too dissimilar to cluster together.  $\Delta ppb5$ /TR\_21256 and  $\Delta ppb5$ -2/TR\_124001 did not appear in a common cluster, which supports the hypothesis that *ppb5*-2 assumed complementary functions to *ppb5* and that a subfunctionalization of these paralogs has happened. Moreover, we could not detect sets of protein phosphatases acting similarly across conditions (Fig. 6A–C). Although this was unexpected, it highlights the complex regulation of phosphorylation states by protein phosphatases and their regulatory subunits.

With the aim to put the functions of the non essential phosphatases in context, we investigated whether the genomic locations of the encoding genes are within or close to known functional clusters. We found no PP encoding genes within CAZyme clusters<sup>64,90</sup> or in light regulated clusters on cellulose<sup>55,64</sup>. However, *dsp4*/TR\_64938 is located within cluster 20 of induction associated genes (supplemental dataset 4 of reference<sup>56</sup>), which also comprises *egl5/cel45a* encoding endoglucanase<sup>56</sup>. *dsp4* shows a significant decrease in biomass formation upon growth on cellulose in light and darkness, which suggests a role in support of normal growth on cellulose.

## Conclusions

Protein phosphatases constitute, together with protein kinases, key players as the currency of signaling in the cell. The set of deletion strains analyzed in this article showed that only some of them displayed defects in growth and development compared to wildtype. In contrast, most of the strains showed alterations in stress response. With respect to enzyme production, several strains showed decreased specific cellulase activity and protease production, albeit no correlation of increased cellulase production and decreased protease activity or *vice versa* was found. In many cases the defect caused by deletion of individual PPs concerned more than one functional pathway (Fig. 6D,E). Considering the interconnection between cellulase production and secondary metabolism that was postulated recently<sup>87,88</sup>, we found several strains with alterations in both pathways. For example, deletion of *rgc1* causes an increase in specific cellulase activity (Fig. 4A) as well as appearance of additional secondary metabolite bands (Fig. 5A, bands a and g). Band c (Fig. 5A) appears in  $\Delta ppb5$ -2/TR\_124001, which shows decreased cellulase activity. In case of *pp2a* we saw several additional bands (Fig. 5A bands d, e, g, i, l and m) and a decrease in *cbh1* transcript levels, while cellulase activity appears to be complemented by other cellulolytic enzymes. Consequently, the connection between secondary metabolism and enzyme formation turns out to be a broad mechanism not limited to one or a few transcription factors or to a cluster. Rather, this connection appears as a general phenomenon, where detection of a regulatory impact on cellulases has to be expected to include also an influence on secondary metabolism.

Our results confirm a central role of PP2A catalytic and regulatory subunits for primary and secondary metabolism. The homologue of PP2A/TR\_56872 in *N. crassa* (PP2A/NCU06563) also showed several defects in growth, development and stress response<sup>67</sup>. Moreover, in *N. crassa* PP2A is involved in the OS-2 pathway, confirming the involvement in stress response<sup>67</sup>. A homologue of the putative regulatory subunit of PP2A, RGB1/TR\_120545 in *S. cerevisiae* Cdc55 is involved in Rho signaling, that regulates cell growth and stress response<sup>91</sup>. The homologue in *N. crassa* (RGB-1/NCU09377) is responsible for dephosphorylation of WCC<sup>75</sup> and the WCC controls phospho-OS-2<sup>92</sup>. In *T. reesei*, the mutant  $\Delta rgb1$ /TR\_120545 shows different phenotypes depending on the light conditions, which is in agreement with a role in light dependent physiology. It remains to be determined



Analysis of specific cellulase activity showed that PPs influence the production of cellulases. Involvement of protein phosphatases in cellulase production was previously studied in *A. nidulans*. In this respect, homologs



of PP2A/TR\_56872 (Ppf1A/AN0164), PZL1/TR\_79535 (PpzA/AN3793), PPH9/TR\_58587 (PtcA/AN6892) and PTY4/TR\_23417 (LtpA/AN10570), defined as non-essential protein phosphatases, were described to be involved in cellulase production in *A. nidulans*<sup>27</sup>. In *T. reesei* deletions of these protein phosphatases caused decreased growth on cellulose in  $\Delta pp2a$ /TR\_56872,  $\Delta pty4$ /TR\_23417 and  $\Delta pzl1$ /TR\_79535. Therefore, the role of those protein phosphatases is consistent between *A. nidulans* and *T. reesei* with the exception of *pph9*. Besides their role in endocellulase production, the function of these proteins was inferred via homology to *S. cerevisiae* if not characterized already in *Aspergilli*<sup>27</sup>. Among them, one has a role in the cell cycle, which is homologous to a low-molecular-weight protein tyrosine phosphatase (LtpA/AN10570, homologue: PTY4/TR\_23417). Furthermore, a homolog to one PP has a role in MAPK regulation (PtcA/AN6892, PPH9/TR\_58587)<sup>93</sup>. A PP2A homologue in *S. cerevisiae*, Ppg1p (homologous to Ppg1A/AN0164 in *A. nidulans* and PP2A/TR\_56872 in *T. reesei*), is involved in glycogen accumulation<sup>27</sup>. Tap42 (TAP42/YMR028W) and Sit4 (SIT4/YDL047W) homologous to the PSP TNG/TR\_48910 are associated with Ppg1p in *S. cerevisiae*, being involved in TOR signaling<sup>27</sup>.

$\Delta dsp1$ /TR\_80292 and  $\Delta rgc1$ /TR\_123502 showed an increased specific cellulase activity compared to wild-type.  $\Delta rgc1$ /TR\_123502 showed a growth defect in liquid culture on cellulose. However, decreased growth was also detected on plates with carboxymethylcellulose and malt extract, indicating that the effect on growth is general and is not caused by the altered cellulase production. Interestingly, RGC1/TR\_123502 was recently shown to be significantly phosphorylated upon induction with sophorose<sup>11</sup>, and this protein harbors a starch/glycogen-binding module. Taken together, these results suggest that the regulatory subunit RGC1/TR\_123502 is required for normal growth and negatively regulates cellulase production in *T. reesei*.

Bidirectional best hit analysis and phylogenetic analysis indicates that PPH5-2/TR\_124001 arose from a duplication event of *pph5*/TR\_21256. PPH5-2/TR\_124001 is only found in *Trichoderma* spp.<sup>46</sup>. In the genome, *pph5* and *pph5-2* are both located on chromosome 5, but not close to each other<sup>94</sup>. Transcriptome data showed that transcript levels of *pph5-2*/TR\_124001 are decreased upon growth on cellulose compared to glucose, glycerol or lactose while *pph5*/TR\_21256 shows an opposite trend in regulation<sup>64</sup>. Hence, both genes displayed opposite transcription patterns.

Our analyses clearly showed different phenotypic patterns for  $\Delta pph5$ /TR\_21256 and  $\Delta pph5-2$ /TR\_124001 in many cases. However, the homolog in *N. crassa* (NCU01767, PPH-5) is required for normal growth of basal hyphae and asexual development<sup>67</sup>, which is not the case for the *T. reesei* homologues. Only  $\Delta pph5-2$ /TR\_124001 shows a significant decrease in conidiation. The *S. cerevisiae* homologue PTC5 is also required for normal vegetative growth<sup>95</sup>. Hence, PPH5 and PPH5-2 assume distinct functions in *T. reesei*, which are not entirely conserved in *N. crassa* and *S. cerevisiae*.

In summary, we demonstrated a broad diversity of functions of protein phosphatases in *T. reesei*, organized in a complex network of target pathways, which is in many cases specific for a given light condition and a carbon source.

## Methods

**Microbial strains and culture conditions.** *Trichoderma reesei* (syn. *Hypocrea jecorina*) strain QM6a (ATCC13631) and the non-homologous end joining (NHEJ) deficient strain QM6a $\Delta tmus53$ <sup>96</sup> were used throughout this study. For genotypes of the strains used and prepared for this study see Table 1. All analyses done in constant darkness including harvesting mycelia were done with red safety light (darkroom lamp, Dr. Fischer 230 V, red, E27, 15 W).

**Generation of deletion strains.** The target phosphatase genes for deletion in *T. reesei* were annotated previously<sup>46</sup>. For deletion vector construction of protein phosphatase genes in *T. reesei*, the method of yeast mediated recombination cloning into the plasmid pRS426 with the hygromycin resistance cassette was used as described previously<sup>97,98</sup>. Deletion cassettes (around 3500 bp) were amplified by PCR using pre-designed primers<sup>98</sup> and transformed to QM6a $\Delta tmus53$  strain by the polyethylene glycol-mediated protoplast transformation method<sup>99,100</sup>. Selection of colonies was performed on 3% (w/v) malt extract agar plates containing 100  $\mu$ g ml<sup>-1</sup> hygromycin B (Roth, Karlsruhe, Germany). After two rounds of single spore isolation, stable transformants were obtained. Deletion strains were confirmed by PCR using primers binding within the deleted region as described previously using the screening primers specified in<sup>98</sup>. Only if no amplicon was obtained with the screening primers binding within the deleted region, strains were considered for further analysis. The copy number of the deletion cassettes integrated in the deletion strains was analyzed using RT-qPCR as described previously<sup>56</sup>. For subsequent analyses, up to three different transformants (if available) were analyzed per deletion to exclude random effects due to the transformation process. Additionally, copy numbers of the deletion cassettes were analyzed to assess the potential of off target effects. Results for strains bearing more than one copy (Additional file 1; Supplementary Table S2) of the deletion cassette were only considered if consistent with the other strains.

**Bioinformatic analysis.** Transcriptome data used in this study were obtained and analyzed previously<sup>55,56,64,66</sup> and are deposited at NCBI Gene Expression Omnibus with accession numbers GSE36448, GSM683732, 683733, 683734 and 683735. Hierarchical clustering was done using the open source software HCE 3.5<sup>101</sup> with default settings using the Pearson correlation algorithm.

Statistical significance of differences to the wildtype was evaluated using the open source software SPSP 1.0.1 (version August 2017). Statistical data for the phenotypic assays shown in Figs 2, 3 and 4 are provided in Additional file 2.

**Analysis of growth.** Deletion strains were grown on plates with 3% (w/v) malt extract (Merck, Darmstadt, Germany) or Mandels-Andreotti medium<sup>102</sup> containing 1% (w/v) carboxymethylcellulose (CMC) as carbon source, for 2–4 days at 28 °C in constant light ( $\approx$ 1800 Lx) or constant darkness. The growth in constant darkness



Strain	Characteristics	Source/Reference
QM6a	Wildtype (ATCC13631)	<sup>90</sup>
QM6a $\Delta$ tmus53		<sup>96</sup>
FF1	Female fertile derivative of QM6a Mat 1-1	<sup>104</sup>
$\Delta$ pp2a	QM6a $\Delta$ tmus53 $\Delta$ 56872::hph <sup>+</sup>	This study
$\Delta$ rgb1	QM6a $\Delta$ tmus53 $\Delta$ 120545::hph <sup>+</sup>	This study
$\Delta$ sit4a	QM6a $\Delta$ tmus53 $\Delta$ 35316::hph <sup>+</sup>	This study
$\Delta$ rgc1	QM6a $\Delta$ tmus53 $\Delta$ 123502::hph <sup>+</sup>	This study
$\Delta$ pzl1	QM6a $\Delta$ tmus53 $\Delta$ 79535::hph <sup>+</sup>	This study
$\Delta$ pph5	QM6a $\Delta$ tmus53 $\Delta$ 21256::hph <sup>+</sup>	This study
$\Delta$ pph8	QM6a $\Delta$ tmus53 $\Delta$ 81164::hph <sup>+</sup>	This study
$\Delta$ pph5-2	QM6a $\Delta$ tmus53 $\Delta$ 124001::hph <sup>+</sup>	This study
$\Delta$ pph9	QM6a $\Delta$ tmus53 $\Delta$ 58587::hph <sup>+</sup>	This study
$\Delta$ pty7	QM6a $\Delta$ tmus53 $\Delta$ 111754::hph <sup>+</sup>	This study
$\Delta$ pty8	QM6a $\Delta$ tmus53 $\Delta$ 65499::hph <sup>+</sup>	This study
$\Delta$ dsp4	QM6a $\Delta$ tmus53 $\Delta$ 64938::hph <sup>+</sup>	This study
$\Delta$ dsp1	QM6a $\Delta$ tmus53 $\Delta$ 80292::hph <sup>+</sup>	This study
$\Delta$ pty5	QM6a $\Delta$ tmus53 $\Delta$ 53525::hph <sup>+</sup>	This study
$\Delta$ pty4	QM6a $\Delta$ tmus53 $\Delta$ 23417::hph <sup>+</sup>	This study

**Table 1.** Strains used in this study.

was analyzed after two days by measuring the colony diameter. The mutant strains were inoculated in the center of the plate and colony diameter reflecting hyphal extension was measured every 24 hours for two to four days. QM6a $\Delta$ tmus53 was used as control for every set. At least three biological replicates were analyzed for every strain.

**Evaluation of development.** For evaluation of sexual development, crossing experiments were prepared on 3% (w/v) malt extract at 22 °C under daylight conditions (cycles of 12 h light-12 h dark), which is the preferred condition for sexual development to happen<sup>80,103</sup>. Briefly, strains were inoculated on opposite sides of petri dishes and the female fertile strain FF1<sup>66,104</sup> was used as crossing partner. Fruiting body formation, size and quantity of fruiting bodies, as well as the ability for ascospore discharge and corresponding quantity were considered. QM6a $\Delta$ tmus53 was used as control.

For assessment of asexual development, deletion strains were grown on 3% (w/v) malt extract for eight days at 28 °C either in constant light ( $\approx$ 1800 Lx) or constant darkness. Three plugs per plate from three independent plates were cut and spores were suspended in 4 ml of spore solution (0.8% (w/v) NaCl with 0.05% (v/v) Tween 80 (Merck, Darmstadt, Germany)). The amount of spores was determined by measurement of the optical density with a spectrophotometer (OD at 600 nm, spectrophotometer from Hitachi, model U-2900) using a standard curve based on microscopic counting. QM6a $\Delta$ tmus53 was used as control for every set. At least three biological replicates were analyzed per strain.

**Osmotic and oxidative stress assays.** For assessment of oxidative stress response, strains were cultivated on Mandels-Andreotti minimal medium with carboxymethylcellulose (CMC) (Sigma Aldrich, St. Louis, USA) (1% w/v) as carbon source, supplemented with 0.25 mM menadione (Sigma, St. Louis, USA). To analyze osmotic stress response, mutant strains were grown on solid Mandels-Andreotti minimal medium containing 1% (w/v) carboxymethylcellulose (CMC) as carbon source, supplemented with 1 M NaCl (Merck, Darmstadt, Germany) or 1 M D-Sorbitol (Sigma Aldrich, St. Louis, USA).

Growth was monitored for three days either in constant light ( $\approx$ 1800 Lx) or constant darkness. Mutant strains were inoculated in the center of the plate and colony diameters were measured every 24 hours for three days at 28 °C. QM6a $\Delta$ tmus53 was used as control for every set. At least three biological replicates were analyzed per strain.

**Analysis of protease activity.** Protease secretion was determined after growing the strains on 1/5 TSA (15 g/L Tryptone (Roth, Karlsruhe, Germany), 5 g/L Soytone (Merck, Darmstadt, Germany), 5 g/L NaCl, 15 g/L Agar-Agar (Roth, Karlsruhe, Germany)) amended with 10% (w/v) powdered milk (Roth, Karlsruhe, Germany) at 28 °C in constant darkness or constant light (1800 lux). The mutant strains were inoculated in the center of the plate and colony diameter as well as halo diameter were measured every 24 hours for 2 days at 28 °C. A halo appearing around the inoculated area is indicative for exoprotease activity<sup>105</sup>. Calculations are based on the ratio halo/mycelium. QM6a $\Delta$ tmus53 was used as control for every set. At least three biological replicates were analyzed per strain.

**Biomass determination.** For analysis of biomass production in the presence of cellulose, strains were grown on Mandels-Andreotti minimal medium with 1% (w/v) microcrystalline cellulose (Alfa Aesar, Karlsruhe, Germany) in constant light and constant darkness as described previously<sup>106</sup>. Cultivations were done in triplicates.

In all cases, plates for inoculation were grown in constant darkness for 14 days to avoid interference by circadian rhythmicity, and harvesting was done after 72 hours of growth either in light or under a red safety light (darkroom lamp, Dr. Fischer 230 V, red, E27, 15 W) for cultures grown in constant darkness. QM6a $\Delta$ tmus53 was used as control for every set. At least three biological replicates were analyzed for every strain.

Due to the insoluble cellulose present in the culture, the indirect method of biomass determination was used as described previously<sup>107</sup>. Briefly, mycelium was snap frozen in liquid nitrogen and ground in a Retsch Mill (Retsch MM301, Haan, Germany) in pre-cooled jars for 30 seconds with an oscillation frequency of 30 Hz and transferred to tubes containing 5 ml 0.1 M NaOH (Merck, Darmstadt, Germany). Samples were sonicated with 70% amplitude three times for 30 seconds and incubated at room temperature for 3 h. After centrifugation for 10 minutes at full speed, the protein content of the supernatant as a corresponding measure of biomass was measured by the Bradford method (Roti-Quant, Roth, Karlsruhe, Germany). At least three biological with two technical replicates were analyzed.

**Cellulase activity.** Strains were grown in liquid medium as described above, and endo-1,4- $\beta$ -d-glucanase activity from culture filtrates harvested after 72 hours was measured using the azo-CMC-cellulose kit (S-ACMC-L, Megazyme, Wicklow, Ireland) according to the manufacturer's instructions. For specific cellulase activity, enzyme activity was correlated to biomass. At least three biological with two technical replicates were analyzed.

**Isolation of total RNA and RT-qPCR.** For isolation of RNA, mycelia grown for 72 hours with cellulose as carbon source in constant light or constant darkness as described above were used. Isolation and quality control of total RNA as well as RT-qPCR were performed as described previously, with the ribosomal gene *l6e* as reference gene<sup>108</sup>. Primers used are taken from<sup>56</sup>. RT-qPCR was performed for three biological replicates with three technical replicates each. Data analysis was done using the software package CFX Maestro (Biorad, Hercules, CA, USA).

**Analysis of secondary metabolites production.** High performance thin-layer chromatography (HPTLC) analysis was performed as described earlier<sup>104,109–111</sup> with modifications. Supernatants from the cultures used for analysis of specific cellulase activity were used. The volume of supernatants used for each sample corresponds to equal amounts of biomass. One volume of supernatant was mixed with 1 volume of acetone (1:1; v/v) (Roth, Karlsruhe, Germany). After shaking for 1 hour (400 rpm at RT), 1 volume of chloroform (Roth, Karlsruhe, Germany) was added (1:1 v/v). After shaking again for 30 minutes (400 rpm at RT) and centrifugation for 1 minute at 500 g, the organic phase (chloroform-lower phase) was transferred to a conical tube. After complete evaporation of the solvent, the samples were resuspended in 140  $\mu$ l chloroform and 9  $\mu$ l were spotted on a HPTLC plate (HPTLC silica gel 60 F254s, Merck 1.15696.0001) using the CAMAG Automatic TLC sampler 4 (CAMAG, Muttenz, Switzerland). This amount was chosen in order to detect smaller changes in metabolite patterns without overloading the plate. Separation was performed in a saturated twin trough chamber with chloroform:trifluoroacetic acid 7:1 (v/v). The plates were analyzed under ultraviolet light (254 nm and 366 nm) using a CAMAG visualizer (CAMAG). Additionally, the plates were derivatized with p-anisaldehyde:sulfuric acid and evaluated again with white and ultraviolet light. Results were visualized using the software visionCATS 1.4.14017.1 (CAMAG).

## Data Availability

All data generated during this study are included in this published article and its supplementary file. GenBank Accession numbers for datasets analyzed for this study are given in Methods and described in the respective cited articles.

## References

1. Turra, D., Segorbe, D. & Di Pietro, A. Protein kinases in plant-pathogenic fungi: conserved regulators of infection. *Annu Rev Phytopathol* **52**, 267–288, <https://doi.org/10.1146/annurev-phyto-102313-050143> (2014).
2. Bischof, R. H., Ramoni, J. & Seiboth, B. Cellulases and beyond: the first 70 years of the enzyme producer *Trichoderma reesei*. *Microb Cell Fact* **15**, 106, <https://doi.org/10.1186/s12934-016-0507-6> (2016).
3. Stappler, E., Rodriguez-Iglesias, A., Bazafkan, H., Li, G. & Schmoll, M. In *Gene expression systems in fungi: Advancements and applications* (eds Schmoll, M. & Dattenböck, C.) 309–334 (Springer International Publishing, 2016).
4. Benocci, T., Aguilar-Pontes, M. V., Zhou, M., Seiboth, B. & de Vries, R. P. Regulators of plant biomass degradation in ascomycetous fungi. *Biotechnol Biofuels* **10**, 152, <https://doi.org/10.1186/s13068-017-0841-x> (2017).
5. Glass, N. L., Schmoll, M., Cate, J. H. & Coradetti, S. Plant cell wall deconstruction by ascomycete fungi. *Annu Rev Microbiol* **67**, 477–498, <https://doi.org/10.1146/annurev-micro-092611-150044> (2013).
6. Cohen, P. The regulation of protein function by multisite phosphorylation - a 25 year update. *Trends Biochem Sci* **25**, 596–601 (2000).
7. Manning, G., Whyte, D. B., Martinez, R., Hunter, T. & Sudarsanam, S. The protein kinase complement of the human genome. *Science* **298**, 1912–1934, <https://doi.org/10.1126/science.1075762> (2002).
8. Cziferszky, A., Mach, R. L. & Kubicek, C. P. Phosphorylation positively regulates DNA binding of the carbon catabolite repressor Cre1 of *Hypocrea jecorina* (*Trichoderma reesei*). *J Biol Chem* **277**, 14688–14694, <https://doi.org/10.1074/jbc.M200744200> (2002).
9. Stricker, A. R., Mach, R. L. & de Graaff, L. H. Regulation of transcription of cellulases- and hemicellulases-encoding genes in *Aspergillus niger* and *Hypocrea jecorina* (*Trichoderma reesei*). *Appl Microbiol Biotechnol* **78**, 211–220 (2008).
10. Tisch, D. & Schmoll, M. In *Biofuel Production - Recent developments and prospects* (ed. dos Santos Bernardes, M. A.) 199–224 (Intech, 2011).
11. Nguyen, E. V. et al. Quantitative site-specific phosphoproteomics of *Trichoderma reesei* signaling pathways upon induction of hydrolytic enzyme production. *J Proteome Res* **15**, 457–467, <https://doi.org/10.1021/acs.jproteome.5b00796> (2016).
12. Zeilinger, S., Ebner, A., Marosits, T., Mach, R. & Kubicek, C. P. The *Hypocrea jecorina* HAP 2/3/5 protein complex binds to the inverted CCAAT-box (ATTGG) within the *cbh2* (cellobiohydrolase II-gene) activating element. *Mol Genet Genomics* **266**, 56–63 (2001).
13. Saloheimo, A., Aro, N., Ilmen, M. & Penttilä, M. Isolation of the *ace1* gene encoding a Cys(2)-His(2) transcription factor involved in regulation of activity of the cellulase promoter *cbh1* of *Trichoderma reesei*. *J Biol Chem* **275**, 5817–5825 (2000).

14. Karimi Aghcheh, R. *et al.* The VELVET A orthologue VEL1 of *Trichoderma reesei* regulates fungal development and is essential for cellulase gene expression. *PLoS One* **9**, e112799, <https://doi.org/10.1371/journal.pone.0112799> (2014).
15. Ivanova, C. *et al.* Genome sequencing and transcriptome analysis of *Trichoderma reesei* QM9978 strain reveals a distal chromosome translocation to be responsible for loss of *vib1* expression and loss of cellulase induction. *Biotechnol Biofuels* **10**, 209, <https://doi.org/10.1186/s13068-017-0897-7> (2017).
16. Xiong, Y., Sun, J. & Glass, N. L. VIB1, a link between glucose signaling and carbon catabolite repression, is essential for plant cell wall degradation by *Neurospora crassa*. *PLoS Genet* **10**, e1004500, <https://doi.org/10.1371/journal.pgen.1004500> (2014).
17. Derntl, C., Rassinger, A., Srebotnik, E., Mach, R. L. & Mach-Aigner, A. R. Xpp1 regulates the expression of xylanases, but not of cellulases in *Trichoderma reesei*. *Biotechnol Biofuels* **8**, 112, <https://doi.org/10.1186/s13068-015-0298-8> (2015).
18. Le Crom, S. *et al.* Tracking the roots of cellulase hyperproduction by the fungus *Trichoderma reesei* using massively parallel DNA sequencing. *Proc Natl Acad Sci USA* **106**, 16151–16156, <https://doi.org/10.1073/pnas.0905848106> (2009).
19. Liu, G. *et al.* Long-term strain improvements accumulate mutations in regulatory elements responsible for hyper-production of cellulolytic enzymes. *Sci Rep* **3**, 1569, <https://doi.org/10.1038/srep01569> (2013).
20. Porciuncula Jde, O. *et al.* Identification of major facilitator transporters involved in cellulase production during lactose culture of *Trichoderma reesei* PC-3-7. *Biosci Biotechnol Biochem* **77**, 1014–1022 (2013).
21. de Assis, L. J. *et al.* Multiple phosphatases regulate carbon source-dependent germination and primary metabolism in *Aspergillus nidulans*. *G3 (Bethesda)* **5**, 857–872, <https://doi.org/10.1534/g3.115.016667> (2015).
22. Gancedo, J. M. Yeast carbon catabolite repression. *Microbiol Mol Biol Rev* **62**, 334–361 (1998).
23. Rande-Gil, F., Sanz, P., Entian, K. D. & Prieto, J. A. Carbon source-dependent phosphorylation of hexokinase PII and its role in the glucose-signaling response in yeast. *Mol Cell Biol* **18**, 2940–2948 (1998).
24. Sanz, P. Snf1 protein kinase: a key player in the response to cellular stress in yeast. *Biochem Soc Trans* **31**, 178–181, 10.1042/ (2003).
25. Zabrocki, P. *et al.* Protein phosphatase 2A on track for nutrient-induced signalling in yeast. *Mol Microbiol* **43**, 835–842 (2002).
26. Noguchi, Y., Tanaka, H., Kanamaru, K., Kato, M. & Kobayashi, T. Xylose triggers reversible phosphorylation of XlnR, the fungal transcriptional activator of xylanolytic and cellulolytic genes in *Aspergillus oryzae*. *Biosci Biotechnol Biochem* **75**, 953–959, <https://doi.org/10.1271/bbb.100923> (2011).
27. Brown, N., de Gouvea, P., Krohn, N., Savoldi, M. & Goldman, G. Functional characterisation of the non-essential protein kinases and phosphatases regulating *Aspergillus nidulans* hydrolytic enzyme production. *Biotechnology for Biofuels* **6**, 91 (2013).
28. Tisch, D. & Schmoll, M. Light regulation of metabolic pathways in fungi. *Appl Microbiol Biotechnol* **85**, 1259–1277, <https://doi.org/10.1007/s00253-009-2320-1> (2010).
29. Schmoll, M. Regulation of plant cell wall degradation by light in *Trichoderma*. *Fungal Biol Biotechnol* **5**, 10, <https://doi.org/10.1186/s40694-018-0052-7> (2018).
30. Diernfellner, A. C. & Schafmeier, T. Phosphorylations: Making the *Neurospora crassa* circadian clock tick. *FEBS Lett* **585**, 1461–1466, <https://doi.org/10.1016/j.febslet.2011.03.049> (2011).
31. Tataroglu, O. & Schafmeier, T. Of switches and hourglasses: regulation of subcellular traffic in circadian clocks by phosphorylation. *EMBO Rep* **11**, 927–935, <https://doi.org/10.1038/embor.2010.174> (2010).
32. Schmoll, M., Tian, C., Sun, J., Tisch, D. & Glass, N. L. Unravelling the molecular basis for light modulated cellulase gene expression - the role of photoreceptors in *Neurospora crassa*. *BMC Genomics* **13**, 127, <https://doi.org/10.1186/1471-2164-13-127> (2012).
33. Stappler, E., Walton, J. D. & Schmoll, M. Abundance of secreted proteins of *Trichoderma reesei* is regulated by light of different intensities. *Front Microbiol* **8**, 2586 (2017).
34. Castellanos, F. *et al.* Crucial factors of the light perception machinery and their impact on growth and cellulase gene transcription in *Trichoderma reesei*. *Fungal Genet Biol* **47**, 468–476, <https://doi.org/10.1016/j.fgb.2010.02.001> (2010).
35. Baker, C. L., Kettenbach, A. N., Loros, J. J., Gerber, S. A. & Dunlap, J. C. Quantitative proteomics reveals a dynamic interactome and phase-specific phosphorylation in the *Neurospora* circadian clock. *Mol Cell* **34**, 354–363, <https://doi.org/10.1016/j.molcel.2009.04.023> (2009).
36. Cha, J., Zhou, M. & Liu, Y. Mechanism of the *Neurospora* circadian clock, a FREQUENCY-centric view. *Biochemistry* **54**, 150–156, <https://doi.org/10.1021/bi5005624> (2015).
37. Janssens, V. & Goris, J. Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochem J* **353**, 417–439 (2001).
38. Ahn, C. S., Han, J. A., Lee, H. S., Lee, S. & Pai, H. S. The PP2A regulatory subunit Tap46, a component of the TOR signaling pathway, modulates growth and metabolism in plants. *Plant Cell* **23**, 185–209, <https://doi.org/10.1105/tpc.110.074005> (2011).
39. Bollen, M. Combinatorial control of protein phosphatase-1. *Trends Biochem Sci* **26**, 426–431 (2001).
40. Cohen, P. T. Protein phosphatase 1-targeted in many directions. *J Cell Sci* **115**, 241–256 (2002).
41. Terrak, M., Kerff, F., Langsetmo, K., Tao, T. & Dominguez, R. Structural basis of protein phosphatase 1 regulation. *Nature* **429**, 780–784, <https://doi.org/10.1038/nature02582> (2004).
42. Lokhandwala, J. *et al.* Structural biochemistry of a fungal LOV domain photoreceptor reveals an evolutionarily conserved pathway integrating light and oxidative stress. *Structure* **23**, 116–125, <https://doi.org/10.1016/j.str.2014.10.020> (2015).
43. Esquivel-Naranjo, E. U. *et al.* A *Trichoderma atroviride* stress-activated MAPK pathway integrates stress and light signals. *Mol Microbiol* **100**, 860–876, <https://doi.org/10.1111/mmi.13355> (2016).
44. Friedl, M. A., Schmoll, M., Kubicek, C. P. & Druzhinina, I. S. Photostimulation of *Hypocrea atroviridis* growth occurs due to a cross-talk of carbon metabolism, blue light receptors and response to oxidative stress. *Microbiology* **154**, 1229–1241 (2008).
45. Ptacek, J. *et al.* Global analysis of protein phosphorylation in yeast. *Nature* **438**, 679–684 (2005).
46. Schmoll, M. *et al.* The genomes of three uneven siblings: footprints of the lifestyles of three *Trichoderma* species. *Microbiol Mol Biol Rev* **80**, 205–327, <https://doi.org/10.1128/MMBR.00040-15> (2016).
47. Shi, Y. Serine/threonine phosphatases: mechanism through structure. *Cell* **139**, 468–484, <https://doi.org/10.1016/j.cell.2009.10.006> (2009).
48. Kerk, D. *et al.* The complement of protein phosphatase catalytic subunits encoded in the genome of Arabidopsis. *Plant Physiol* **129**, 908–925, <https://doi.org/10.1104/pp.004002> (2002).
49. Ramponi, G. & Stefani, M. Structure and function of the low Mr phosphotyrosine protein phosphatases. *Biochim Biophys Acta* **1341**, 137–156 (1997).
50. Kosti, I., Mandel-Gutfreund, Y., Glaser, F. & Horwitz, B. A. Comparative analysis of fungal protein kinases and associated domains. *BMC Genomics* **11**, 133 (2010).
51. Stark, M. J. Yeast protein serine/threonine phosphatases: multiple roles and diverse regulation. *Yeast* **12**, 1647–1675 (1996).
52. Häkkinen, M. *et al.* Screening of candidate regulators for cellulase and hemicellulase production in *Trichoderma reesei* and identification of a factor essential for cellulase production. *Biotechnol Biofuels* **7**, 14, <https://doi.org/10.1186/1754-6834-7-14> (2014).
53. Stricker, A. R., Grosstessner-Hain, K., Wurleitner, E. & Mach, R. L. Xyr1 (Xylanase Regulator 1) Regulates both the Hydrolytic Enzyme System and D-Xylose Metabolism in *Hypocrea jecorina*. *Eukaryot Cell* **5**, 2128–2137 (2006).
54. Stricker, A. R., Trefflinger, P., Aro, N., Penttilä, M. & Mach, R. L. Role of ACE2 (Activator of Cellulases 2) within the *xyn2* transcriptome of *Hypocrea jecorina*. *Fungal Genet Biol* **45**, 436–445, <https://doi.org/10.1016/j.fgb.2007.08.005> (2008).
55. Tisch, D. & Schmoll, M. Targets of light signalling in *Trichoderma reesei*. *BMC Genomics* **14**, 657, <https://doi.org/10.1186/1471-2164-14-657> (2013).

56. Tisch, D., Kubicek, C. P. & Schmoll, M. The phosducin-like protein PhLP1 impacts regulation of glycoside hydrolases and light response in *Trichoderma reesei*. *BMC Genomics* **12**, 613 (2011).
57. Cabrera, I. E. *et al.* Global analysis of predicted G protein-coupled receptor genes in the filamentous fungus, *Neurospora crassa*. *G3 (Bethesda)* **5**, 2729–2743, <https://doi.org/10.1534/g3.115.020974> (2015).
58. Schmoll, M., Schuster, A., do Nascimento Silva, R. & Kubicek, C. P. The G-alpha protein GNA3 of *Hypocrea jecorina* (anamorph *Trichoderma reesei*) regulates cellulase gene expression in the presence of light. *Eukaryot Cell* **8**, 410–420, <https://doi.org/10.1128/EC.00256-08> (2009).
59. Seibel, C. *et al.* Light-dependent roles of the G-protein subunit GNA1 of *Hypocrea jecorina* (anamorph *Trichoderma reesei*). *BMC Biol* **7**, 58 (2009).
60. Kim, H. *et al.* Roles for receptors, pheromones, G proteins, and mating type genes during sexual reproduction in *Neurospora crassa*. *Genetics* **190**, 1389–1404, <https://doi.org/10.1534/genetics.111.136358> (2012).
61. Li, L., Wright, S. J., Krystofova, S., Park, G. & Borkovich, K. A. Heterotrimeric G protein signaling in filamentous fungi. *Annu Rev Microbiol* **61**, 423–452 (2007).
62. Seibel, C., Tisch, D., Kubicek, C. P. & Schmoll, M. The role of pheromone receptors for communication and mating in *Hypocrea jecorina* (*Trichoderma reesei*). *Fungal Genet Biol* **49**, 814–824, <https://doi.org/10.1016/j.fgb.2012.07.004> (2012).
63. Van Dijk, P. *et al.* Nutrient Sensing at the Plasma Membrane of Fungal Cells. *Microbiol Spectr* **5**, <https://doi.org/10.1128/microbiolspec.FUNK-0031-2016> (2017).
64. Stappeler, E., Dattenböck, C., Tisch, D. & Schmoll, M. Analysis of light- and carbon-specific transcriptomes implicates a class of G-protein-coupled receptors in cellulose sensing. *mSphere* **2**, e00089–00017, <https://doi.org/10.1128/mSphere.00089-17> (2017).
65. Dattenböck, C. *et al.* Gene regulation associated with sexual development and female fertility in different isolates of *Trichoderma reesei*. *Fungal Biol Biotechnol* **5**, 9 (2018).
66. Tisch, D. *et al.* Omics Analyses of *Trichoderma reesei* CBS999.97 and QM6a indicate the relevance of female fertility to carbohydrate-active enzyme and transporter levels. *Appl Environ Microbiol* **83**, <https://doi.org/10.1128/AEM.01578-17> (2017).
67. Ghosh, A., Servin, J. A., Park, G. & Borkovich, K. A. Global analysis of serine/threonine and tyrosine protein phosphatase catalytic subunit genes in *Neurospora crassa* reveals interplay between phosphatases and the p38 mitogen-activated protein kinase. *G3 (Bethesda)* **4**, 349–365, <https://doi.org/10.1534/g3.113.008813> (2014).
68. Young, C., Mapes, J., Hanneman, J., Al-Zarban, S. & Ota, I. Role of Ptc2 type 2C Ser/Thr phosphatase in yeast high-osmolarity glycerol pathway inactivation. *Eukaryot Cell* **1**, 1032–1040 (2002).
69. Hirasawa, T. *et al.* Comparison of transcriptional responses to osmotic stresses induced by NaCl and sorbitol additions in *Saccharomyces cerevisiae* using DNA microarray. *J Biosci Bioeng* **102**, 568–571, <https://doi.org/10.1263/jbb.102.568> (2006).
70. Nikolaou, E. *et al.* Phylogenetic diversity of stress signalling pathways in fungi. *BMC Evol Biol* **9**, 44, <https://doi.org/10.1186/1471-2148-9-44> (2009).
71. Posas, F. *et al.* The gene PPG encodes a novel yeast protein phosphatase involved in glycogen accumulation. *J Biol Chem* **268**, 1349–1354 (1993).
72. Wang, M. *et al.* A mitogen-activated protein kinase Tmk3 participates in high osmolarity resistance, cell wall integrity maintenance and cellulase production regulation in *Trichoderma reesei*. *PLoS One* **8**, e72189, <https://doi.org/10.1371/journal.pone.0072189> (2013).
73. Cheng, A., Ross, K. E., Kaldis, P. & Solomon, M. J. Dephosphorylation of cyclin-dependent kinases by type 2C protein phosphatases. *Genes Dev* **13**, 2946–2957 (1999).
74. Marsolier, M. C., Roussel, P., Leroy, C. & Mann, C. Involvement of the PP2C-like phosphatase Ptc2p in the DNA checkpoint pathways of *Saccharomyces cerevisiae*. *Genetics* **154**, 1523–1532 (2000).
75. Schafmeier, T. *et al.* Transcriptional Feedback of *Neurospora* Circadian Clock Gene by Phosphorylation-Dependent Inactivation of Its Transcription Factor. *Cell* **122**, 235–246, <https://doi.org/10.1016/j.cell.2005.05.032> (2005).
76. Posas, F., Bollen, M., Stalmans, W. & Arino, J. Biochemical characterization of recombinant yeast PPZ1, a protein phosphatase involved in salt tolerance. *FEBS Lett* **368**, 39–44 (1995).
77. Yenuso, L., Mulet, J. M., Arino, J. & Serrano, R. The Ppz protein phosphatases are key regulators of K<sup>+</sup> and pH homeostasis: implications for salt tolerance, cell wall integrity and cell cycle progression. *EMBO J* **21**, 920–929, <https://doi.org/10.1093/emboj/21.5.920> (2002).
78. Muszkiet, L. *et al.* The protein phosphatase PhzA of *A. fumigatus* is involved in oxidative stress tolerance and fungal virulence. *Fungal Genet Biol* **66**, 79–85, <https://doi.org/10.1016/j.fgb.2014.02.009> (2014).
79. Bloemendal, S. *et al.* A homologue of the human STRIPAK complex controls sexual development in fungi. *Mol Microbiol* **84**, 310–323, <https://doi.org/10.1111/j.1365-2958.2012.08024.x> (2012).
80. Seidl, V., Seibel, C., Kubicek, C. P. & Schmoll, M. Sexual development in the industrial workhorse *Trichoderma reesei*. *Proc Natl Acad Sci USA* **106**, 13909–13914, <https://doi.org/10.1073/pnas.0904936106> (2009).
81. Liu, X. *et al.* The putative protein phosphatase MoYvh1 functions upstream of MoPdeH to regulate the development and pathogenicity in *Magnaporthe oryzae*. *Mol Plant Microbe Interact* **29**, 496–507, <https://doi.org/10.1094/MPMI-11-15-0259-R> (2016).
82. Metz, B. *et al.* Expression of biomass-degrading enzymes is a major event during conidium development in *Trichoderma reesei*. *Eukaryotic Cell* **10**, 1527–1535, <https://doi.org/10.1128/EC.05014-11> (2011).
83. Braaksma, M., Smilde, A. K., van der Werf, M. J. & Punt, P. J. The effect of environmental conditions on extracellular protease activity in controlled fermentations of *Aspergillus niger*. *Microbiology* **155**, 3430–3439, <https://doi.org/10.1099/mic.0.031062-0> (2009).
84. van den Hombergh, J. P., van de Vondervoort, P. J., Fraissinet-Tachet, L. & Visser, J. *Aspergillus* as a host for heterologous protein production: the problem of proteases. *Trends Biotechnol* **15**, 256–263 (1997).
85. Landowski, C. P. *et al.* Enabling low cost biopharmaceuticals: A systematic approach to delete proteases from a well-known protein production host *Trichoderma reesei*. *PLoS One* **10**, e0134723, <https://doi.org/10.1371/journal.pone.0134723> (2015).
86. Mukherjee, P. K., Horwitz, B. A. & Kenerley, C. M. Secondary metabolism in *Trichoderma* - a genomic perspective. *Microbiology* **158**, 35–45, <https://doi.org/10.1099/mic.0.053629-0> (2012).
87. Monroy, A. A., Stappeler, E., Schuster, A., Sulyok, M. & Schmoll, M. A CRE1-regulated cluster is responsible for light dependent production of dihydrotrichotetrone in *Trichoderma reesei*. *PLoS One*, e0182530 (2017).
88. Derntl, C. *et al.* Transcription factor Xpp1 is a switch between primary and secondary fungal metabolism. *Proc Natl Acad Sci USA* **114**, E560–E569, <https://doi.org/10.1073/pnas.1609348114> (2017).
89. Manfiolli, A. O. *et al.* *Aspergillus fumigatus* protein phosphatase PpzA is involved in iron assimilation, secondary metabolite production, and virulence. *Cell Microbiol* **19**, <https://doi.org/10.1111/cmi.12770> (2017).
90. Martinez, D. *et al.* Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*). *Nat Biotechnol* **26**, 553–560 (2008).
91. Jonasson, E. M. *et al.* Zds1/Zds2-PP2ACdc55 complex specifies signaling output from Rho1 GTPase. *J Cell Biol* **212**, 51–61, <https://doi.org/10.1083/jcb.201508119> (2016).
92. Lamb, T. M., Goldsmith, C. S., Bennett, L., Finch, K. E. & Bell-Pedersen, D. Direct transcriptional control of a p38 MAPK pathway by the circadian clock in *Neurospora crassa*. *PLoS One* **6**, e27149, <https://doi.org/10.1371/journal.pone.0027149> (2011).



93. Tatjer, L. *et al.* Wide-Ranging Effects of the Yeast Ptc1 Protein Phosphatase Acting Through the MAPK Kinase Mkk1. *Genetics* **202**, 141–156, <https://doi.org/10.1534/genetics.115.183202> (2016).
94. Marie-Nelly, H. *et al.* High-quality genome (re)assembly using chromosomal contact data. *Nat Commun* **5**, 5695, <https://doi.org/10.1038/ncomms6695> (2014).
95. Yoshikawa, K. *et al.* Comprehensive phenotypic analysis of single-gene deletion and overexpression strains of *Saccharomyces cerevisiae*. *Yeast* **28**, 349–361, <https://doi.org/10.1002/yea.1843> (2011).
96. Steiger, M. G. *et al.* Transformation system for *Hypocrea jecorina* (*Trichoderma reesei*) that favors homologous integration and employs reusable bidirectionally selectable markers. *Appl Environ Microbiol* **77**, 114–121, <https://doi.org/10.1128/AEM.02100-10> (2011).
97. Colot, H. V. *et al.* A high-throughput gene knockout procedure for *Neurospora* reveals functions for multiple transcription factors. *Proc Natl Acad Sci USA* **103**, 10352–10357, <https://doi.org/10.1073/pnas.0601456103> (2006).
98. Schuster, A. *et al.* A versatile toolkit for high throughput functional genomics with *Trichoderma reesei*. *Biotechnol Biofuels* **5**, 1, <https://doi.org/10.1186/1754-6834-5-1> (2012).
99. Gruber, F., Visser, J., Kubicek, C. P. & de Graaff, L. H. The development of a heterologous transformation system for the cellulolytic fungus *Trichoderma reesei* based on a *pyrG*-negative mutant strain. *Curr Genet* **18**, 71–76 (1990).
100. Rodriguez-Iglesias, A. & Schmoll, M. In *Genetic Transformation systems in Fungi* Vol. 1 (eds M. A. Van den Berg & K. Maruthachalam) Ch. 2, 21–40 (Springer International Publishing, 2015).
101. Seo, J., Gordish-Dressman, H. & Hoffman, E. P. An interactive power analysis tool for microarray hypothesis testing and generation. *Bioinformatics* **22**, 808–814, <https://doi.org/10.1093/bioinformatics/btk052> (2006).
102. Mandels, M. & Andreotti, R. Problems and challenges in the cellulose to cellulase fermentation. *Proc Biochem* **13**, 6–13 (1978).
103. Schmoll, M. & Wang, T. F. In *The Mycota (Vol. I): Growth, Differentiation and Sexuality The Mycota* (ed. J. Wendland) 457–474 (Springer International Publishing, 2016).
104. Bazafkan, H. *et al.* Mating type dependent partner sensing as mediated by VEL1 in *Trichoderma reesei*. *Mol Microbiol* **96**, 1103–1118, <https://doi.org/10.1111/mmi.12993> (2015).
105. Brown, M. R. & Foster, J. H. A simple diagnostic milk medium for *Pseudomonas aeruginosa*. *J Clin Pathol* **23**, 172–177 (1970).
106. Schmoll, M., Franchi, L. & Kubicek, C. P. Envoy, a PAS/LOV domain protein of *Hypocrea jecorina* (Anamorph *Trichoderma reesei*), modulates cellulase gene transcription in response to light. *Eukaryot Cell* **4**, 1998–2007 (2005).
107. Schuster, A., Kubicek, C. P. & Schmoll, M. Dehydrogenase GRD1 represents a novel component of the cellulase regulon in *Trichoderma reesei* (*Hypocrea jecorina*). *Appl Environ Microbiol* **77**, 4553–4563, <https://doi.org/10.1128/AEM.00513-11> (2011).
108. Tisch, D., Kubicek, C. P. & Schmoll, M. New insights into the mechanism of light modulated signaling by heterotrimeric G-proteins: ENVOY acts on *gna1* and *gna3* and adjusts cAMP levels in *Trichoderma reesei* (*Hypocrea jecorina*). *Fungal Genet Biol* **48**, 631–640, <https://doi.org/10.1016/j.fgb.2010.12.009> (2011).
109. Bazafkan, H. *et al.* SUB1 has photoreceptor dependent and independent functions in sexual development and secondary metabolism in *Trichoderma reesei*. *Mol Microbiol* **106**, 742–759, <https://doi.org/10.1111/mmi.13842> (2017).
110. Bok, J. W. & Keller, N. P. LaeA, a regulator of secondary metabolism in *Aspergillus* spp. *Eukaryot Cell* **3**, 527–535 (2004).
111. Reyes-Dominguez, Y. *et al.* Heterochromatic marks are associated with the repression of secondary metabolism clusters in *Aspergillus nidulans*. *Mol Microbiol* **76**, 1376–1386, <https://doi.org/10.1111/j.1365-2958.2010.07051.x> (2010).

## Acknowledgements

We want to thank Stefan Böhmendorfer and Josua Oberlerchner (University of Life Sciences BOKU) for technical assistance with HPTLC analysis and Sabrina Beier for support of statistical analysis. The work of ARI was funded by the Austrian Science Fund (FWF) grants P24350 and P26935 to MS.

## Author Contributions

A.R.I. performed experimental work and drafted the manuscript. M.S. conceived of the study, supervised work of A.R.I., participated in data analysis and wrote the final version of the manuscript. All authors read the final version of the manuscript.

## Additional Information

**Supplementary information** accompanies this paper at <https://doi.org/10.1038/s41598-019-47421-z>.

**Competing Interests:** The authors declare no competing interests.

**Publisher's note:** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



**Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2019