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ORIGINAL PAPER

Nutrition supply affects the activity of pathogenesis-related β-1,3-glucanases and chitinases in wheat

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Abstract Nitrogen (N) is an essential mineral for plants and both its deficiency and excess causes serious problems in agriculture. As stress-inducible defense is costly, N conditions likely affect the trade-off between the growth and defense. Previous studies identified a few defense-related enzymes dependent on N nutrition. Chitinases (EC 3.2.1.14) and glucanases (EC 3.2.1.39) are typical plant defense enzymes belonging to the group of pathogenesis-related (PR) proteins with multiple functions in plants. Since a comprehensive study on the impact of N nutrition on their activity is missing, we studied their profiles and activities at isoforms level in wheat plants grown hydroponically at N doses corresponding to limited (0, 0.75 and 5.25 mM N), optimal N (7.5 mM N) as well as excess (15, 30 and 35 mM N) N supply in the form of nitrate. Our results show that several isoforms of both enzymes in wheat leaves and/or shoots clearly depended on N supply, while their activities rather depended on organ type. Furthermore, glucanases and chitinases appeared to be regulated in an opposite way. The activities of particular chitinases and glucanases correlated with a proline content that has multiple functions in plants. Proline typically accumulated with increasing the N supply when certain excessive N doses induced the gene for proline synthase (*P5CS*) in shoots and that for ornithine aminotransferase (*OAT*) in roots. This work points to a N-dependent activity of several defense-related compounds suggesting the possibly of altered plant defense potential under various N regimes.

Keywords Defense cost · Glucanhydrolases · Nitrogen · PR-proteins · Trade-off

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Introduction

Up to 90 % of the nitrogen (N) content of cereals can be remobilized from the vegetative plant parts to the grain (Gregersen et al. 2008); this affects both yield and bread quality. Consequently, nitrogen is one of the mineral nutrients that limits plant productivity, and a considerable amount of knowledge has been collected on the mechanisms of nitrate uptake, transport and N remobilization under the conditions of N shortage or deficiency. In recent decades, however, the overuse of N fertilizers has become a serious issue. Only 30–40 % of the N applied for crop production is utilized by plants; the rest is decomposed by soil microbial flora to nitrous oxide or dinitrogen (N_2) causing

a series of environmental and economic problems. It is therefore imperative to study the adaptation mechanisms to low-N and/or high-N availability in crops, as these are key for coping with a low N availability in the soil or a surplus of N fertilizer supply.

The impact of both N deficiency and excess on plants is well known, with low N reducing photosynthesis and limiting plant growth. While plants can cope reasonably well with a short-term N shortage or deficiency, a longer period of N starvation interferes with the synthesis of nucleic acids and enzymes (Hörtensteiner and Feller 2002). On the other hand, N concentration in a stagnant nutrient solution exceeding 10 mM nitrate generally slows plant growth, while a value of 25 mM is considered the threshold of toxicity for some species (Sánchez et al. 2001 and therein). Several studies have shown that both types of non-optimal N conditions alter the plants ability to withstand environmental stresses. For example, constitutive defense components such as various phenolic compounds were generally more strongly expressed under N-limiting conditions, while observations indicated variable correlations with N supply for other compounds such as terpenes or alkaloids (Dietrich et al. 2004 and therein). There are few studies reporting on inducible defense components responsive to nutrient availability. A positive correlation with excessive N was found for the activity of chitinases (Dietrich et al. 2004; Konotop et al. 2012) and peroxidases (Dietrich et al. 2004). More recently, several high-throughput studies have focused on plant responses to different fertilizer regimes (Hakeem et al. 2012; Chandna and Ahmed 2015) and identified that defense-related enzymes such as chitinases respond to unfavorable N doses.

Chitinases (EC 3.2.1.14) together with β-1,3-glucanases (EC 3.2.1.39) are hydrolytic enzymes that often act together in plants. They have been widely studied in the context of plant pathogenesis, assigning them to the family of pathogenesis-related (PR) proteins. Moreover, their role in the defense against abiotic stresses such as heavy metals (Gálusová et al. 2014; Mészáros et al. 2013), cold (Zur et al. 2013), and drought (Gregorová et al. 2015) has been proven. These enzymes are involved in many morphological and developmental processes too (for review, see Kasprzewska 2003) and this renders them crucial for plant development under a range of environmental conditions (Gálusová et al. 2014).

This work studied the activity of glucanases and chitinases in wheat under different N supply treatments. As components of primary but also secondary stress (Mészáros et al. 2014), these enzymes represent a good system to monitor environmental changes. Although a dependence of chitinase induction on N has been shown in *Arabidopsis* (Dietrich et al. 2004), the data only relate to total enzyme activity but not individual isoforms. A chitinase homologue responsive to N supply has been identified in wheat (Chandna and Ahmad 2015) and peach (Wang et al. 2012a). For β-1,3-glucanases (for simplicity henceforth glucanases), a single enzyme dependent on N supply in flax has been identified with a role in fiber formation (Wang et al. 2009). We performed an experiment to assess the profile and activity of these enzymes in plants at low N, optimal N and for a series of excessive N concentrations in hydroponic growth media. We analyzed some parameters of growth, photosynthesis and the accumulation of proline as a typical plant defense metabolite under different stress types. Proline has also been suggested as a good indicator of N excess (Sánchez et al. 2001). Our work brings novel results on N-dependent activities of several glucanase and chitinase isoforms, indicating their role in plant adaptation to various N supplies.

Materials and methods

Plant material, cultivation and experimental design

Wheat seeds (*Triticum aestivum* cv. Genoveva) were pregerminated in Petri dishes on moist filter paper for two days. Uniformly germinated seeds (15, roots ca. 1.5 cm) were placed under hydroponic conditions into plastic containers (15 × 15 × 20 cm) with 700 ml of modified Hoagland solution (0.2 mM NH_4NO_3, 5.0 mM KNO_3, 2.0 mM $Ca(NO_3)_2$, 2.0 mM $MgSO_4$, 0.5 mM Na_2SiO_3, 0.1 mM KH_2PO_4, 50 µM NaFe(III)EDTA, 50 µM H_3BO_3, 5 µM $MnCl_2$, 5 µM $ZnSO_4$, 0.5 µM $CuSO_4$, and 0.1 µM Na_2MoO_3; Shavrukov et al. 2006). The modifications included replacement of KNO_3 with K_2SO_4 and $Ca(NO_3)_2$ with $CaCl_2$, and supplementing with different amounts of nitrogen in the form of NH_4NO_3 to achieve final nitrogen concentrations of 0, 0.75 and 5.25 mM N (starvation, suboptimal doses), 7.5 mM (optimal; the standard concentration in the basic Hoagland solution), and 15, 25, 30 and 35 mM N (excess of N nutrition). The containers were constantly aerated and cultivated in a temperature-controlled chamber at 18 °C between 6 p.m. and 6 a.m. and 22 °C between 6 a.m. and 6 p.m. Relative humidity was 60 % with a 16/8 h photoperiod, irradiance ranging from 300 to 400 µmol/m^2/s of PAR. Plants were sampled and analyzed individually after 10 days of growth. Roots were rinsed in distilled water and dried using paper towels. All material (unless analyzed immediately) was frozen in liquid nitrogen and stored at −80 °C for further analysis. The experiment in a randomized complete block design was performed nine times with one container of 15 plants per N concentration. The growth rate and photosynthesis-related parameters were determined for each replicate. The number of replicates for other analyses is indicated in the corresponding data.

Assays of basic physiology, lipid peroxidation and nutrient content

Growth rate was determined by measurements of fresh and dry weight and length of both shoots and roots. The content of assimilation pigments in 50 mg of fresh tissue was determined in acetone extracts according to Lichtenthaler and Wellburn (1983) in 8–10 leaves per container. The measured parameters were: chlorophylls Chl *a*, Chl *b*, total chlorophylls Chl ($a+b$), Chl a/b ratio (as an indicator of functional pigment equipment), total carotenoids *Car*, Chl $(a+b)/Car$ ratio. Minimum (F_0) and maximum fluorescence (F_{max}) as well as maximum quantum yield of photosystem II photochemistry (Fv/Fm) was determined in another three leaves per container using a Handy FluorCam FC 1000-H as an indicator of photoinhibition (photosystem II complexes injury). The data from individual containers were averaged to represent a biological replicate.

The level of malondialdehyde (MDA) in shoot and root tips (100 mg fresh weight) was spectrophotometrically estimated by the thiobarbituric acid assay using a molar extinction coefficient 155×10^5 mM/cm (Karabal et al. 2003).

For quantification of tissue nitrogen and phosphorus content in leaf tips, analyses in acid mineralizates were performed colorimetrically with an automatic FIAstar 5010 Analyzer (Tecator, Sweden) (for details, see Adamec 2002). Since most of the nitrate taken up by roots is transported to the shoots (Gojon and Gaymard 2010), roots were not examined in this respect.

Analyses of proline

Free proline content in root and leaf tips (~1 cm long, a total of 200 mg fresh weight) was quantified spectrophotometrically based on light absorption of the benzene phase at 515 nm using a standard curve (Sánchez et al. 2001).

For proline-related expression analyses, genes for proline synthase (*P5CS*) and ornithine amino transferase (*OAT*) were analysed using published primer pairs (Saltzmann et al. 2008): Ta_P5CS: GCACCCTCGAATTTGTT GATG/ACAATCTGTGTGTGCACTTCCAT, Ta_OAT: GGCACGGAGGCAAATGAG/AGTGAAATAATGTCA TGGGAACCA. The cDNA was made from 1 µg RNA (Békésiová et al. 1999) using the Maxima H Minus First Strand cDNA Synthesis Kit and OligodT primers (Thermo Scientific) as per the manufacturer's instructions and stored at −20 °C until use. The gene for β-tubulin (U76895) was used for normalization. The reaction mixtures contained 5 µl 2× SYBR® Green PCR Master Mix (Life Technologies), 0.3 µl 10 µM of both forward and reverse gene specific primers, nuclease-free water, and 1 µl 1:3 diluted cDNA. The qPCR was carried out in LightCycler® Nano (Roche) real-time PCR system in a duplicate in eight well PCR strips. The PCR programme started with initial incubation at 50 °C for 2 min, followed by denaturation at 95 °C for 10 min. Forty-five cycles of amplification were performed using a thermal cycling profile of 95 °C for 10 s and 60 °C for 30 s. Subsequently, a melting curve was recorded by holding at 95 °C for 10 s, cooling to 60 °C and then heating at 0.1 °C/s up to 95 °C. The amplification and melting curve data were collected and analyzed using the LightCycler Nano software 1.0.

Assay of chitinase and glucanase proteins

Total proteins were extracted from roots and leaves (Hurkman and Tanaka 1986). Aliquots (20 µg) were separated in 12.5 % polyacrylamide gels to detect total enzyme profiles, profiles of acidic/neutral and basic/neutral isoforms (respectively) under standard conditions. For detection of chitinases, the gels contained 0.01 % (w/v) glycol chitin as the enzyme substrate. After separation, the gels were renatured in 50 mM sodium acetate buffer (pH 5.0) with 1 % (v/v) Triton X-100 overnight. The enzyme profiles were stained for enzyme activity with 0.01 % (w/v) Fluorescent Brightener 28 (Sigma) as described by Pan et al. (1991), and photographed under UV light. The glucanase activities of protein fractions were detected in gels with 0.01 % (w/v) laminarin (Sigma), subsequent re-naturation with Triton X-100 and staining with 2,3,5-triphenyltetrazolium chloride (Sigma) as described by Pan et al. (1991). Enzyme profiles in gels were scanned.

The background-corrected integrated density (ID) of the bands was taken as a measure of the enzyme activity using Scion Image software (http://www.scioncorp.com) (Gálusová et al. 2014). The molecular size of isoforms was determined using co-separated marker proteins after Coomassie-staining of protein profiles.

The total chitinase activity in samples was measured fluorimetrically using 4-methylumbelliferyl-β-D-*N,N′,N″*-triacetylchitotrioside [4-MU-(GlcNAc)3] as a substrate, as described previously (Libantová et al. 2009). The reaction mixture contained 20 µl of protein extracts, 30 µl of 300 µM substrate in 0.1 M sodium citrate buffer (pH 3.0). After incubation at 37 °C for 1 h, the reaction was stopped by adding 150 µl of 0.2 M Na_2CO_3 and fluorescence was measured by Fluoroskan II microtiterplate reader (TITERTEK, Finland) using excitation and emission filters 355 nm/450 nm. The chitinase activity was calculated based on the standard curve as picomoles of methylumbelliferone generated per hour per microgram of soluble protein at 37 °C.

The activity of total β-1,3-glucanases in protein samples was determined with a 3,5-dinitrosalicylic acid (DNS) method (Miller 1959).

Statistical analyses

Experiments were performed in three or more independent replicates, as indicated. One-way ANOVA was performed to test the effects of nitrogen concentration in growth media on the tested plant parameters. Multiple comparisons were performed using a Tukey post hoc test, separately for shoots and roots. The relationship between the parameter values was examined using a Spearman correlation coefficient. The statistical analyses were conducted with the statistical package STATISTICA 8 (StatSoft Inc. 2007). For transparent presentation of results, the data for individual parameters were standardized (z) as $z=(x-\mu)/\sigma$, while x is the actual value of the parameter, μ is the average value and σ is the standard deviation for the given parameter. Importantly, the standardization had no effect on the analyses of variance. Standardized values of all measured parameters were plotted as star icon plots to show the impact of N concentration. Each ray (plotted from the twelve o' clock position) represents a different variable. The length of each ray represents the relative value of a particular variable.

Results

Nitrogen at sub- and supra-optimal concentrations was supplied to the growth media under hydroponic conditions. The N concentrations in the media were readily reflected in the foliar N contents; quadratic regression (at $P<0.05$, $F=3.88$) revealed a convex relationship with the highest values between 5 and 25 mM of nitrate (Fig. 1a). Simultaneously, foliar P content remained unaltered in the analyzed plants (Fig. 1b).

The N availability strongly affected the plant growth. The dry biomass of both shoots and roots was significantly lower at starvation as well as excessive N (>25 mM for shoots and >15 mM for roots) (Fig. 1c, d). Surprisingly, 30 mM N had a comparable effect to the standard concentration.

Out of the nine photosynthetic parameters measured, most were significantly dependent on the N supply (Fig. 1e, f, g). Chlorophyll fluorescence showed a linear increase of F_{max} and Fv/Fm with the increasing N dose (Fig. 1g), while the F_0 did not change significantly (data not shown). The contents of chlorophyll pigments [Chl *a*, Chl (*a*+*b*), *Car*, Chl (*a*+*b*)/*Car*] were more variable, only that of Chl *b* was unaffected by N dosing. Generally, a linearly increasing positive effect was observed with concentration of N in the media (the strongest at 30 mM N), but this was interrupted by a non-significant drop at 25 mM N. The weight ratio of Chl *a* and Chl *b* as well as the Chl *a*/Chl *b* to total carotenoids ratio were affected at this dose only (Online resource 1). Thus, even at the highest N doses, the plants seem to photosynthesize efficiently.

Common biochemical signs of stress, such as the rate of membrane lipid peroxidation (determined as level of MDA) and proline contents significantly increased in the roots at 30 mM N, indicating a stress (Fig. 2a, b). In shoots, the moderate accumulation of proline with the increasing N concentration was significant ($P<0.05$) (Fig. 2b). Since two proline synthesis pathways are known (the glutamate and the ornithine pathway), we studied the activity of proline synthase *P5CS* and ornithine δ-aminotransferase *OAT* genes. The *P5CS* was only detected in the shoots and was strongly induced at two excessive N doses (15 and 30 mM) (Fig. 2c). In contrast, the *OAT* gene was expressed only in the roots, with a strong induction at 35 mM N (Fig. 2d). This suggests that elevated proline levels in shoots relate to the expression of *P5CS*, but in roots it is the *OAT* gene. Nevertheless, the levels of proline and membrane peroxidation rate correlate with each other ($R=0.7$, $P<0.05$) and suggest that extreme N values causes stress, in an organ-specific manner (Table 1).

Total activities of glucanases and chitinases reflected variable responses in a dose- and tissue-dependent manner (Fig. 2e, f). In the absence of N in the media, total chitinase activities in the leaves were almost fivefold higher than in roots (Fig. 2e). At higher N concentrations, however, the activities in both organs were comparable, while a gradual (non-significant) increase was detected with increasing N (Fig. 2e). The total activities of glucanases at zero N were similar in both organs (Fig. 2f). With increasing N doses, the activities in both organs decreased, however this trend was interrupted by relatively high values at 15 mM N in roots and 30 mM N in shoots (Fig. 2f). Thus, the data support a strong effect of N nutrition on overall activity of selected PR-enzymes.

Total enzyme activities, however, do not necessarily provide relevant data for making general conclusions. Our results show that they result from the action of several isoforms of at least four sizes in shoots (65, 48, 40 and 35 kDa) as well as roots (70, 50, 45 and 35 kDa) (Online resource 2; Fig. 3). These comprise at least four acidic and four basic isoforms in both organs, respectively (Online resource 2). The icon plots of all quantified isoforms (ten in both organs, respectively) suggest their different regulation in the two organs, responding more sensitively to N excess than deficiency (especially in shoots) (Fig. 3a). In roots, a single acidic (*A*) and two basic (*a* and *b*) isoforms are significantly induced with an increasing N dose (Fig. 3b, c). In shoots only the 35 kDa isoform fraction was activated at 30 mM N, while a single acidic chitinase (*B*) was significantly inhibited by increased N doses (Fig. 3e, f; data corresponding to isoforms unaffected by N are not shown).

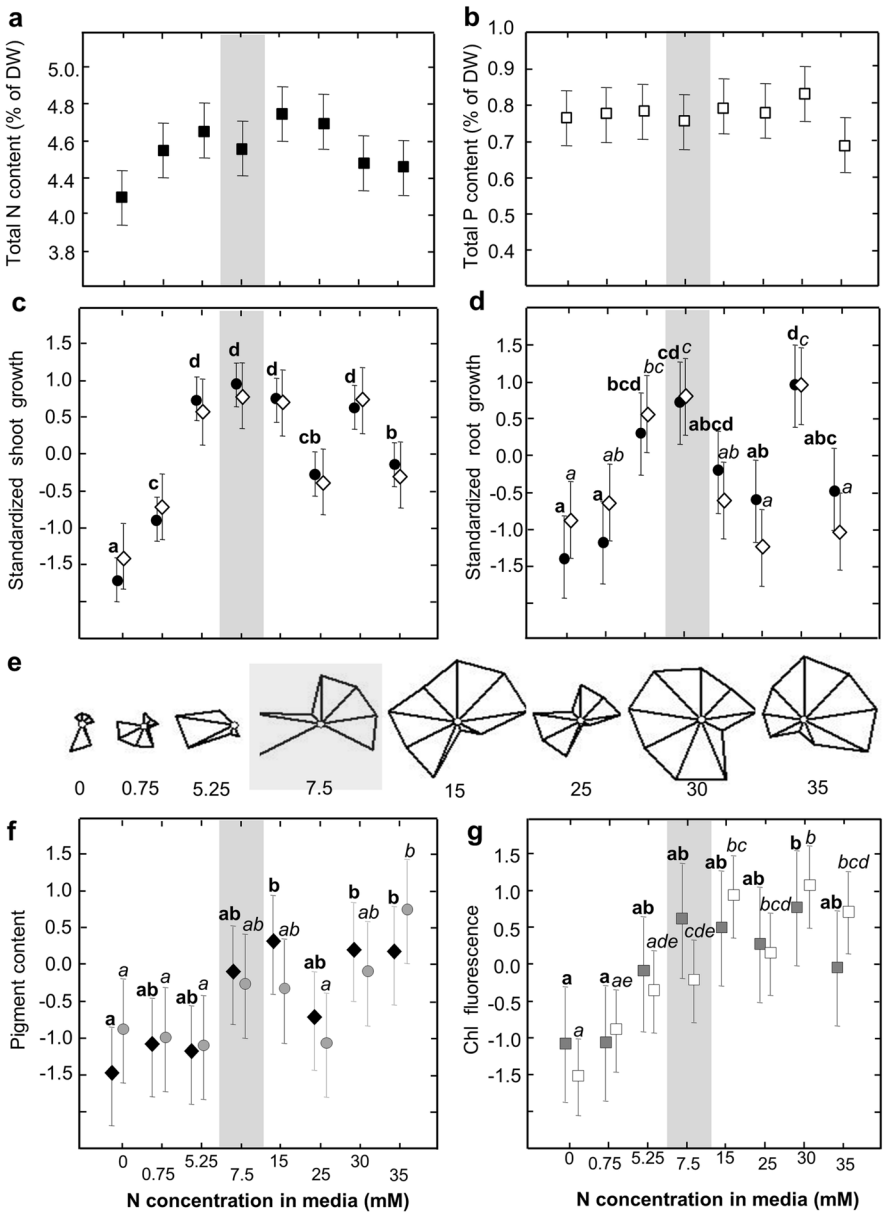

Fig. 1 Influence of different N concentrations in growth media on plant parameters. Foliar N content (**a**), and P content (**b**) were determined in shoots. Dry weight (*filled circles, bold letters*) and length (*empty rectangles, italics*) was determined for shoots (**c**) and roots (**d**). The *icon plot* (**e**) shows the data of nine photosynthetic parameters (clockwise from twelve o' clock position: content of Chl *a*, Chl *b*, total Chl (*a* + *b*), total carotenoids *Car*, Chl *a*/Chl *b*, Chl (*a* + *b*)/*Car*, minimal fluorescence F_0, maximal fluorescence F_{max} and quantum yield Fv/Fm). The length of each ray in the icon plot represents the relative value of the particular variable. All pigment parameters were affected except for Chl *b*; the typical trend of the data is shown for Chl *a* (*black symblos, bold letters*) and *Car* (*grey symbols, italics*) (**f**). N-dependent chlorophyll fluorescence parameters were the F_{max} (*gray symbols, bold letters*) and the Fv/Fm (*empty symbols, italics*) (**g**). Data in graphs are standardized average values with 95 % confidential intervals (n = 4 for **a, b**; n = 9 for **c, d** and n = 5 for **f, g**). *Different letters* indicate significance at $P < 0.05$

For the glucanases, up to four different isoforms in roots (150, 68, 45, and 35 kDa) and five in shoots (150, 68, 50, 38 and 30 kDa) were detected in the early stages for the wheat plants (Online resource 2). The icon plots of the quantified isoforms (nine from roots and seven from shoots) display larger activity changes with changing N in roots when compared with shoots (especially at lower N doses) (Fig. 4a). Only the 68 kDa isoform in roots (Fig. 4b) and the 150 kDa isoform in shoots (Fig. 4e) were significantly N-responsive; they were both highly active during starvation but were inhibited or unaffected at excessive N. Separation of differently charged proteins identified an acidic root glucanase (isoform *B*) that was inhibited by excessive N in the media (Fig. 4c), and two acidic glucanases in shoots that were affected by excessive N (isoforms *A, B*) and also by N starvation (*A*) (Fig. 4f). (Data corresponding to isoforms unaffected by N are not shown).

Discussion

The nutritional status of plants influences the plant growth and availability of resources for defense. The highest applied N doses caused the foliar N to reach the full accumulation capacity of the plants (Fig. 1), probably due to inhibition of enzymes involved in nitrate metabolism (Sánchez et al. 2001). Nevertheless, shortage as well as high excess N caused growth inhibition, as observed for *Lotus japonicus* (Márquez et al. 2005). In contrast to our results, 4 and 12 mM NO_3^- had no effect on wheat

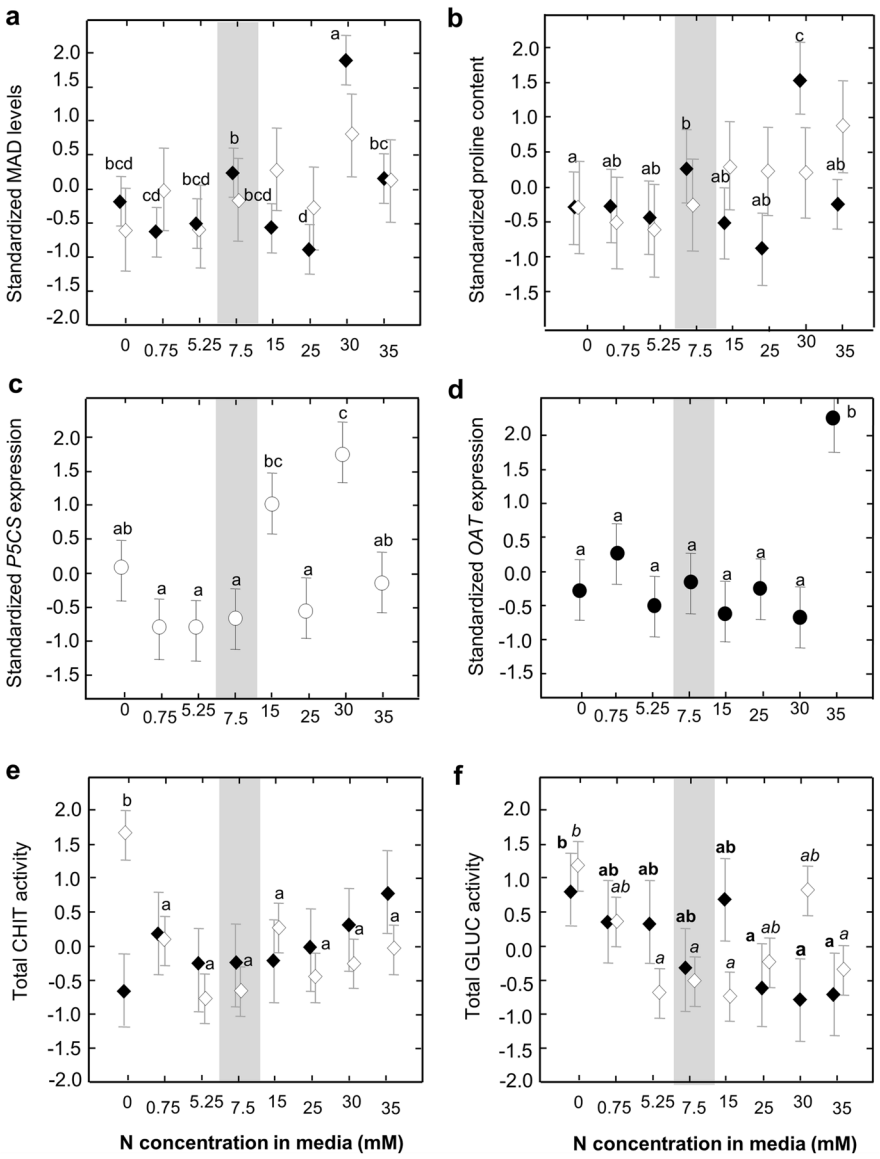

Fig. 2 Influence of different N concentrations in growth media (0–35 mm) on levels of membrane lipid peroxidation (**a**), proline (**b**), transcripts for *P5CS* (**c**) and *OAT* genes (**d**), total enzyme activities of chitinases (CHIT) (**e**) and glucanases (GLUC) (**f**). Shown are data for roots (*black symbols, bold letters*) and shoots (*empty symbols, italics*) as standardized average values with 95 % confidential intervals (n = 9 for **a, b** and n = 3 for **c–f**). *Different letters* indicate significance at $P < 0.05$

(*T. aestivum*) and maize (*Zea mays*) plants (Cramer and Lewis 1993), and limited N supply resulted in positive root growth of poplar (Wei et al. 2013). Contradictions are believed to result from the different N buffering abilities of the growth media, which is usually low for culture media in comparison to soil (Wang et al. 2004). It is noteworthy that the curvilinear pattern of the growth parameter data were repeatedly distorted at certain (mainly 30 mM) N doses (Fig. 1c, d). Moreover, the results of some downstream analyses of the same plants showed a similar distortion (e.g. some photosynthesis parameters, proline content in roots, total chitinase activity in shoots), while others did not (e.g. proline content in shoots, mineral content) indicating specificity of responses to the N conditions. This phenomenon likely relates to variable nitrate uptake by plants in response to external nitrate supply fluctuations (Malagoli and Le Deunff 2014a, b). Furthermore, the four known nitrate transport systems in plants exhibit different sensitivities to nitrate and operate at a different NO_3^- concentration ranges (Wang et al. 2012a; Migocka et al. 2013). This affects nitrate uptake and consequent plant growth. Re-evaluation of the linearity of nitrate uptake and transport in plants within wider ranges of concentration has been suggested (Wang et al. 2012b).

The N concentration in media positively correlated with the content of Chl *a*, total Chl (*a* + *b*) and carotenoids in plant tissue (Online resource 3) as observed previously in tropical woody seedlings during acclimation to nitrogen limitation (Kitajima and Hogan 2003). On the other hand, correlation with Chl *a*/*b* ratio, an indicator of the functional pigment equipment and adaptation of the photosynthetic apparatus, was low (especially for concentrations below

Table 1 Correlation coeficient (R) and corresponding significance (P) for selected parameters

Parameter 1	Parameter 2	R	P
Proline_shoot	N in media	0.81	0.014
Chlorophyll a	N in media	0.81	0.014
Basic GLUC_shoot_b	N in media	0.88	0.004
Basic CHIT_shoot_IF_d	N in media	0.95	0.001
CHIT_shoot_IF_65 kDa	N in media	0.76	0.028
GLUC_shoot_IF_38 kDa	N in tissue	−0.83	0.010
GLUC_shoot_IF_30 kDa	N in tissue	−0.83	0.010
Proline_shoot	MAD	0.62	0.101
Chlorophyll a	MAD	0.83	0.010
Acid GLUC_shoot_IF_A	Proline	0.74	0.036
Basic GLUC_shoot_IF_A	Proline	0.64	0.085
Basic CHIT_shoot_IF_d	Proline	0.86	0.006
CHIT_shoot_IF_65 kDa	Proline	0.76	0.028
Acid CHIT_root_IF_A	N in media	0.83	0.010
Basic CHIT-root_IF_a	N in media	0.76	0.028
Basic CHIT-root_IF_b	N in media	0.93	0.001
GLUC total_root	N in media	−0.83	0.010
Proline_root	MAD	0.74	0.036
GLUC total_root	CHIT total_root	−0.81	0.015

7.5 mM) (Online resource 1). Alterations of the Chl *a*/*b* ratio can indicate changes in the thylakoid membrane structure under starvation and/or synthesis deficiency. The relationship of photosynthetic parameters with excessive N in media was not linear, possibly for the same reasons as discussed above. Balancing a limitation elsewhere in the photosynthetic system has been suggested e.g. through an activation state of Rubisco that might serve as a storage protein at high N concentrations (Cheng and Fuchigami 2000). The foliar P content in shoots was unaffected by N.

Dose of N in growth media as a stressing factor

The N concentration in the media significantly increased the rate of membrane lipid peroxidation in roots at the 30 mM excess level. Levels of MDA correlated in both organs with levels of proline (Table 1). Elevated proline content is common in stressed plants but under variable nitrate supplythe actual state is more complex. Since proline is also a nitrogenous storage compound, the correlation between the N availability and the accumulation of proline is usually positive (Sánchez et al. 2001 and therein). Here, an accumulation of proline was observed for elevated (but not limited) N doses. Under stress conditions, such as high salinity and N limitation, the proline biosynthesis probably occurs predominantly *via* the glutamate pathway (Sánchez et al. 2001) as activation of the *P5CS* gene has only been observed in shoots but responses of the *OAT* to stresses are contradictory (reviewed in Verslues and Sharma 2010). Similarly, in our study, the *P5CS* gene was active only in the shoots but the response to stress can be applied only for high N doses. In contrast, the gene for an alternative proline synthesis pathway, the *OAT*, was active in roots and induced at the highest N excess. Previously in bean plants exposed to N excess, the P5CS enzyme activity dropped in both roots and leaves, and the OAT activities significantly rose reflecting the enhanced ornithine pathway (Sánchez et al. 2001). Based on our data we conclude that wheat roots at 30 mM N suffered from a stress. Here it is also important to note that the data of proline content and corresponding gene expression can be affected by post-translational regulation and/or metabolic flux rates, as well as by the presence of highly similar homologues.

Effect of N concentration on defense-related enzymes

The results for root samples show an increase of total chitinase activity with N supply, as observed previously (Dietrich et al. 2004). Total chitinase activity has been positively correlated with tolerance to pathogens (Zur et al. 2013), salt stress (Mészáros et al. 2014) and metals (Mészáros et al. 2014). In contrast, the total glucanase activity of samples displayed a negative correlation with N concentration in media ($R=-0.83$, $P=0.010$) (Table 1). Correlation analyses revealed that these two enzymes might act in roots (but not shoots) in the opposite way in response to various N doses ($R=-0.81$, $P=0.015$) (Table 1), supposedly due to different regulation mechanisms and functions in plants (Wu and Bradford 2003). Different tissues have different sets and functions of PRs (Gálusová et al. 2014; this study); e.g. in roots they are likely part of a pre-existing mechanism against soil-borne pathogens (Gregorová et al. 2015).

Among enzyme profiles, and similar to those described previously (Gregorová et al. 2015), we identified several chitinase isoforms (three in roots and one in shoots) dependent on the N concentration in media. Previously, a single basic chitinase has been identified in wheat as (positively) responsive to fertilization regime (Chandna and Ahmad 2015), while others identified no chitinase response to nitrate availability in the wheat proteome (Hakeem et al. 2012). Wang et al. (2012a) detected a chitinase precursor in peach roots at elevated nitrate levels, but the protein accumulation was strongly repressed. Similarly, several glucanases exerted N-dependent behavior in wheat. None of the above-mentioned high-throughput studies in wheat, rice and peach detected any glucanase responsive to N supply. A β-1,3-glucanase activity has been shown to affect fiber strength in cotton depending on N-fertilization rate, possibly by releasing monomers for cellulose synthesis and by facilitating cell expansion for cellulose deposition (Wang

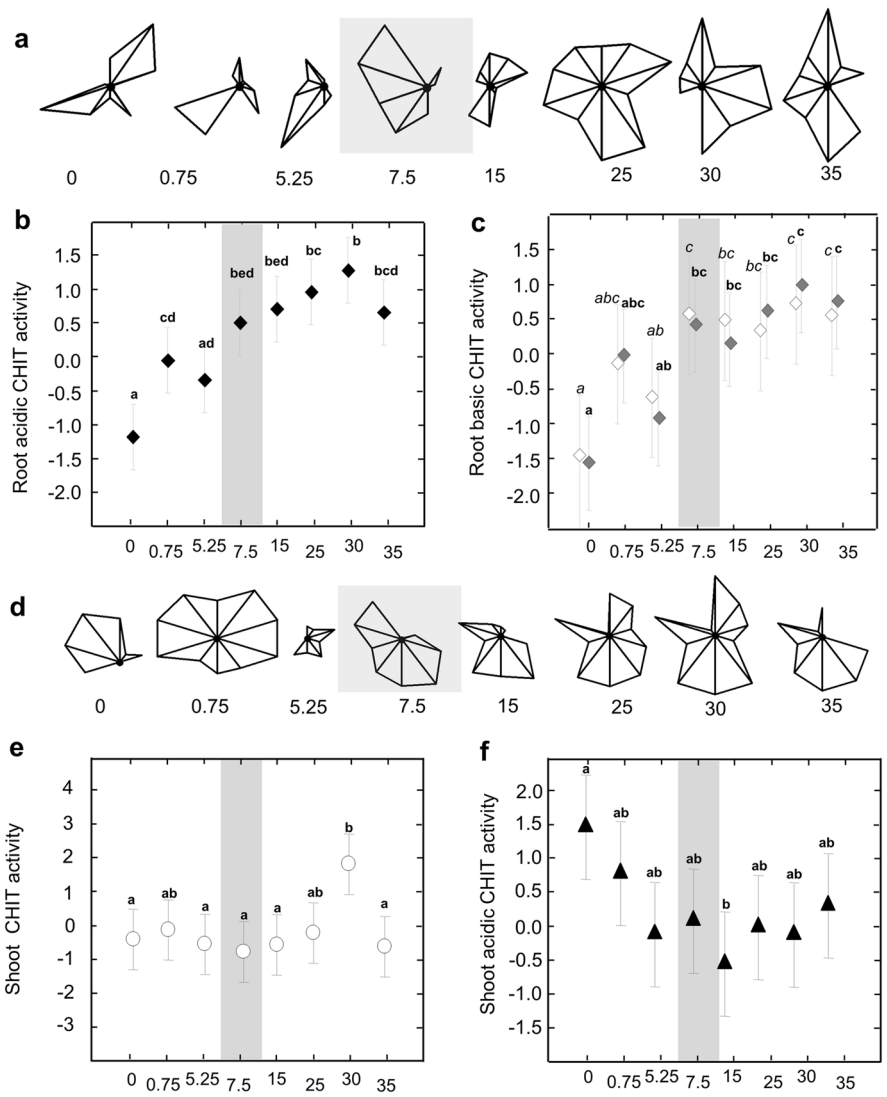

Fig. 3 Influence of different N concentrations in growth media (0–35 mM) on activity of chitinase (CHIT) isoforms in roots (**a–c**) and shoots (**d–f**). The icon plot displays the activities of the all ten isoforms quantified in roots (clockwise from twelve o'clock position 70, 50, 45, 35 kDa, basic *a* and *d*, acidic *A–D*) (**a**), pattern of the significantly affected acidic isoform *A* (**b**), and the two basic isoforms *b* (*grey symbols, bold letters*) and *d* (*empty symbols, italics*) (**c**), icon plot with activities of the all CHIT isoforms quantified in shoots (clockwise: 65, 48, 40, 35 kDa, basic isoforms *a* and *d*, acidic *A–D*) (**d**). The N supply affected the shoot isoform of 35 kDa (**e**) and the acidic chitinase *B* (**f**). Data are standardized average values with 95 % confidential intervals (n = 5). *Different letters* indicate significance at $P < 0.05$. The length of each ray in the icon plot represents the relative value of particular variable

et al. 2009). At lower as well as higher N doses the enzyme was comparatively less active (in contrast to the glucanase isoform in this study; Fig. 4) and resulted in lower fiber strengths (Wang et al. 2009).

Interplay and possible role of the studied PR enzymes under different N availability

We identified several chitinase and glucanase isoforms that seem to contribute to solving the trade-off between the growth and defense. They display different functional and/or spatial specificity of individual isoforms in different organs, as described recently in adult wheat plants in response to long-term drought (Gregorová et al. 2015) and during germination of *Solanaceous* seeds (Petruzzelli et al. 1999). Nevertheless, under starving conditions, their functioning might consume resources initially dedicated to growth (Smakowska et al. 2016).

Our data show that the N-responsive glucanases and chitinases are regulated in an opposed way to meet the cell requirements under non-optimal nutrition resources. The role of chitinases in the N response is not clear; it is proposed that they alter the flexibility-elasticity ratio of the cell wall, enabling it to withstand the adjustment of the size of cells during stress or growth (Konno et al. 2008). Since chitinases have no known endogenous substrate in plants (except for non-specific arabinogalactans), they might represent merely (or mainly) a resistant trait (Dietrich et al. 2004). They can also generate signal molecules triggering downstream defense responses. Since some detected chitinases are clearly favoured by N supply (Fig. 3a, b), they might also serve as storage proteins (Avice et al. 2003). In contrast to chitinases, some glucanases appear to be induced at both N extremes (Fig. 4f); N-responsive but not N-conditioned activity might indicate an important morphological rather than a (costly) defense role. On the

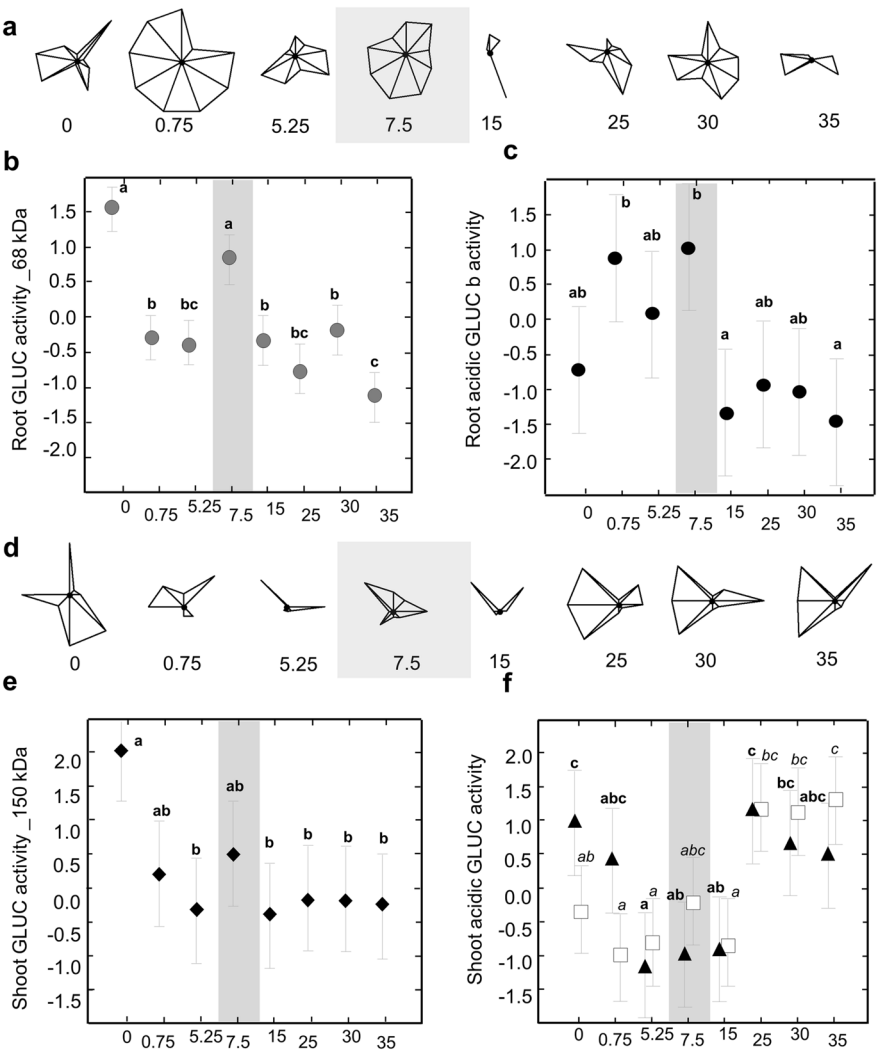

Fig. 4 Influence of different N concentrations in growth media (0–35 mM) on activity of individual glucanase (GLUC) isoforms in roots (**a–c**) and shoots (**d–f**). The icon plot shows data for the all nine root isoforms quantified (clockwise from twelve o'clock position 150, 68, 45, 35 kDa, acidic *A–C*, basic *a, b*) (**a**). Pattern of the significantly affected 68 kDa isoform (**b**), and the acidic isoform *B* (**c**). Icon plot with data for quantified shoot isoforms (clockwise: 150, 68, 50, 38, 30 kDa, acidic *A*-*B* and basic isoform *a*) (**d**), pattern of the significantly affected isoforms of 150 kDa (**e**), and of the two acidic isoforms *A* (*empty square, italics*) and *B* (*black triangle, bold letters*) (**f**). Data are standardized average values with 95 % confidential intervals (n = 5). *Different letters* indicate significance at $P < 0.05$

other hand, the drop of their activity with N concentration (Fig. 4b) might contribute to the regulation of symplastic trafficking, gradient formation and signalling through degradation of strategically localized callose (β-1,3-glucan) (Piršelová and Matušíková 2013). The relevance of these mechanisms for N utilization or stress, however, has been poorly studied. Recently, Zhang et al. (2014) described a signalling module formed by ethylene, jasmonate and low affinity nitrate transporters (ET/JA/NRT) that enables a cross-talk between stress-initiated nitrate allocation to roots and the environment. This module further coordinates nitrate allocation and the trade-off between the growth and environmental adaptation. Given that the ET/JA signalling pathway also regulates many PR proteins (Wu and Bradford 2003; Smakowska et al. 2016), this model is probably responsible for nitrate-dependent activity of specific glucanase/chitinase isoforms, too (Table 1). We might speculate that the behaviour of these particular isoforms over the applied N-range reflects the altered energetic conditions in plants (e.g. the acidic and the basic chitinase isoforms in roots, Fig. 3b, c), a response to stressing environment (e.g. the 150 kDa glucanase in shoots or the biphasic acidic glucanase in roots, Fig. 4c), or both. Although the exact role of individual isoforms in cells is unknown, those with enhanced activities at nutrition extremes appear to trade-off growth processes. An experiment with additional (biotic or abiotic) stress types might bring further knowledge on nutrient-dependent PR responses in plants. The multiple functions of these enzymes render them good candidates for the existing cross-talk between the defense and growth/developmental processes (Walters and Heil 2007).

Conclusion

Data on physiological and growth parameters indicate that plant responses are not linearly dependent on N doses in the growth media. We also showed that nutrition availability

affected the activities of PRs, and several chitinase and glucanase isoforms responded to extreme N fertilization regimes in an organ-specific manner. The possible roles of these enzymes in the adaptation to N supply are largely unknown, and their role as defense components remains to be elucidated. A more complex study on the effects of different fertilization regimes on PRs activity under additional stresses might bring valuable knowledge for efficient fertilization and plant protection strategies.

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Compliance with ethical standards

Conflict of interest The authors report no conflict of interest.

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