Growth Stimulatory Effects and Genome-Wide Transcriptional Changes Produced by Protein Hydrolysates in Maize Seedlings

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Protein hydrolysates are an emerging class of crop management products utilized for improving nutrient assimilation and mitigating crop stress. They generally consist of a mixture of peptides and free amino acids derived from the hydrolysis of plant or animal sources. The present work was aimed at studying the effects and the action mechanisms of a protein hydrolysate derived from animal residues on maize root growth and physiology in comparison with the effects induced by either free amino acids or inorganic N supply. The application of the protein hydrolysate caused a remarkable enhancement of root growth. In particular, in the protein hydrolysate-treated plants the length and surface area of lateral roots were about 7 and 1.5 times higher than in plants treated with inorganic N or free amino acids, respectively. The root growth promoting effect of the protein hydrolysate was associated with an increased root accumulation of K, Zn, Cu, and Mn when compared with inorganic N and amino acids treatments. A microarray analysis allowed to dissect the transcriptional changes induced by the different treatments demonstrating treatment-specific effects principally on cell wall organization, transport processes, stress responses and hormone metabolism.

Keywords: biostimulant, ionomic analysis, hormone metabolism, maize, microarray analysis, protein hydrolysates, root, transport

INTRODUCTION

The availability of mineral nutrients in the soil represents one of the most important limiting factors for crop productivity which is therefore highly dependent on the vast use of fertilizers (Tilman et al., 2002). However, excessive application of fertilizers as well as agrochemicals is causing severe environmental problems resulting in massive ecological degradation throughout the world (Tilman et al., 2002). Hence, the improvement of crop nutrient use efficiency that could reduce the use of fertilizers without any penalty on productivity, is worldwide an important goal (Baligar et al., 2001).

In this scenario, biostimulants are an emerging class of crop management products aiming at the mitigation of crop stress and improvement of nutrient assimilation (Halpern et al., 2015). The European Biostimulant Industry Council (EBIC) define these products as “materials which contain substance(s) and/or microorganisms whose function when applied to plants or the rhizosphere is to stimulate natural processes to benefit nutrient uptake, nutrient use efficiency, tolerance to abiotic stress, and/or crop quality, independently of its nutrient content” (http://www.biostimulants.eu). The emerging biostimulants market is estimated to grow of 10.4% from 2016 to 2021, reaching a
value of 2.91 billion USD and an area of application of 24.9 million hectares by 2021 (http://www.marketsandmarkets.com/search.asp?Search=biostimulants).

A variety of biostimulant compounds are available in the market (reviewed in Calvo et al., 2014). They are classified as microbial inoculants, humic substances, fulvic acids, protein hydrolysates and amino acids, and seaweed extracts. These formulations are usually composed by different molecules and therefore their effect can be the result of many components that may work synergistically. The positive effects of biostimulants on plants include: yield increase (Ertani et al., 2009), increase of abiotic stress tolerance (Zhang et al., 2003; El Hadrami et al., 2010) and nutrient assimilation (Varanini and Pinton, 1995; Canellas et al., 2002), enhancement of fruit quality (Masny et al., 2004; Karakurt et al., 2009) and soil microbial activity (Chen et al., 2002). Their positive influence on plant growth is not due to a direct fertilization effect because they are active at very low concentration (Calvo et al., 2014). They indeed exhibit auxin-like and gibberellin-like activities and thus they are thought to function as signaling molecules (Ertani et al., 2009, 2013).

The protein hydrolysates have been proven to stimulate root growth and leaf biomass of several crops. du Jardin (2015) reviewed various effects resulting from the application of these compounds to crops. Direct effects on plants include modulation of N uptake and assimilation by regulation of enzymes involved in N metabolism and by acting on the signaling pathway of N acquisition in roots (Ertani et al., 2009, 2013). They can also regulate enzymes of the TCA cycle, contributing to the interplay of C and N metabolisms (Schiavon et al., 2008). Protein hydrolysates can improve plant antioxidant defense against free radicals thus mitigating environmental stress (du Jardin, 2015). They are also known to increase microbial biomass and activity, soil respiration and soil fertility (du Jardin, 2015). The application of protein hydrolysates can modify the morphology of the roots, facilitating nutrient uptake as a consequence of the increased absorptive surface area (Ertani et al., 2013). Moreover, the chelating and complexing activities of specific amino acids and peptides in the substrates are supposed to enhance nutrients availability and acquisition by roots (Colla et al., 2014).

In perspective of a circular economy, the use of protein hydrolysates can contribute to environment protection. Indeed, protein hydrolysates are generally produced from industrial and agricultural organic waste, turning them into high value-added products and, at the same time, reducing the costs derived from their disposal.

Although the effects of protein hydrolysates on crop performance have been documented, the scientific basis of their action has partially been elucidated mainly due to the complex nature of these products. The present work aims at shedding light on the effects and the action mechanisms of a commercial protein hydrolysates. The product used in this work is obtained by chemical hydrolysis of animal by-products and consists of a mixture of small peptides and a low percentage of free amino acids. We assessed the effects of the protein hydrolysates on maize root growth in comparison with the effects produced by an equal amount of N supplied either as a free amino acids mixture mimicking the biostimulant composition, or as N inorganic compound (NH₄H₂PO₄). It is noteworthy that free amino acids are also included among the biostimulant compounds (Calvo et al., 2014). In order to elucidate the mechanisms underlying the effects observed in the root apparatus, root micro- and macro-nutrient accumulation was evaluated. Furthermore, we performed a transcriptome analysis that allowed identifying differential gene expression patterns in maize roots in response to the different forms of N supply highlighting global changes in gene transcription across multiple metabolic processes.

**MATERIALS AND METHODS**

### Plant Materials and Growth Conditions

Maize seeds (PO423 Hybrid, Pioneer Italia S.p.A.) were soaked in water for 24 h and germinated in the dark on wet filter paper for 72 h. The seedlings were then transferred to plastic pots containing 2 L of a 0.05 mM CaSO₄ solution and grown for 24 h under a 16/8 h light/dark regime at 22–26°C, 40–50% relative humidity, 125 µE m⁻²s⁻¹ light intensity. Each pot contained 12 seedlings that were grown in a diluted nutrient solution (Pinton et al., 1999) containing 100 mM MgSO₄, 5 µM KCl, 200 µM K₂SO₄, 175 µM KH₂PO₄, 400 µM CaSO₄, 25 µM NH₄H₂PO₄, 2.5 µM H₂BO₃, 0.2 µM MnSO₄, 0.2 µM ZnSO₄, 0.05 µM CuSO₄, 0.05 µM NaMoO₄, 2 µM Fe-EDTA and supplemented with either protein hydrolysates (SICIT2000 S.p.A.) or inorganic nitrogen (NH₄H₂PO₄) or a mixture of free amino acids mimicking the amino acids content of the protein hydrolysates (see also Results and Discussion section for treatment description). In all the treatments, the total N amount was kept constant at 5.65 or 11.3 mgL⁻¹. After 3 days, roots of 24 seedlings for each treatment (protein hydrolysate, inorganic N and free amino acids) at 5.65 or 11.3 mgL⁻¹ total N dose were collected for further analysis. The experiment was run three times obtaining three independent biological replicates.

### Phenotypic Analysis of Maize Seedlings

Primary, seminal and lateral root average length was evaluated using ImageJ software. For the measurement of lateral roots length, the 10 longest roots per plant were considered. Primary, seminal and lateral root total length and surface area were measured with the aid of WinRHIZO™ scanner and automated software (Arsenault et al., 1995).

### Macro- and Micro-Nutrients Quantification

The nitrogen concentration of the root samples was determined using the EA-IRMS Delta V (Thermo Fisher Scientific). The calibration curve for %N determination in dried tissues was performed using atropine (%N = 4.84).

Other macro- and micro-nutrients were quantified by ICP-MS analysis. Dried root samples (about 5 mg) were weighted and digested in a TFM microsampling insert using 250 µl of 69% ultrapure HNO₃. Three inserts were put into 100-ml oven vessel containing 10 ml of water (milliQ, 18.2 M cm) and 1 ml of 30% H₂O₂. In addition, 5 mg of the following reference material were digested: NIST 1515 (apple leaves). Sample digestion was performed using a microwave oven (Milestone StartD® microwave). A 20-min ramping period was used to...
reach a digestion temperature of 180°C, which thereafter was maintained for 20 min. At the end, samples were diluted with water (milliQ, 18.2 M cm) to a final concentration of 3% HNO3. Multi-elemental analysis was carried out using the Agilent 7500cx ICP-MS (Agilent). The instrument was tuned using tuning solution (Agilent tuning solution 1 ppb) in a standard mode checking the sensitivity of masses 7Li, 89Y, and 205Tl and the oxide and double charged ion levels (< 2%). Each macro- and micronutrient were quantified using a multi-element standard solution.

**RNA Extraction and Microarray Analyses**

Total RNA was isolated from plants treated with protein hydrolysates, inorganic N and free amino acid mixture at the highest N concentration (11.3 mg L−1) using the Spectrum™ Plant Total RNA kit (Sigma-Aldrich) and quantified by spectrophotometry using NanoDrop™ 1000 (Thermo Scientific). RNA quality was evaluated using Agilent 2100 Bioanalyzer (Agilent). For each sample, the reactions of cRNA synthesis and labeling were carried out using 200 ng of total RNA and the Low Input Quick Amp Labeling Kit, One-Color (Agilent) and Cyanine 3 (Cy3)-CTP fluorescent dye according to the Agilent technical manual (http://www.agilent.com). Cy3-labeled cRNA (1.65 µg) of each sample was hybridized on a custom 4x44K Agilent array according to manufacturer's manual for 17 h at 65°C and scanned on Agilent G2565CA Microarray Scanner System (Agilent). Array hybridizations and washing were performed according to manufacturer's manual (One-Color Microarray-Based Gene Expression Analysis—Low Input Quick Amp Labeling—Protocol). Each “subarray” allow analyzing the expression of 39,372 maize transcripts between Bio vs. N, Aa vs. N, and Bio vs. Aa were identified through Student’s t-test using MeV software (http://mev.tm4.org/#/welcome) setting with the following parameters: p-value based on permutation with critical p-value of 0.01 and adjusted Bonferroni correction. Differentially expressed transcripts were filtered on the basis of fold changes value ([FC]≥2). All microarray expression data are available at the GEO (http://www.ncbi.nlm.nih.gov/geo) under the series entry (GSE89535).

### Quantitative RT-PCR Analysis

For the quantitative RT-PCR (qRT-PCR) we used the same RNA samples extracted as described above. Three cDNA samples derived from 3 independent RNA samples were analyzed. DNase treatment and reverse transcription were performed as described in Molesini et al. (2014). cDNA amplification and PCR cycling conditions and product dissociation curve were also performed as indicated in Molesini et al. (2014). Data from qRT-PCR experiments were analyzed according to the 2−ΔΔCt method. The list of primers adopted for qRT-PCR is reported in Supplementary Table 1. UBCE gene, coding for ubiquitin-conjugating enzyme, was used as reference gene (Manoli et al., 2012).

### RESULTS AND DISCUSSIONS

#### Protein Hydrolysates and Free Amino Acids Display Different Stimulatory Effects on Root Growth

To investigate the effects of protein hydrolysates, we grew maize seedlings for 72 h after the emergence of the primary root in a N-free nutrient solution supplemented with the protein hydrolysate (Bio), inorganic nitrogen (NH4H2PO4·N) or a mixture of amino acids (Aa) mimicking the composition in amino acids of the protein hydrolysate. The protein hydrolysate is a liquid formulate derived from the hydrolysis of cow connective tissue, a by-product of tanning industry. It contains 30% (w/w) organic matters (C), 11.3% (w/w) total nitrogen (N), 10% (w/w) organic N, of which 62.5% (w/w) total amino acids and 10% (w/w) free amino acids (the detailed amino acids composition is summarized in Supplementary Table 2). The molecular weights of the peptides present in the protein hydrolysates range from 1,500 to 2,000 Da. To evaluate the dose response and the relative effects of Bio treatment on roots and shoots, we supplied the seedling with increasing doses of the biostimulant from 0.001 to 0.1 mL L−1. The growth of the shoots was not affected by the treatments, whereas the protein hydrolysate at 0.05 and 0.1 mL L−1 promoted the growth of the roots (Supplementary Figure 1). To analyze the effects of Bio treatments in terms of their contribution to N supply in the growth medium, we compared the root growth of maize seedlings treated with Bio at 0.05 and 0.1 mL L−1 and seedlings treated with equivalent amounts of total N (5.65 and 11.3 mg L−1, respectively) supplied either as inorganic N (NH4H2PO4) or free Aa. The Aa treatment consisted of a mixture of free amino acids identical in composition and concentration to the amino acids present in the protein hydrolysates described in Supplementary Table 2. Both Bio treatments induced root growth (Figures 1A–C), this effect was particularly evident for the lateral roots whose average length was approximately 2 and 3 times higher than that of seedlings supplied with 5.65 and 11.3 mg L−1 inorganic N, respectively. Also the Aa treatments showed the capacity to promote root growth compared to N. The effect was detectable in the primary and seminal roots at the higher Aa concentration, whereas the average length of lateral roots was increased also with the lower concentration (Figures 1A–C). Interestingly, the protein hydrolysates, containing only 10% of free amino acids, had always a stronger effect on root growth than a treatment consisting of free amino acids only.

A rapid and efficient growth of root apparatus can be advantageous during the first phases of seedling emergence after sowing, increasing the seedling capacity to absorb water and mineral elements (Lynch, 1995). Therefore, we calculated
Protein Hydrolysates Increase the Uptake of Specific Nutrients

To study the effects of Bio and Aa on root nutrient accumulation, we quantified the macro- and micro-nutrient concentrations in the roots of seedlings treated with Bio, N, and Aa; in all the treatments, the total N supply was equal to 11.3 mg L⁻¹ (Figure 2). The root concentration of Ca, Mg, Na, and P did not show statistically significant variations irrespectively from the treatment applied (Figure 2). Among the macronutrients, only K concentration was significantly increased in Bio- and Aa-treated seedlings compared with N-treated ones (Figure 2). Regarding the micronutrients, the concentration of Fe and Mo was not modified by the different treatments, whereas Cu, Mn, and Zn concentrations increased Bio-treated roots, but not in Aa-treated roots. The strongest effect of the Bio treatment was observed for Mn whose concentration was more than 8-fold higher in Bio-treated than in N-treated roots (Figure 2). The increased level of K in roots treated with organic N might be related to its function in maintaining ion balance and stabilizing cellular pH. The improved accumulation of Cu, Mn, and Zn in protein hydrolysate-treated roots might be the result of a specific action on metal trasporters (see Section Transport Processes) or the consequence of the...
peptide metal binding capacity that might facilitate nutrient availability.

**Global Changes in the Root Transcriptome in Response to Protein Hydrolysates and Free Amino Acids**

The transcriptional changes in maize roots subjected to 3-day treatment with Bio, N, or Aa were analyzed by means of genome-wide microarray hybridization analysis. For this aim, we used an Agilent chip that allowed to analyse the expression of 39,372 among the predicted maize transcripts (Schnable et al., 2009; Release 5b; http://www.maizesequence.org/index.html).

Differentially expressed transcripts between roots supplied with different N forms were identified through a $t$-test (adjusted $p$-value $\leq 0.01$ and $|FC| \geq 2$). The analysis revealed that 995 transcripts were differentially expressed between Aa- and N-treated (Supplementary Table 3), 587 between Bio- and
N-treated roots (Supplementary Table 4) and 431 between Bio- and Aa-treated roots (Supplementary Table 5) (Figure 3A), indicating high dissimilarity between the three transcriptional profiles. Moreover, 79 transcripts were differentially expressed both in the comparisons Aa vs. N and Bio vs. Aa, 51 were in common between Bio vs. Aa and Bio vs. N and 190 between Bio vs. N and Aa vs. N (Figure 3A) (Supplementary Tables S3–S5). Only two transcripts were differentially expressed in all the three comparisons (Supplementary Tables S3–S5). The transcriptional profile of 5 differentially expressed transcripts (GRMZM2G347457_T01, GRMZM2G096958_T01, GRMZM2G429955_T01, GRMZM2G030036_T01, and GRMZM2G024996_T01, coding respectively for peptide transporter, nicotianamine aminotransferase1, chlorophyll a-b binding protein 2, nicotianamine synthase 1, and glycine-rich cell wall structural protein genes) was validated through qRT-PCR (Supplementary Figure 2). The annotation of all the up- and down-regulated transcripts was hand-curated, assigning them a “Gene Ontology” (GO) biological process term on the basis of a BlastP analysis. The transcripts were then grouped in main functional categories. In all the comparisons about 50% of the transcripts encode proteins with an unknown function and were assigned to the “biological process” class (Figure 3B, Supplementary Tables S3–S5). The other most abundant categories are “regulation of biological process,” “organic substance metabolic process” and “cellular metabolic process.” Interestingly, the transcripts belonging to “response to stress” were highly represented in Aa vs. N comparison while they were less abundant in Bio vs. Aa and Bio vs. N. Noticeably, the “nitrogen compound metabolic process” category was poorly represented, whereas the transcripts related to cellular transport (“establishment of localization”) were quite abundant.

Transcription Factors

The “regulation of biological process” category included several transcripts encoding transcription factors (TFs). We identified 61 TF transcripts in the Bio vs. N, 35 in Bio vs. Aa and 89 in the Aa vs. N comparisons (Supplementary Tables S3–S5).

Concerning the distribution of these transcripts in TF gene families, AP2-EREB, bHLH, MYB, WRKY, NAC were the most represented families (Figure 4). Recently, AP2-EREBP, bHLH, MYB, and WRKY TF families have been shown to participate in the response to nutrient stress playing a major role in controlling regulatory network related to root development and

![Figure 3](image-url)

**FIGURE 3** | Distribution of differentially regulated genes in the three comparisons and main functional categories of differentially expressed transcripts. (A) Venn diagrams showing the shared and the specific differentially regulated transcripts in the different treatments (|FC| ≥ 2; adjusted p-value ≤ 0.01). (B) Distribution of differentially regulated transcripts in the three comparisons Bio vs. Aa, Bio vs. N and Aa vs. N grouped into main functional categories. For each functional category, the transcript percentage is calculated on the total of the differentially expressed transcripts minus those belonging to the “biological process” category.
N-deficiency response (Zhao et al., 2005; Rushton et al., 2012; Takehisa et al., 2013; He et al., 2016). Tai et al. (2016) also reported that members of some of these TF families were differentially expressed in primary, crown and seminal roots of maize, suggesting functional specialization of the different root types.

AP2-EREB transcripts were both up- and down-regulated in the different comparisons, whereas those encoding WRKYs were mostly down-regulated in all the three comparisons. A member of AP2-EREB family, preferentially expressed in coleoptile nodes during maize root development, plays a role in the formation of crown roots (Mutreireh et al., 2013). PLETHORA2 in Arabidopsis (babyboom1, the homolog in maize) encodes another AP2-EREBP TF required for the formation of root stem cells (Aida et al., 2004). AP2-EREBP and WRKY family were proven to be involved in the differential root response to N limitation of two Chinese maize inbred lines (Chen et al., 2015). WRKY transcripts were also induced in rice root after N, P, and K deficiency (Takehisa et al., 2013).

MYB proteins are important regulator of different physiological processes in plants, including development, metabolism and responses to environmental stresses (Li et al., 2015). In our work, a large number of MYB transcripts were modulated in response to the different N sources, resulting all up-regulated in the comparisons Bio vs. Aa, whereas in the comparison Aa vs. N, 11 members of this family were down-regulated. Some members of MYB family in Arabidopsis and rice regulate lateral root development and modulate auxin-mediated progression of lateral root development (Dai et al., 2012; Gibbs et al., 2014). MYB TFs can also respond to N deficiency; a member of this family in cucumber showed a rapid induction after N deprivation and functions in ethylene and auxin signaling (Zhao et al., 2015). Interestingly, these results well fit with the enhanced lateral growth in roots of Bio-treated seedlings (Figure 1C).

Gene family encoding bHLH TFs was largely up-regulated in all the three comparisons. This TF family is implicated in the control of various biological pathways, including the plant response to nutrient deprivation (Yang et al., 2016). A rice bHLH family member (OsPTF1) mediates tolerance to P deprivation in rice (Yi et al., 2005). Another member of this family plays an important role in adaptation to the P- and N-starvation in Triticum aestivum regulating genes involved in the uptake of P and N (Yang et al., 2016).

Several NAC transcripts were also differentially modulated in all three treatments. A variety of NAC members isolated from different species participate in both biotic and abiotic stress signaling pathways enhancing drought and salt stress tolerance (Liu et al., 2014; Su et al., 2015). Lu et al. (2015) analyzed the function of 7 maize NAC demonstrating their role in ABA-dependent abiotic stress responses. Interestingly, a NAC member of Populus tremula x Populus alba, is implicated in roots response to N deficiency, probably regulating root growth under low N conditions (Wei et al., 2013). Similarly, NAC29 in Arabidopsis showed an elevated expression after N deprivation, and it was also responsive to chronic low N (Peng et al., 2007).

An interesting TF gene family is that of the LATERAL ORGAN BOUNDARIES domain (LBD) proteins. Intriguingly, every transcript belonging to this family was down-regulated in all the pairwise comparisons (AC149818.2_FGT009 and GRMZM2G092542_T01 in Bio vs. N; GRMZM2G092483_T01 in Bio vs. Aa; GRMZM2G092542_T01, AC234149.1_FGT002, AC149818.2_FGT009 and GRMZM2G073044_T01 in Aa vs. N) (Supplementary Tables S3–S5). These proteins display high functional diversity including regulation of lateral root formation and N metabolism (Xu et al., 2016). In Arabidopsis, LBD16,
LBD18, and LBD29 regulate lateral root organogenesis acting on auxin signaling pathway (Feng et al., 2012). The maize LBD protein RTCS (down-regulated in Aa vs. N and Bio vs. N) is the closest homologs of AtLBD16/29, is involved in seminal and shoot-borne root initiation (Taramino et al., 2007). Moreover, lateral root emergence requires LDB-dependent activation of EXPANSIN (Kim and Lee, 2013). Members of the LBD family were shown to negatively regulate genes involved in response to N limitation in Arabidopsis (Rubin et al., 2009).

Finally, two members of TEOSINTE BRANCHED1/CYCO IDEA/PROLIFERATING CELL FACTOR (TCP) family were up-regulated in the comparisons Bio vs. N (GRMZM2G180568_T01) and Bio vs. Aa (GRMZM2G060319_T01). A member of this family (TCP20) expressed in root tips and vascular tissue of Arabidopsis, was shown to modulate lateral root growth in response to N supply and to regulate the nitrate transporter NRT1.1 expression (Guan et al., 2014).

Overall, this analysis may suggest that the different N sources produce different effects on root growth and metabolism by distinct modulation of TFs involved in the control of root development and N availability.

**Cell Wall Components**

In the present work, we found numerous differentially expressed genes belonging to “cellular component organization” category in all the pairwise comparisons (18 in Aa vs. N, 6 in Bio vs. N and 7 in Bio vs. Aa) (Table 1). Almost all the differentially expressed transcripts of this category encode extensins, expansins, pectinesterases, Casparian strip membrane proteins, xylolucanendotransglucosylases/hydrolases, and glycine-rich cell wall structural proteins. In particular, the transcript GRMZM2G024996_T01, coding for a glycine-rich cell wall structural protein (GRP), showed the highest level of down-regulation in Bio vs. N (-19) and Aa vs. N (-63). This transcript showed high homology with a Petunia hybrida gene encoding GRP1 that is expressed during cell expansive growth and repressed during lignification (Condit, 1993). Genes involved in cell wall remodeling can regulate root growth and lateral root formation. Transcripts coding for expansins were modulated in all the three comparisons. It is well documented that these enzymes loosen the network of wall polysaccharides, allowing turgor-driven cell enlargement (Cosgrove, 2000).

**Table 1** | Differentially expressed transcripts involved in cell wall organization.

| Genome ID       | UniProt ID | Description             | Aa vs. N (FC) | Bio vs. N (FC) | Bio vs. Aa (FC) |
|-----------------|------------|-------------------------|---------------|---------------|-----------------|
| GRMZM2G070913_T01 | K7VZR1     | Pectinesterase          | 3.77          | 2.81          |                 |
| GRMZM2G112619_T01 | K7U9J0     | Xyloglucan endotransglucosylase/hydrolase | 3.28          | 2.93          |                 |
| GRMZM2G083888_T01 | CSY9J6     | Casparian strip membrane protein 4 | 2.80          |              |                 |
| GRMZM5G0886185_T01 | A0A096UH62 | Xyloglucan endotransglucosylase/hydrolase | 2.80          |              |                 |
| GRMZM5G0858456_T01 | B6SRP0     | Fucosyltransferase 7   | 2.80          |              |                 |
| GRMZM2G455564_T01 | K7TZ03     | Pectinesterase          | 2.41          |              |                 |
| GRMZM2G127184_T01 | B6SRP0     | Fucosyltransferase 7   | 2.39          |              |                 |
| GRMZM2G073079_T01 | Q9ZT66     | Endo-1,3;1,4-beta-D-glucanase | 2.24          |              |                 |
| GRMZM2G110832_T01 | B6T969     | Casparian strip membrane protein 1 | 2.23          |              |                 |
| GRMZM2G144888_T01 | Q1ZYQ8     | Expansin-B10            | 2.00          |              |                 |
| GRMZM2G114432_T01 | Q8H274     | Expansin-like A3        | -2.02         | -2.41         |                 |
| GRMZM2G435380_T01 | M8BPN6     | Polygalacturonase       | -2.06         |              |                 |
| GRMZM2G168651_T01 | P14918     | Extensin                | -2.11         |              |                 |
| GRMZM5G0870571_T01 | M7ZV5Y     | Galactoside 2-alpha-L-fucosyltransferase | -2.16         |              |                 |
| GRMZM2G392125_T01 | A0A096TJQ7 | Xyloglucan endotransglucosylase/hydrolase | -2.22         |              |                 |
| GRMZM2G167637_T01 | A0A096SUN3 | Pectinesterase          | -2.37         |              |                 |
| GRMZM2G113761_T01 | A0A096RU6S | Xyloglucan endotransglucosylase/hydrolase | -2.74         |              |                 |
| GRMZM2G024996_T01 | P27483     | Glycine-rich cell wall structural protein | -63.07        | -19.32        | -19.32          |
| GRMZM2G127072_T01 | B6UAK6     | Expansin-like 3         | 2.06          |              |                 |
| GRMZM2G043943_T01 | A0A096QHT7 | Pectinesterase          | -2.01         |              |                 |
| GRMZM2G109842_T01 | P35082     | Profilin-2              | -3.45         |              |                 |
| GRMZM2G164785_T01 | POC1Y5     | Expansin-B11            | 5.68          |              |                 |
| GRMZM2G152189_T01 | B6STF8     | Vegetative cell wall protein gp1 | 2.71          |              |                 |
| GRMZM2G153666_T01 | B6UBJ39    | Polygalacturonase       | 2.54          |              |                 |
| GRMZM2G413006_T01 | A0A096TNC3 | Xyloglucan endotransglucosylase/hydrolase | -2.19         |              |                 |
| GRMZM2G118759_T01 | B6TEE0     | Glycine-rich cell wall structural protein 2 | -2.24         |              |                 |
| GRMZM2G0499701_T01 | MTZJ85     | Expansin-A22            | -3.29         |              |                 |
| GRMZM5G0870571_T01 | K7V5L2     | Uncharacterized protein | -3.58         |              |                 |

Genome ID, Maize transcript ID (ZmB73_5b_FGS_cDNA.fasta.gz); FC, fold change value.
were up-regulated specifically in Aa vs. N. These proteins apparently regulate the transition of the lateral root primordia from flat to rounded morphology during root development (Lucas et al., 2013). Four and one transcripts coding for xylanogluconendotransglucosylases/hydrolases were differentially expressed in Aa vs. N and Bio vs. Aa, respectively. These enzymes play an important role in the remodeling of the xylanoglucon/cellulose framework in the wall during cell growth and differentiation (Hara et al., 2014). Similarly, pectinesterase enzymes (modulated in Aa vs. N and Bio vs. N) are involved in the process of cell wall extension.

Taken together these results suggest that transcriptional changes in genes encoding cell wall modifying enzymes induced by Bio and Aa, may result in cell wall remodeling that in turn affects root growth and architecture.

**Stress-Related Transcripts**

A relatively high number (38) of stress-related transcripts were differentially regulated in Aa vs. N comparison, whereas they were 14 and 6 in Bio vs. N and Bio vs. Aa, respectively (Table 2). About 50% of the stress related transcripts in Aa vs. N were represented by those coding for peroxidases (12 up-regulated, 5 down-regulated). At first glance, it is clear how the Aa treatment compared with N caused a higher stress response in the roots than Bio did. A possible explanation is that Aa caused an increase in ROS activity followed by an increase in expression of peroxidase genes for preventing H2O2 damage. Up-regulation of stress-related genes such as those for peroxidases and superoxide dismutases was observed in the root transcriptome of rice grown under P, K, and N deficiency (Takehisa et al., 2013). Moreover, ROS, such as H2O2 and O2- are known secondary messages in several pathways associated with responses to biotic and abiotic stresses in plants (Apel and Hirt, 2004). Aside from their anti-oxidative activity, peroxidases are the most abundant enzymes in the cell wall where they display a multifunctional activity, also related to growth regulation (Vuletic et al., 2014). Peroxidases exist in multiple forms exhibiting different cellular localization and playing numerous biological functions. Therefore, to identify the specific involvement of peroxidases in the response of roots to Aa and Bio would require further investigation. Among the other differentially expressed genes in this category, the majority are transcripts involved in biotic stress response (Table 2).

**Transport Processes**

Differentially expressed transcripts grouped in “establishment of localization” are involved in several transport processes (Table 3). We focused on genes playing a role in transport processes of amino acids, peptides, NO3- and NH4+. Considering amino acid transport, only in the Aa vs. N comparison, we found the up-regulation of a transcript encoding a putative amino acid permease (AAP; GRMZM2G180547_T01) that could be involved into transport across membranes. This result suggests that amino acid supply as N-source positively affected components involved in their uptake and/or translocation (Tegeder, 2012; Tegeder and Ward, 2012). Members of Amino Acid-Polyamine-Organocation (APC), Drug/Metabolite Transporter (DMT), ATP Binding Cassette (ABC), and Major Facilitator (MFS) super families play a role in amino acid export from the cytosol to apoplastic space or into intracellular compartments such as the vacuole (Okumoto and Pilot, 2011). Aa and Bio treatments modulated the expression of transcripts encoding protein belonging to DMT, ABC, and MFS families that can mediate these transport processes (Supplementary Tables S3, S4). In addition, members of the ABC and PTR families can be involved also in peptide transport (Koh et al., 2002; Waterworth and Bray, 2006). Other peptide transporters belong to the oligopeptide transporter (OPT) family (Koh et al., 2002; Waterworth and Bray, 2006). Concerning the PTR transporters, we observed a prevailing negative modulation of transcripts caused by Aa and Bio (GRMZM2G026523_T01, GRMZM2G122712_T01, GRMZM2G347457_T01, GRMZM2G057611_T01, GRMZM2G015767_T01). Focusing on OPT family, only the comparison Bio vs. N underlined the up-regulation of transcripts encoding a putative transporter (GRMZM2G152555_T01) suggesting a role of this gene in transport of peptides in Bio-treated maize roots.

Aa and Bio treatments caused also different modulations of transcripts involved in the uptake of N inorganic forms (NO3- and NH4+). Aa up-regulated (Aa vs. N) the ZmNRT2.2 (GRMZM2G010251_T01; Plett et al., 2010), a well-known gene involved in the inducible high affinity transport systems (iHATS) in maize roots (Garnett et al., 2013; Zamboni et al., 2014; Pii et al., 2016). Both Aa and Bio treatments down-regulated the ZmNRT1.2 transcript encoding a low affinity NO3- transporter (GRMZM2G137421_T01; Garnett et al., 2013), while the expression of another low affinity NO3- transporter, ZmNRT1.4B (GRMZM2G476069_T01) which is very low expressed during plant development (Garnett et al., 2013), seems to be Bio-specific (up-regulated both in Bio vs. Aa and Bio vs. N). Another Bio-specific transcript encodes a putative NH4+ transporter (AMT2; GRMZM2G335218_T01, down-regulated both in Bio vs. Aa and Bio vs. N).

Our analysis showed that transcripts involved in the uptake systems of other mineral nutrients were selectively affected by Aa and Bio. Organic N sources stimulated the expression of transcripts encoding putative stripe-like (YSL) transporters (GRMZM2G156599_T01, GRMZM2G024196_T01, GRMZM2G135291_T01) involved into the uptake of iron-phytosiderophore and distribution of metals within the whole plant (Curie et al., 2009). ZmYSL1 (GRMZM2G156599_T01) encodes a protein that acts as a proton-coupled symporter of metals chelated to phytosiderophore and to nicotianamine (Schaaf et al., 2004) playing a key role in Strategy II of Fe acquisition utilized by the graminaceous species such as maize (Kobayashi and Nishizawa, 2012). A noticeable difference in the responses to organic N forms relies in the behavior of genes involved in nicotianamine and phytosiderophore biosynthesis. In particular, only in the Aa vs. N comparison we could observe a positive modulation of transcripts involved into nicotianamine synthesis (nicotianamine synthase, NAS; GRMZM2G030036_T01, AC233955.1_FGT003, GRMZM2G124785_T01, GRMZM2G034956_T01, GRMZM2G38520_T01, GRMZM2G312481_T01) and into phytosiderophore biosynthesis (deoxymugineic acid synthase, DMAS; GRMZM2G060952_T01) (Kobayashi and Nishizawa, 2012). However, in the
### TABLE 2 | Differentially expressed stress-related transcripts.

| Genome ID | UniProt ID | Description | Aa vs. N (FC) | Bio vs. N (FC) | Bio vs. Aa (FC) |
|------------|------------|-------------|---------------|---------------|----------------|
| GRMZM2G138450_T01 | A0A096SAM6 | Peroxidase | 19.08 | | |
| GRMZM2G10175_T01 | D7NLB3 | Peroxidase | 15.97 | | |
| GRMZM2G150731_T01 | B4FYH1 | Peroxidase | 6.40 | | |
| GRMZM2G407740_T01 | C0P3T3 | Peroxidase | 5.32 | | |
| GRMZM2G133475_T01 | A5H454 | Peroxidase | 6.64 | 4.86 | |
| GRMZM2G010640_T01 | A0A096PWP5 | Peroxidase | 4.27 | | |
| GRMZM2G0404676_T01 | A0A096TLS6 | Peroxidase | 4.19 | | 2.11 |
| GRMZM2G023840_T01 | K7VCN5 | Peroxidase | 3.46 | | |
| GRMZM2G09792_T01 | A0A096PVZ5 | Uncharacterized protein | 3.41 | | |
| GRMZM2G037156_T01 | K7UT08 | Peroxidase | 3.29 | | |
| GRMZM2G089982_T01 | B6T3V1 | Peroxidase | 2.17 | | |
| GRMZM2G405459_T01 | A0A096TLS6 | Peroxidase | 2.16 | | |
| GRMZM2G038665_T01 | CSXLZ2 | MLO-like protein | -2.06 | -2.54 | |
| GRMZM2G017116_T01 | B6TR53 | Defense-related protein | -2.07 | -2.75 | |
| GRMZM2G103442_T01 | A0A096RM9 | Peroxidase | -2.18 | | |
| GRMZM2G108487_T01 | A3FMA3 | Putative serine type endopeptidase inhibitor | -2.22 | -2.01 | |
| GRMZM2G112538_T01 | B6TR53 | Pathogenesis-related protein 10 | -2.33 | | |
| GRMZM2G112488_T01 | D4HR93 | Pathogenesis-related protein 10 | -2.45 | | |
| GRMZM2G112524_T01 | B6TR52 | Pathogenesis-related protein | -2.52 | -2.11 | |
| GRMZM2G430500_T01 | A0A096TRQ6 | Uncharacterized protein | -2.60 | | |
| GRMZM2G374971_T01 | P33679 | Zeamatin | -2.61 | -3.07 | |
| AC197758.3_FGT004 | Q6Z5J6 | Ent-pimara-8(14),15-diene synthase | -2.72 | | |
| GRMZM2G108207_T01 | B4G197 | 16.9 kDa class I heat shock protein 1 | -2.73 | | |
| GRMZM2G061766_T01 | K7V347 | Uncharacterized protein | -2.88 | | |
| GRMZM2G171078_T01 | A0A096SWX6 | Peroxidase | -2.89 | | |
| GRMZM2G089988_T01 | Q08275 | 17.0 kDa class II heat shock protein | -3.36 | | |
| AC214360.3_FG001 | Q6Z5J6 | Ent-pimara-8(14),15-diene synthase | -3.44 | | |
| GRMZM2G176085_T01 | B4FYD8 | Peroxidase | -3.46 | | |
| GRMZM2G158232_T01 | B4G197 | 16.9 kDa class I heat shock protein 1 | -3.49 | -2.22 | |
| GRMZM2G335242_T01 | B6TDQ6 | 17.4 kDa class I heat shock protein | -3.50 | | |
| GRMZM2G28306_T01 | O1EG72 | (S)-beta-macrocarpene synthase | -3.78 | -4.81 | |
| GRMZM2G073510_T01 | B6UFC1 | Mating-type switching protein sw10 | -3.79 | | |
| GRMZM2G149675_T01 | B6TR55 | Win1 | -4.10 | -5.31 | |
| GRMZM2G117989_T01 | B6TR55 | Win1 | -4.64 | | |
| GRMZM2G078013_T01 | W0NT67 | NBS-LRR disease resistance protein | -3.25 | | |
| GRMZM2G037146_T01 | Q8W0O8 | Small heat shock-like protein | 2.45 | | |
| GRMZM2G168447_T01 | M8B2N3 | Pathogenesis-related protein 1A | 2.14 | | |
| GRMZM2G080183_T01 | B4FT74 | Peroxidase | 2.12 | | |
| GRMZM2G080183_T01 | B4FT74 | Peroxidase | 2.11 | | |
| GRMZM2G085943_T01 | B6U175 | 17.5 kDa class II heat shock protein | 2.12 | | |
| GRMZM2G063438_T01 | AAMT3 | Anthranilate O-methyltransferase 3 | 2.12 | | |
| GRMZM2G080183_T01 | B4FT74 | Peroxidase | 2.09 | | |
| GRMZM2G080183_T01 | B4FT74 | Peroxidase | 2.14 | | |

Genome ID, Maize transcript ID (ZmB73_5b_FGS_cdna.fasta.gz); FC, fold change value.
### TABLE 3 | Differentially expressed transcripts involved in transport processes.

| Genome ID                        | UniProt ID | Description                                           | Aa vs. N (FC) | Bio vs. N (FC) | Bio vs. Aa (FC) |
|----------------------------------|------------|-------------------------------------------------------|---------------|---------------|-----------------|
| GRMZM2G010251_T01               | B4FSV9     | High affinity nitrate transporter                     | 9.37          |               |                 |
| GRMZM2G156599_T01               | Q9AY27     | Iron-phytosiderophore transporter yellow stripe 1     | 8.60          | 2.25          |                 |
| GRMZM2G000614_T01               | Q7FMW4     | ABC transporter G family member 38                    | 3.94          |               |                 |
| GRMZM2G135291_T01               | G3XDL3     | Putative iron-phytosiderophore transporter            | 3.82          |               |                 |
| GRMZM2G180547_T01               | Q5SLH2     | Amino acid carrier, putative, expressed               | 3.53          |               |                 |
| GRMZM2G090932_T01               | B6T0F4     | Tonoplast dicarboxylate transporter                   | 3.30          |               |                 |
| GRMZM2G072071_T01               | B6U4J2     | ATP-binding cassette sub-family B member 10           | 2.76          |               |                 |
| GRMZM2G148060_T01               | K7VD92     | Putative ferroportin-domain family protein            | 2.70          |               |                 |
| GRMZM2G118507_T01               | K7VD86     | Uncharacterized protein                               | 2.65          |               |                 |
| GRMZM2G024196_T01               | Q7XKF4     | Probable metal-nicotianamine transporter YSL13        | 2.53          | 3.89          |                 |
| GRMZM2G064437_T01               | B6TDG1     | Proton myo-inositol cotransporter                    | 2.53          |               |                 |
| GRMZM2G059465_T01               | K7TWC7     | Calcium-transporting ATPase                           | 2.49          |               |                 |
| GRMZM2G129843_T01               | B6U7Q9     | Lipid binding protein                                | 2.41          |               |                 |
| GRMZM2G0362848_T01              | V9SBV7     | Nucleobase cation symporter 1                        | 2.32          |               |                 |
| GRMZM2G029951_T01               | A0A096Q8Z7 | Uncharacterized protein                               | 2.29          |               |                 |
| GRMZM2G123884_T01               | Q5V8E3     | Probable sodium/metabolite cotransporter BAS1, chloroplastic | 2.21        |               |                 |
| GRMZM2G056908_T01               | Q9ATL8     | Aquaporin TIP2-2                                     | 2.19          |               |                 |
| GRMZM2G053991_T01               | Q5V7C1     | Nuclear transport factor 2 (NTF2) family protein       | 2.16          |               |                 |
| AC186166.3_FGT008               | A0A096PGB1 | Uncharacterized protein                               | 2.16          |               |                 |
| GRMZM2G112456_T01               | QOHIN0     | Oligopeptide transporter 4                           | 2.17          |               |                 |
| GRMZM2G047431_T01               | Q69EY5     | GDP dissociation inhibitor protein                    | 2.17          |               |                 |
| GRMZM2G080178_T01               | B6UC24     | Solute transporter 1.2                               | 2.21          |               |                 |
| GRMZM2G122712_T01               | Q6AUG7     | Putative proton-dependent oligopeptide transporter (POT) | 2.26        | −2.96         |                 |
| GRMZM2G009344_T01               | B4FET6     | ATPUp3                                               | 2.28          |               |                 |
| GRMZM2G026523_T01               | B4FQ14     | Peptide transporter PTR2                             | 2.30          |               |                 |
| GRMZM2G167758_T01               | Q9FMG7     | Nuclear transport factor 2 (NTF2) family protein       | 2.32          |               |                 |
| AC185254.4_FGT002               | O62V9      | Putative ABC transporter                              | 2.39          |               |                 |
| GRMZM2G311401_T01               | A0A096TSV7 | Uncharacterized protein                               | 2.48          |               |                 |
| GRMZM2G027891_T01               | QQLB23     | Os04g0561000 protein                                 | 2.54          |               |                 |
| GRMZM2G091147_T01               | K7TV7     | Uncharacterized protein                               | 2.68          |               |                 |
| GRMZM2G0396831_T01              | K7VPL8     | Uncharacterized protein                               | 2.69          |               |                 |
| AC235441.1_FGT004               | Q2QP91     | Dors1-like family protein, expressed                 | 2.75          |               |                 |
| GRMZM2G040871_T01               | B6SKF6     | Hexose transporter                                   | 2.77          |               |                 |
| GRMZM2G139639_T01               | B6TJ37     | Inorganic phosphate transporter 1-5                 | 2.78          | 2.72          |                 |
| GRMZM2G3191781_T01              | M8CDO8     | Phosphatidylinositol transfer protein 2               | 2.85          |               |                 |
| GRMZM2G048726_T01               | B4FJ28     | Signal recognition particle 9 kDa protein             | 2.93          | −2.73         |                 |
| GRMZM2G137108_T01               | Q9ATN2     | Aquaporin NIP2-2                                     | 3.02          |               |                 |
| GRMZM2G154845_T01               | A0A096SLG9 | Protein DETOXIFICATION                               | 3.19          | −2.31         |                 |
| GRMZM2G168439_T01               | B4F9E1     | Aquaporin TIP1-2                                     | 3.49          | −3.14         |                 |
| GRMZM2G168385_T01               | B6T9U6     | Bidirectional sugar transporter SWEET                | 3.57          |               |                 |
| GRMZM2G010779_T01               | B6U9K03    | Vacuolar cation/proton exchanger 2                    | 3.60          | −2.44         |                 |
| GRMZM2G0351347_T01              | C4J1B8     | Calcium-activated outward-rectifying potassium channel 1 | 3.67        |               |                 |
| GRMZM2G0305446_T01              | Q9ATL7     | Aquaporin TIP3-1                                    | 3.70          |               |                 |

(Continued)
### TABLE 3 | Continued

| Genome ID          | UniProt ID | Description                              | Aa vs. N (FC) | Bio vs. N (FC) | Bio vs. Aa (FC) |
|--------------------|------------|------------------------------------------|---------------|---------------|---------------|
| GRMZM2G144581_T01  | A0A096SEB4 | Bidirectional sugar transporter SWEET4   | −4.01         |               |               |
| GRMZM2G060742_T01  | B6SS8      | Citrate transporter family protein       | −4.44         | −2.55         |               |
| GRMZM2G101958_T01  | B6SGP7     | Non-specific lipid-transfer protein      | 7.01          |               |               |
| GRMZM2G037229_T01  | A0A096GDL2 | Probable magnesium transporter            | 5.44          |               |               |
| GRMZM2G107239_T01  | Q0IQZ4     | Os11g0695900 protein                     | 4.87          |               |               |
| GRMZM2G173699_T01  | B4FTL9     | Sugar transporter SWEET                  | 3.38          |               |               |
| GRMZM2G476069_T01  | M7ZNB4     | Nitrate transporter 1.4                  | 3.01          |               |               |
| GRMZM2G098088_T01  | K7UTZ0     | Hexose transporter                       | 2.75          |               |               |
| GRMZM2G384861_T01  | K7WBE5     | Uncharacterized protein                  | 2.71          |               |               |
| GRMZM2G425683_T01  | B6TCP1     | Carbohydrate transporter/sugar porter/transporter | 2.37 |               |               |
| GRMZM2G152555_T01  | W9R1V3     | Oligopeptide transporter 1               | 2.28          |               |               |
| GRMZM2G0902780_T01 | CQ4H8      | Phosphate transporter                    | 2.22          |               |               |
| GRMZM2G130454_T01  | B6UDW9     | Lipid transfer protein                   | 2.07          |               |               |
| GRMZM2G0005293_T01 | B6U0S4     | Patellin-5                               | −2.06         |               |               |
| GRMZM2G150488_T01  | Q0J9C7     | Os04g0660900 protein                     | −2.09         | −2.55         |               |
| GRMZM2G093276_T01  | E3WCP2     | Zinc transporter                         | −2.32         |               |               |
| GRMZM2G047762_T01  | A0A096QK7  | Uncharacterized protein                  | −2.32         |               |               |
| GRMZM2G090945_T01  | Q67UA2     | Putative phosphate transport protein, mitochondrial | −2.46 |               |               |
| GRMZM2G477872_T01  | A845G      | ABC transporter G family member 45       | −2.70         |               |               |
| GRMZM2G075150_T01  | Q7XEN0     | Exocyst complex component Sec15          | −2.71         |               |               |
| GRMZM2G335218_T01  | K7V706     | Ammonium transporter                     | −2.80         | −4.26         |               |
| GRMZM2G055545_T01  | A0A096QK0  | Uncharacterized protein                  | −2.88         |               |               |
| GRMZM2G0347457_T01 | B6TSV4     | Peptide transporter PTR2                 | −3.47         |               |               |
| GRMZM2G070500_T01  | B6TX8      | Nodulin-like protein                     | 3.62          |               |               |
| GRMZM2G061495_T01  | Q7XNZ2     | OSJNBa0027G07.3 protein                 | 3.20          |               |               |
| GRMZM2G0865543_T01 | B6SUBS     | Electron carrier/electron transporter/iron ion binding protein | 3.16 |               |               |
| GRMZM2G4776069_T01 | M8C905     | Nitrate/chlorate transporter             | 2.89          |               |               |
| GRMZM2G057611_T01  | Q67VA9     | Putative oligopeptide transporter        | 2.85          |               |               |
| GRMZM2G423884_T01  | K7V9U9     | Protein detoxification                   | 2.79          |               |               |
| GRMZM2G0519761_T01 | K7UHM7     | Uncharacterized protein                  | 2.64          |               |               |
| GRMZM2G055883_T01  | Q852B2     | Os05g0823500 protein                     | 2.64          |               |               |
| GRMZM2G020859_T01  | B6SV43     | Potassium channel AKT2/3                 | 2.36          |               |               |
| GRMZM2G153981_T01  | M7YYS5     | ABC transporter B family member 11       | 2.34          |               |               |
| GRMZM2G0806774_T01 | K3XV44     | Glutamate receptor                       | 2.23          |               |               |
| GRMZM2G328999_T01  | K7V706     | Ammonium transporter                     | 2.20          |               |               |
| GRMZM2G011636_T01  | A0A096PX7D | 25.3 kDa vesicle transport protein       | −2.12         |               |               |
| GRMZM2G0850455_T01 | B6T290     | Lipid transfer protein                   | −2.15         |               |               |
| GRMZM2G015767_T01  | B8U0T7     | Peptide transporter PTR2                 | −2.21         |               |               |
| AC234152.1_FGT007  | K7UH5      | Potassium channel2                      | −2.28         |               |               |
| AC203966.5_FGT006  | M8CDC1     | 25.3 kDa vesicle transport protein       | −5.56         |               |               |

Genome ID, Maize transcript ID (ZmB73_5b_FGS_cdna.fasta.gz); FC, fold change value.

Comparison Bio vs. N only one gene related to phytosiderophore biosynthesis (nicotiamine aminotransferase, NAAT; GRMZM2G096958_T01) was found up-regulated. Taken together, even with defined differences these results indicate a strong impact of Aa and Bio with respect to Fe-stress responses of maize roots. Furthermore, besides Fe, nicotiamine forms transport coordination complexes with divalent transition metal cations, i.e., Mn²⁺, Zn²⁺, Cu²⁺ (Benes et al., 1983) whose concentrations were increased in Bio-treated roots.

The category of “establishment of localization” grouped also transcripts involved in S, P, and K transport processes. We observed a down-regulation of a transcript for a sulfate transporter (SULTR; GRMZM2G080178_T01; Hawkesford, 2002) when we compared the transcriptional profile of Aa- with that of N-treated roots. Concerning P, transcripts encoding putative Pi transporters (PHT; Raghothama, 2000) were down-regulated by Bio (GRMZM2G09045_T01) and Aa treatment (GRMZM2G139639_T01). Only in the comparison

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Bio vs. Aa two transcripts encoding for K channel AKT (Hirsch et al., 1998) were up- (GRMZM2G020859_T01) and down-regulated (AC234152.1_FGT007), respectively. Due to the presence of several members belonging to transporter families that have specific roles in nutrient uptake and translocation, and considering that transporter proteins are subjected to multiple forms of regulation (e.g., post-translational modifications), the transcriptional data do not allow a full explanation of the observed changes in tissue nutrient concentrations displayed by the different treatments.

Interestingly, a glutamate receptor (GRMZM5G806774_T01) functioning as non-selective cation channel is induced in Bio vs. Aa. This receptor, regulated by a broad range of amino acids, is involved in different physiological processes such as C/N sensing, resistance against fungal infection, root growth and response to wounding (De Bortoli et al., 2016).

### Hormonal Metabolism and Signaling

A number of genes related to hormonal metabolism and signaling displayed expression changes in Bio- and Aa-treated seedlings vs. seedlings supplied with N (Table 4).

| Genome ID | UniProt ID | Description | Aa vs. N (FC) | Bio vs. N (FC) | Bio vs. Aa (FC) |
|-----------|------------|-------------|---------------|---------------|----------------|
| GRMZM2G046669_T01 | M8AKK4 | Gibberellin 3-beta-dioxygenase 1 | 4.77 | 3.07 | |
| GRMZM2G035156_T01 | Q0DUR2 | Transcription factor ILI6 | 4.68 | | |
| GRMZM2G011463_T01 | B4F68 | SAUR37-auxin-responsive SAUR family member | 3.83 | | |
| GRMZM2G462883_T01 | N1R055 | Putative gibberellin receptor GID1L3 | 2.63 | | |
| GRMZM2G093173_T01 | K7U96 | WAT-1-related protein | 3.23 | 2.12 | |
| GRMZM2G034917_T01 | N1R055 | Putative gibberellin receptor GID1L3 | 2.10 | | |
| GRMZM2G301932_T01 | B6TXN5 | Gibberellin receptor GID1L2 | 2.06 | | |
| GRMZM2G012546_T01 | M8BF8 | Putative gibberellin receptor GID1L3 | 2.07 | | |
| GRMZM2G422249_T01 | A0A096TQ86 | Uncharacterized protein; response to auxin | 2.0 | | |
| GRMZM2G364328_T01 | B6T2Z6 | WAT1-related protein | −5.57 | | |
| GRMZM2G050997_T01 | Q709Q5 | Cytokinin oxidase 2 | −4.48 | | |
| GRMZM2G164090_T01 | B6TLZ8 | Gibberellin-regulated protein 2 | −4.33 | −3.34 | |
| GRMZM2G150688_T01 | B6TWS8 | Gibberellin-regulated protein 1 | −4.0 | | |
| GRMZM2G303790_T01 | B6TLX4 | Jasmonate-induced protein | −3.18 | | |
| GRMZM2G07025864_T01 | K7V88 | Cytokinin oxidase 3 | −3.04 | 2.0 | |
| GRMZM2G173732_T01 | A2Z6Z0 | Protein BiG GRAIN 1-like | −2.98 | −3.01 | |
| GRMZM2G065230_T01 | B7ZXT3 | WAT-1-related protein | −2.75 | −2.7 | |
| GRMZM2G420812_T01 | B6T2P5 | SAUR31-auxin-responsive SAUR family member | −2.74 | −2.49 | |
| GRMZM2G117978_T01 | B6STN8 | Cytokinin-N-glucosyltransferase 1 | −2.51 | | |
| GRMZM2G013448_T01 | C0PEP2 | 1-aminoacyclopropane-1-carboxylate oxidase 1 | −2.50 | | |
| GRMZM2G025742_T01 | B6RWW | Auxin efflux carrier component | −2.49 | | |
| GRMZM2G062019_T01 | B6TWT9 | Gibberellin receptor GID1L2 | −2.49 | | |
| GRMZM2G3685891_T01 | K7V64 | WAT-1-related protein | −2.44 | −3.26 | |
| GRMZM2G107900_T01 | A0A096RE9 | Uncharacterized protein; response to auxin | −2.39 | | |
| GRMZM2G171822_T01 | Q2Q77 | Protein kinase PINOID | −2.15 | | |
| GRMZM2G022679_T01 | Q8S0S6 | Gibberellin oxidase 2-oxidase | −2.10 | −2.34 | |
| GRMZM2G068701_T01 | Q0D4Z6 | Probable indole-3-acetic acid-amido synthetase GH3.8 | −2.06 | | |
| GRMZM2G141473_T01 | C2B888 | Indole-3-acetaldehyde oxidase | −2.0 | | |
| AC233864.1_FGT009 | Q7XTN9 | OSJNBa0093O08.8 protein; response to auxin | −11.81 | −6.49 | |
| GRMZM2G471931_T01 | K7TM25 | Cytokinin riboside 5′-monophosphate phosphoribohydrolase | 2.38 | | |
| GRMZM2G136567_T01 | K7V88 | WAT-1-related protein | 3.43 | | |
| GRMZM2G300012_T01 | A0A096RE9 | Uncharacterized protein; response to auxin | 3.64 | | |
| GRMZM2G001977_T01 | B6UC04 | Gibberellin receptor GID1L2 | 3.05 | | |
| GRMZM2G053338_T01 | B6U4E2 | Indole-3-acetic acid-amido synthetase GH3.8 | −2.11 | | |
| GRMZM2G050321_T01 | B6SU3 | Jasmonate O-methyltransferase | −2.69 | | |
| GRMZM2G024131_T01 | Q41819 | Indole-3-acetyltransferase beta-glucosyltransferase | −4.34 | | |
| GRMZM2G155680_T01 | A0A096SLZ9 | Cytokinin riboside 5′-monophosphate phosphoribohydrolase | 2.04 | | |
| GRMZM2G384762_T01 | A0A096SLZ9 | Auxin-responsive protein | 16.7 | | |

Genome ID, Maize transcript ID [2mB73.5b_FGS_cDNA fasta.gz]; FC, fold change value.
A set of 9 genes whose expression profiles distinguished the Bio and Aa treatments vs. N represented the signature of organic N. Three of these genes coding for gibberellin 3-beta-dioxygenase 1 (GRMZM2G046669_T01), gibberellin 2-oxidase (GRMZM2G022679_T01) and gibberellin-regulated protein 2 (GRMZM2G164090_T01), respectively were related to gibberellin action. Gibberellin 3-beta-dioxygenase which converts inactive gibberellins (GAs) in their active form was up-regulated, whereas gibberellin 2-oxidase, implicated in GAs deactivation, was down-regulated in both Bio vs. N and Aa vs. N, suggesting that organic N forms induced the increase of active GAs in the roots. The other 5 genes that characterized the organic N supply were all related to auxin signaling or transport and were down-regulated in both Aa vs. N and Bio vs. N.

A characteristic feature of the Bio treatment vs. Aa and N was the induction of genes (GRMZM2G471931_T01 and GRMZM2G155680_T01) coding for the enzyme cytokinin (CK) riboside 5'-monophosphate phosphoribohydrolase that converts CK ribosides in free CK. The up-regulation of these genes would result in an increased release of CKs from conjugates. CKs play an important role in root response to N supply coordinating root growth and N availability in the soil (Kiba et al., 2011; Kiba and Krapp, 2016). CKs also interact with auxin in determining root growth and architecture (Mi et al., 2008; Pacifici et al., 2015; Schaller et al., 2015). A second characteristic feature of the response of root to Bio was the down-regulation of GRMZM2G053338_T01 and GRMZM2G204131_T01 genes coding for an indole-3-acetic acid-amido synthetase and indole-3-acetate beta-glucosyltransferase, respectively. As these enzymes mediate the formation of IAA conjugate, their down-regulation indicates that Bio-treated roots retains a higher level of active indol-3-acetic acid (IAA). The GRMZM2G050321_T01 gene coding for a jasmonate O-methyltransferase was also down-regulated in Bio vs. N. Jasmonate O-methyltransferase catalyzes the formation of volatile methyl jasmonate that plays different roles in plant development and stress-response. In particular, high level of methyl jasmonate inhibits root growth. For instance, maize jasmonic acid-deficient opr7opr8 double-mutant showed much longer lateral roots compared with wild type (Yan et al., 2014), phenotype that resembles the root morphology of Bio-treated plants (Figure 1C).

The analyses of the differentially expressed genes of the Aa vs. N comparison revealed the strong involvement of the gibberellin signaling pathway in the free amino acids action. Four genes (GRMZM2G012546_T01, GRMZM2G462883_T01, GRMZM2G034917_T01, GRMZM2G301932_T01) coding for putative GA receptors were up-regulated and one (GRMZM2G062019_T01) down-regulated in Aa vs. N comparison. Another interesting feature was the down-regulation of two genes involved in auxin transport (GRMZM2G025742_T01 and GRMZM2G171822_T01), the first coding for a component of an auxin efflux carrier and the second coding for a PINOID kinase that regulates the membrane localization of the auxin efflux transporters PIN (Christensen et al., 2000). One of the effects of the Aa treatment was also the restraint of IAA and CK catabolism as the results of the down regulation of a gene coding for indole-3-acetaldehyde oxidase (GRMZM2G141473_T01) and two genes (GRMZM2G702564_T01 and GRMZM2G050997_T01) coding for a CK oxidase 3 and a cytokinin oxidase 2, respectively.

The growth and architecture of the root apparatus is the result of the action of several phytohormones as well as their interplay. Therefore, it is not surprising that the activity of Bio and Aa on the root apparatus led to modifications in the metabolism and signaling pathways of different phytohormones. However, although some transcriptional changes were common to both treatments (i.e., the changes in the expression of genes coding for enzymes involved in GA metabolism), differences in the involvement of hormones in Bio and Aa root growth promoting effects have emerged. For instance, CK release from conjugated and inhibition of IAA conjugation together with a lower synthesis of methyl JA appeared the main effects of Bio, whereas the activity of Aa seems to result principally from altered GA synthesis and signaling and restrain of CK and IAA degradation.

A few transcripts coding for CLE peptide hormones were down-regulated in the analyzed comparisons (GRMZM2G468688_T01 in Bio vs. N and Aa vs. N, GRMZM2G165836_T01 in Bio vs. A and GRMZM2G466532_T01 in Aa vs. N). On the other hand, GRMZM2G438840_T01, coding for CLE receptor kinase CLAVATA1 (CLV1) was up-regulated in Bio vs. N and Aa vs. N. CLE signaling peptides and CLV1 play a notable role in sensing N (Miyawaki et al., 2013). It has been shown that lateral roots stop growing under severe deficiency of N, while the expression of CLE peptides is induced (Araya et al., 2014). This regulation serves as a mechanism to prevent the expansion of the lateral root system into N-poor environments (Gruber et al., 2013). The clv1 mutant exhibits progressive growth of lateral roots under N-deficient conditions (Wang et al., 2016).

Interestingly, the transcripts GRMZM2G055607_T01, coding for a sulfotransferase which catalyzes post-translational tyrosine sulfation of secreted peptides, was over-expressed in the Bio vs. Aa comparison. In Arabidopsis, the loss-of-function mutant for tyrosyl-protein sulfotransferase, shows a short-root phenotype (Shinohara et al., 2016). Thus, this finding may represent another clue to unravel the action mechanisms of root growth stimulation exerted by Bio.

CONCLUSIONS

The present study dissected the biostimulatory activity of short peptides and free amino acids on maize seedlings. We demonstrated that protein hydrolysates containing peptides and a very low fraction of free amino acids were more efficient in stimulating root growth and micronutrient accumulation than free amino acid mixture with the same amino acid composition, suggesting a specific role for small peptides in controlling root growth. The genome-wide transcriptional analysis of maize root responses to Bio and Aa as compared with inorganic N, allowed to shed light on the similarities and differences in the mechanisms of action of the two biostimulants. The Aa produced a stronger modification of transcriptional networks than Bio, 995 and 587 differentially expressed genes were detected in Aa vs. N and...
Bio vs. N, respectively. Both treatments displayed effects on genes related to oligopeptide and induced modifications of genes involved in NO$_3^-$ transport, demonstrating that N organic forms can interfere with inorganic N uptake, although the root total N was unchanged. On the other hand, a specific action of Bio seemed to be related to the regulation of the glutamate receptor which is involved in root growth and C/N signaling. Modification in genes implicated in metal ion transport were also detected in both treatments, although with some distinct features. Even if plants were grown in the presence of Fe-EDTA and the content of Fe in the roots was unmodified, Aa and Bio positively affected components involved in Strategy II responses to Fe deficiency. In particular, Aa treatment caused the up-regulation of several transcripts involved in the synthesis of metal chelators (nicotianamine and mugineic acids) and in their transport. On the contrary, only three genes (one related to phytosiderophore synthesis and two to metal-phytosiderophore uptake and translocation) were up-regulated in Bio-treated roots. We might hypothesize that peptides could chelate metals facilitating their uptake and making in turn the biosynthesis of phytosiderophores less crucial. This might explain the higher contents of Cu, Mn, and Zn detected in maize roots treated with Bio. The stimulation of root growth was associated as expected, with perturbations in hormone balance. Both biostimulants modulated genes involved in GAs metabolism thus likely leading to increased GAs levels, and in auxin signaling and transport. In addition, Bio specifically modulated CKs release from conjugates and jasmonate metabolism. Future investigations aiming at studying the effects of protein hydrolysate and free amino acid applications on the content and distribution of phytohormones in the plant, would be useful to deepen these findings. Noticeably, Aa treatment modified the expression of a high number of genes involved in the response to oxidative stress, whereas Bio caused only a modest modulation of stress-related genes. This might suggest that the lower growth promoting capacity of Aa respect to Bio might also be linked to the different metabolic engagement in stress responses.

**AUTHOR CONTRIBUTIONS**

ZV and TP designed the experimental set up. CS and AZ performed the experiments. CS, AZ, ZV, and TP carried out the analysis of the data and draft the manuscript. TP coordinated the project. All the authors approved the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.00433/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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