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Quantitative Proteomics of Maize Roots Treated with a Protein Hydrolysate: A Comparative Study with Transcriptomics Highlights the Molecular Mechanisms Responsive to Biostimulants

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ABSTRACT: Protein hydrolysate (PH)-based biostimulants offer a cost-effective and sustainable approach for the regulation of physiological processes in plants to stimulate growth and improve stress tolerance. Understanding the mode of action of PHs is challenging, but it is indispensable to improve existing candidates and to develop novel molecules with enhanced stimulatory effects. Hence, the present study aimed to understand the proteome level responses in the B73 maize roots treated with APR, a PH biostimulant, at two increasing concentrations and to compare and integrate it with the transcriptomic data obtained previously under identical experimental conditions. Results indicate that APR induced dose-dependent global changes in the transcriptome and proteome of maize roots. APR treatment altered the expression and abundance of several genes and proteins related to redox homeostasis, stress response, glycolysis, tricarboxylic acid cycle, pentose phosphate pathway, and other metabolic pathways of carbohydrates, amino acids, and lipids. Further, metabolic processes of phytohormone, secondary metabolites, especially phenylpropanoids, flavonoids, and terpenoids and transport, and cytoskeletal reorganization associated mechanisms were stimulated. Our results suggest that APR treatment altered the redox homeostasis and thus triggered an oxidative signal. This could be one of the key regulators of the cascade of downstream events involving multiple signaling, hormonal, and metabolic pathways, resulting in an altered physiological and metabolic state which consequently could lead to improved growth and stress adaptation observed in biostimulant—treated plants.

KEYWORDS: *biostimulant, omics, TMT, RNA-seq, redox, metabolism, stress, secondary metabolites*

INTRODUCTION

Crop productivity is essentially determined by plant growth, development, and alleviation of the negative effects of various abiotic and biotic stresses. Biostimulants offer a potentially novel, cost-effective, sustainable, and environmentally friendly approach for the regulation/modification of physiological processes in plants to stimulate growth, to mitigate stress-induced limitations, and to increase yield (Yakhin et al., 2017). Biostimulants are natural substances or microorganisms, which when applied to plants or the rhizosphere in small amounts, stimulate or enhance nutrient uptake and efficiency, tolerance to abiotic stress, and/or crop quality [adapted from European Biostimulant Industry Council (EBIC).[1] The beneficial effects of various biostimulants on plant growth, production, yield, and abiotic stress tolerance are well known and are extensively reviewed.[2−4]

Biostimulants are often derived from complex sources and based on their constituents, the major classes of biostimulants include humic substances (HS),[5] hormone-containing products (HCP), and amino acid—containing products (AACP).[6] Protein hydrolysates (PHs) are an important class of AACP; they usually comprise mixtures of polypeptides, oligopeptides, and amino acids that are derived from protein sources by chemical, thermal, and/or enzymatic hydrolysis.[7] PHs are mainly produced from agro-industrial byproducts derived from animal and plant sources.[8,9] Because waste streams from food and agricultural industries are recycled in the production of PHs, the problem of waste disposal is also addressed. Therefore, application of PHs is interesting both from the environmental and economical perspectives.[10,11]

Application of PHs has been reported to improve agronomical traits in several horticultural crops with increased biomass and productivity, improved Fe and N metabolism, nutrient and water uptake, and efficiencies (extensively reviewed by Colla et al.[10] 2015). However, understanding the mode of action of PHs is challenging, as they are derived from complex sources and induce multiple responses in plants. While PHs predominantly contain peptides and free amino acids,[3] they might also include carbohydrates and negligible quantities of mineral elements, phenols, phytohormones, and other organic compounds.[10] Hence, the observed stimulatory responses on plant growth and productivity could be ascribed

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to the synergistic effect of all the bioactive components in the biostimulants.

Understanding the mode of action of biostimulants is indispensable to improve existing candidates in the pipeline and to develop novel molecules with enhanced stimulatory effects. Further, based on the recent EBIC recommendations, all biostimulant claims need to be adequately supported by scientific evidence.[12] This forges the need to justify biostimulant claims by experimental data generated under controlled conditions (greenhouse, growth chamber) and/or large field trials. In addition, results from carefully designed small-scale laboratory and growth chamber studies and published peer-reviewed research articles addressing the mode of action of the biostimulants could potentially validate and substantiate conclusions drawn from previous trials.[12]

In the post-genomic era, various advanced omics tools—transcriptomics, proteomics, metabolomics, and ionomics (the study of mineral trace element composition of a particular organism) are being routinely adopted to understand the molecular mechanisms underpinning the biostimulant activity.[13,14] Integrated multi-omics studies have the potential to provide a comprehensive snapshot of the molecular mechanisms and pathways stimulated in response to biostimulant. In particular, transcriptomics[15,16] and proteomics[17,18] have been used to understand the mode of action of biostimulants in the recent years.

Trevisan et al.[19] (2017) examined the transcriptome level changes in roots of B73 maize seedlings treated with APR—a collagen-derived PH-based biostimulant produced by ILSA S.p.A (Arzignano, Vicenza, Italy). Results indicated that the APR treatment altered the expression of genes associated with reactive oxygen species (ROS) homeostasis, and signaling, transcription regulation, stress response, and more generally physiological and metabolic pathways related to plant growth. The present study aims to understand the proteome level responses in the B73 maize roots treated with APR at two increasing concentrations and to integrate it with the transcriptomic data obtained by Trevisan et al.[19] 2017 under identical experimental conditions. To the best of our knowledge, the present is the first study providing a comprehensive comparative insight into the proteome and transcriptome level changes induced by a PH biostimulant.

MATERIALS AND METHODS

Plant Growth and Treatment with APR. APR biostimulant was obtained from ILSA S.p.A (Arzignano, Vicenza, Italy), and *Zea mays* L. (B73) seedlings were treated with APR at two increasing concentrations (A1/2 and A1) as described by Trevisan et al.[19] 2017. Two biological replicates were maintained for control, A1/2, and A1 treatments. Roots harvested from control and APR-treated plants were stored at −80 °C until further analysis.

Protein Extraction and Tandem Mass Tag Labeling. Protein extraction was carried out as described by Lan et al. (2011), and protein concentration was determined by Bradford's assay.[20] Prior to tandem mass tag (TMT) labeling, equal concentration of proteins was electrophoresed until the bromophenol blue dye was completely inside the resolving gel, focusing the protein samples into a narrow band. After staining, the sections containing the bands were manually excised, cut in smaller pieces, and destained. Each gel band was treated with 50 μL of 25 mM dithiothreitol (DTT) in 100 mM NH_4HCO_3 for 1 h at 55 °C to reduce disulfide bonds and was treated subsequently with 55 mM iodoacetamide, for 45 min, at room temperature, and in the dark for alkylation of cysteines. It was then repeatedly washed with 100 mM NH_4HCO_3 and acetonitrile (ACN). After dehydration in a speed-vac system, gel slices were incubated (18 h, 37 °C) with 100 μL of sequencing grade modified trypsin (Promega, 12.5 μg/mL in 50 mM NH_4HCO_3, pH 8.0). Peptides were extracted from the gel for 30 min, under constant agitation by two consecutive additions of 100 μL of 75% ACN and dried under vacuum. Peptide mixtures extracted from each band were labeled with 6-plex TMT reagents (Thermo Scientific, MA, USA) according to the manufacturer's instructions. Briefly, 8 μL of each TMT label (0.8 mg in 40 μL of ACN) was added to each peptide extract. The reaction was carried on for 60 min at room temperature and then quenched by the addition of 8 μL of 5% (w/v) NH_2OH for 15 min.

Strong Cation Exchange. To reduce sample complexity, labeled peptides were fractionated by combining strong cation exchange (SCX) and reversed-phase chromatography. SCX was carried out as described by Tolin et al.[21] 2013. Briefly, peptides were resuspended in 500 μL of a buffer containing 5 mM KH_2PO_4 and 25% ACN (pH ≤ 3, adjusted with H_3PO_4) and loaded in an SCX cartridge (Applied Biosystems, USA) using a syringe pump at a flow rate of 50 μL/min. After thorough washing, a total of six fractions for each batch were collected by eluting the peptides with 500 μL of 50, 100, 150, 200, 250, and 350 mM KCl, respectively, prepared in 5 mM KH_2PO_4, and 25% ACN. A second SCX fractionation was also performed because the first process was not satisfactory. This second time five fractions were obtained by eluting the peptides with the following concentrations of KCl: 40, 80, 120, 200, and 350 mM. ACN was then removed in a speed-vac system. Dried samples were suspended in 400 μL of water/0.1% formic acid (FA) and desalted using C18 cartridges (SepPack, Waters) following manufacturer's instructions. Finally, samples were dried under vacuum and suspended in 0.1% FA to an estimated concentration of 1 mg/mL just prior to liquid chromatography coupled to tandem mass spectrometry (LC−MS/MS) analysis.

Liquid Chromatography Coupled to Tandem Mass Spectrometry. Samples were analyzed with an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Germany), coupled to an Ultimate 3000 NanoLC system (Dionex-Thermo Fisher Scientific). A total of 1 μg of each peptide mixture were loaded into a trap column (C18, 300 μm I.D, 10 mm, ProteCol, Analytical Technology), washed for 6 min at a flow rate of 8 μL/min, and separated at a flow rate of 250 nL/min using a 11 cm pico-frit chromatographic column (75 μm I.D, 10 μm tip, New Objective) packed in-house with C18 material (Aeris Peptide 3.6 μm XB-C18, Phenomenex) and a linear gradient of ACN from 3 to 50% in 90 min. MS spectra were acquired in positive ionization mode, with an m/z scan range from 300 to 1700 Da. The instrument operated in a data-dependent mode: a full MS scan at 60,000 resolution was followed by the MS/MS scan of the three most intense ions. Both CID (for identification) and HCD (for quantification) fragmentations were performed on the same precursors. After a first run of analysis, data were subjected to database search (see below) and all identified peptides were used to generate a static excluding list. All samples (except fractions at 200, 250, and 350 mM of the first set of experiments) were analyzed again using the same chromatographic and instrumental settings except for the application of the excluding list.

Database Search and Quantification. All LC−MS/MS data were processed using Proteome Discoverer (Thermo Fischer Scientific, version 1.4) and analyzed with Mascot version 2.2.4 (Matrix Science, UK) for protein identification. Enzyme specificity was set to trypsin with up to one allowed miss cleavage. Peptide and fragment mass tolerance were set to 10 ppm and 0.6 Da, respectively. Searches were done against the *Z. mays* sequences in the UniProt database (version March 2018, 132486 sequences) concatenated with a database of common contaminants often found in proteomics experiments. Carbamidomethyl-Cys, 6-plex TMT at N-termini and 6-plex TMT at -Lys were set as fixed modifications and oxidation of Met as a variable modification. Data were filtered to exclude MS/MS spectra containing less than five peaks and with a total ion count lower than 50. Percolator was used to assess the confidence of peptide and protein identification: proteins were considered as positively identified if at least two independent, unique peptides per protein

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were identified with a q value ≤ 0.01. Proteins were grouped into protein families according to the principle of maximum parsimony.

For quantification, the reporter mass tolerance was set to 50 ppm. The software detected the reporter ions and performed the quantification of relative peptide abundance after normalization to correct for technical variations between different LC−MS/MS runs. Quantification values of each protein were exported to an excel output file, and the mean protein ratios (treated/control) were calculated dividing the quantification value of each protein in treated samples to that of control samples. A Z-test was performed to identify proteins with a significantly different abundance ($p \leq 0.05$), and significant hits were further filtered to keep into account only those proteins showing a fold change (FC) ≥1.5 and ≤−1.5. All data regarding proteins and peptides identification and quantification are reported in Table S14, Supporting Information. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD017781.

Bioinformatic Analysis. Because the experimental setup was identical, the RNA-seq-based transcriptome data set (Trevisan et al., 2017) was reanalyzed along with the quantitative proteomics data set. Reported differentially expressed genes (DEGs) were filtered based on the log2 FC threshold set to 1.5 (DEGs with FC ≥1.5 or ≤−1.5 were considered as up and down-regulated, respectively) (Tables S1 and S2, Supporting Information). Amino acid sequences of all the DEGs were obtained from the MaizeGDB data base (https://www.maizegdb.org/gene-center/gene#translate). Functional classification of the DEGs and differentially abundant proteins (DAPs) based on biological process and molecular function was carried out with the BLAST2GO v5.2 pipeline (https://www.blast2go.com/). EggNOG-mapper v1 (http://eggnogdb.embl.de/) was used for Clusters of Orthologous Groups (COG)—based classification of DEGs and DAPs. In addition to the biological process—and COG—based categorization, all DEGs and DAPs were manually curated and categorized based on the description of their biological process and function in UniProt. Sub-cellular localization was predicted using DeepLoc v1.0 (http://www.cbs.dtu.dk/services/-DeepLoc1.0/index.php). Gene ontology (GO) and pathway enrichment was performed with g:Profiler (https://biit.cs.ut.ee/gprofiler/gost) with Benjamini−Hochberg false discovery rate (FDR) significance threshold set at 0.05. GO enrichment was analyzed in EnrichmentMap (Merico et al., 2010) and visualized in Cytoscape (Shannon et al., 2003). BLAST-based assigning of K numbers and KEGG pathway mapping were performed with BlastKOALA (https://www.kegg.jp/blastkoala/) (Kanehisa et al., 2016) and KEGG mapper (https://www.genome.jp/) (Kanehisa and Sato, 2020).

Correlation Analysis between Transcriptomics and Proteomics Data set. In addition to direct accession—based comparison between RNA-Seq and TMT data sets, a local BLAST was carried out using BioEdit.[22] Amino acid sequences of DEGs identified in the transcriptomics analyses were considered as the database and sequences of the identified DAPs were queried against the database with E-value set to 0.0001. Significant BLAST hits were then further refined based on sequence identity with a 70% cut-off. Descriptions of the genes and their corresponding BLAST-matched proteins were manually verified. Spearman's correlation coefficient was calculated between the FC values of DEGs and DAPs, and the heat map was generated using Gitools (http://www.gitools.org).

RESULTS

Alterations in the Transcriptome and Proteome of Maize Roots Treated with APR. A total of 1006 DEGs were reported in maize roots treated with APR.[19] Reapplying a stringent threshold (log2 FC ≥1.5 or ≤−1.5), we identified 262 and 608 genes that were differentially expressed in response to APR treatment at concentrations A1/2 and A1, respectively (Tables S1 and S2, Supporting Information). There were 126 DEGs in common between the two concentrations (Figure 1), with 97% of common DEGs

Figure 1. Total number of identified DEGs and DAPs in the transcriptomics and proteomics analyses.

showing the same trend in the expression level. Among the 262 DEGs in roots treated with A1/2, 214 were upregulated and 48 were downregulated. In plants treated with the full dose (A1), an equal number of DEGs were upregulated (304) and downregulated (304) (Figure S1, Supporting Information).

The TMT-labeling-based quantitative proteomics approach resulted in reliable identification of 878 proteins, of which 128 and 242 were identified as DAPs in response to APR treatments A1/2 and A1, respectively (Tables S3 and S4, Supporting Information). A sub-set of proteins (81) were common between the two dosages of APR treatments (Figure 1), and all common DAPs had the same trend in the change in abundance. With A1/2 dose, 60 and 68 DAPs increased and decreased in abundance, respectively. While with the A1 treatment, abundance of 108 DAPs increased, whereas 134 DAPs had reduced abundance (Figure S1, Supporting Information).

GO-Based Functional Classification of DEGs and DAPs. GO-based classification of the biological processes indicated that a major proportion of DEGs and DAPs were related to metabolism, specifically organic substance metabolism, cellular, and primary metabolisms (Figure 2A,B, Tables S5−S8 Supporting Information), followed by nitrogen compound and small molecule metabolic processes and biosynthetic processes at both the APR concentrations. Interestingly, at both A1/2 and A1 concentrations, biological processes related to response to endogenous and external stimulus, abiotic and biotic stimulus, and signal transduction were only represented by DEGs.

There was a remarkable increase in the % of DEGs and DAPs related to cellular response to stimulus, stress, and chemical with the full dose (A1) compared to A1/2. Positive correlations between transcriptomics and proteomics data sets on a biological process level were observed between the % of DEGs and DAPs of A1/2 (R^2 = 0.80) and A1 (R^2 = 0.62) data sets (Figure 2A,B).

Among the various molecular functions, major proportion of the DEGs and DAPs was related to binding, especially binding associated with organic cyclic compounds, small molecules, and heterocyclic compounds (Figure S2A,B, Supporting Information). Unlike biological processes, there was no correlation between the % of DEGs and DAPs with the molecular function.

COG-Based Classification of DEGs and DAPs and Their Sub-cellular Localization. COG-based classification clustered all the DEGs and DAPs into 20 categories (Figure 3, Tables S1−S4, Supporting Information). Excluding the unknown function clusters, majority of DEGs and DAPs clustered under COG-categories representing various metabolisms, followed by cellular processing and signaling and information storage and processing. Among the categories representing metabolism, a major proportion of the DEGs and DAPs were associated with secondary metabolite biosynthesis,

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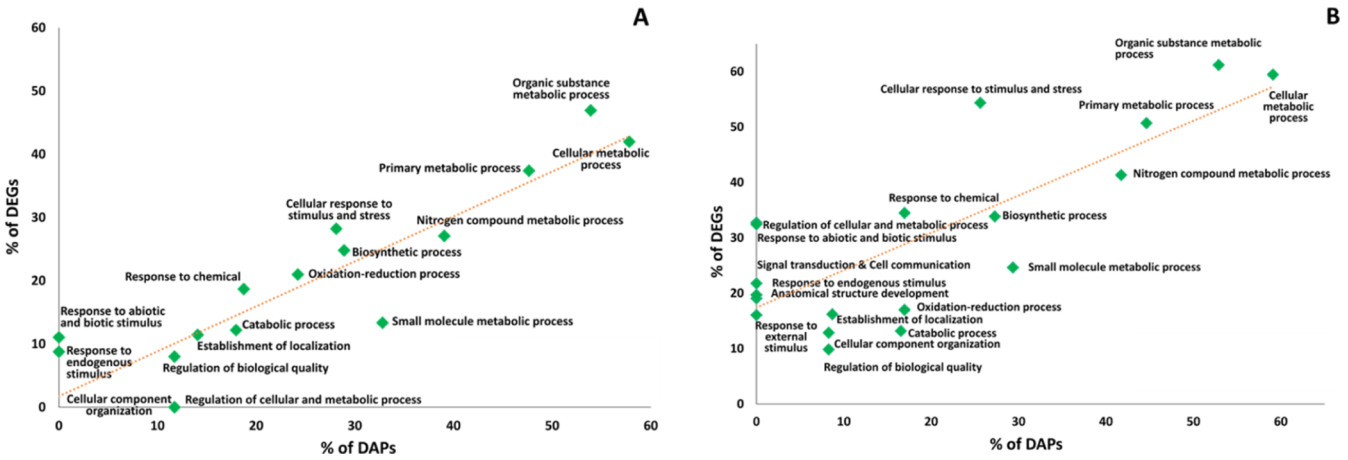

Figure 2. Classification of DEGs and DAPs based on biological processes and the correlation between % of DEGs and DAPs with APR at A1/2 (A) and A1 (B), respectively.

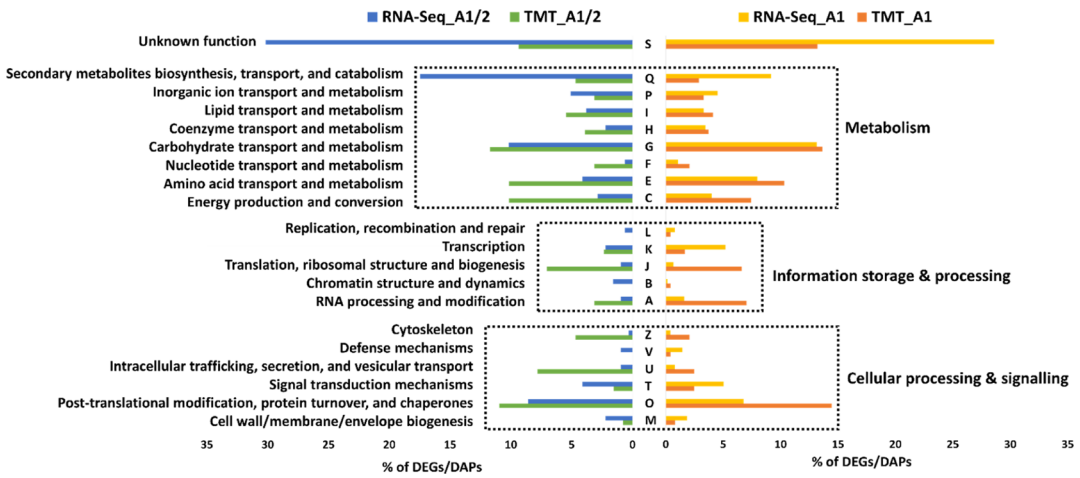

Figure 3. COG-based classification of the identified DEGs and DAPs. Description of the COG categories denoted as letters is shown on the left.

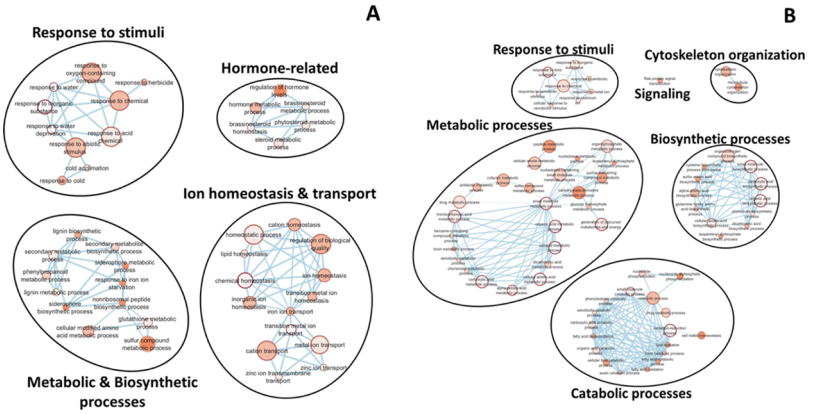

Figure 4. GO enrichment analysis of the transcriptomics (A) and proteomics (B) data sets of A1/2 APR treatment. Nodes (circles) denote the description of the gene set, node size corresponds to the number of associated GO terms within each gene set, node color corresponds to the significance (p value) tested by Benjamini–Hochberg FDR threshold at 0.05, and edge (lines connecting the nodes) size corresponds to the number of GO terms that overlap between two connected nodes.

carbohydrate and amino acid transport, metabolism, energy production, and conversion. Interestingly, at A1/2, percentage of DEGs and DAPs categorized under secondary metabolite biosynthesis was higher than at A1. Similar distributions of DEGs and DAPs representing other categories notably carbohydrate, amino acid transport, metabolism, and energy production and conversion were observed between A1/2 and A1.

The identified DEGs and DAPs were predicted to be localized to 10 different cellular compartments (Figure S3, Tables S1–S4, Supporting Information). The highest proportion of DEGs and DAPs irrespective of the APR

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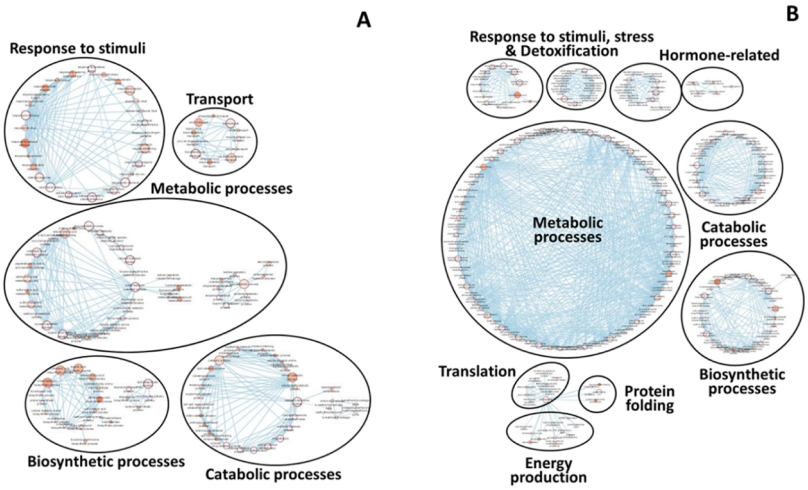

Figure 5. GO enrichment analysis of the transcriptomics (A) and proteomics (B) data sets of A1 APR treatment. Nodes (circles) denote the description of the gene set, node size corresponds to the number of associated GO terms within each gene set, node color corresponds to the significance (p value) tested by Benjamini−Hochberg FDR threshold at 0.05, and edge (lines connecting the nodes) size corresponds to the number of GO terms that overlap between two connected nodes.

concentrations was localized to cytoplasm, followed by secretory proteins. It is also interesting to note that there is an increased number of DEGs localized to the cell membrane and DAPs localized to mitochondria and peroxisome in response to A1/2 compared to A1.

GO Enrichment Analysis. *GO Enrichment Analysis of Transcriptomics and Proteomics Data sets of A1/2 Treatment.* Significantly enriched GO terms of the biological processes responsive to APR treatment could be grouped into four distinct clusters. Within the transcriptomics data set of A1/2 dosage, GO terms associated with response to various stimulus including osmotic and salt stress, regulation of hormone levels, and metabolic and biosynthetic processes of brassinosteroid (BR), glutathione, amino acids, and secondary metabolites such as phenylpropanoids, lignin, and siderophore were enriched. Further, GO terms associated with homeostasis and transports of ions, especially zinc, were also enriched (Figure 4A, Table S9, Supporting Information).

Similarities were found among the GO terms enriched between the transcriptomics and proteomics data sets of A1/2 dosage especially within the response to stimuli cluster. However, additional GO terms associated with response to toxic substance, metal ion, and xenobiotic stimulus were also enriched within the proteomics data set. Interestingly, the number of GO terms associated with metabolic and biosynthetic processes in the proteomics data set was comparatively higher, among which GO terms related to metabolic and biosynthetic processes of carbohydrate derivatives, amino acids, lipids, organophosphate, organonitrogen, sulfur compounds, and auxin (IAA) were significantly enriched. Enrichment of other GO terms associated with cell redox homeostasis and cytoskeleton organization was also observed (Figure 4B, Table S11, Supporting Information).

GO Enrichment Analysis of Transcriptomics and Proteomics Data sets of A1 Treatment. GO terms related to response to stimuli were similar between the A1/2 and A1 transcriptomics data sets. However, higher proportion of GO terms related to various stresses including oxidative, osmotic and salt were enriched within the A1 data set. Correspondingly, GO terms related to redox homeostasis, cellular oxidant detoxification, and metabolic and catabolic processes of ROS, and hydrogen peroxide were significantly enriched. There was higher number GO terms related to primary metabolism and biosynthesis and GO terms associated with biosynthetic processes of terpenoids, BR, strigolactone (SL) and various secondary metabolites were also highly represented (Figure 5A, Table S10, Supporting Information).

With respect to the proteomics data set of the full dose (A1) APR treatment, GO terms related to response to stimulus including cellular response to oxidative stress and ROS were enriched. Several GO terms associated with response to phytohormones especially cytokinin (CK) and BR were found to be enriched. GO terms were related to metabolic processes of organonitrogen compounds, organic acid, oxoacid, carboxylic acid, organophosphate, amino acid, sulfur compounds, ATP and ROS were most represented, so were the GO terms related to biosynthetic processes of various secondary metabolites (Figure 5B, Table S12, Supporting Information).

Pathway Mapping and Enrichment Analysis. Pathway mapping indicated that there were common and exclusive pathways that were responsive at the transcriptome and proteome level in response to APR (Figures S4, S5, Supporting Information). Pathways associated with biosynthesis and metabolism of various amino acids and phenylpropanoid were among the common pathways between the transcriptomics and proteomics data sets of A1/2 dosage (Figure S4A, Supporting Information). Pathways related to biosynthesis of BR, flavonoids, terpenoids, and, plant hormone signal transduction, and nitrogen metabolism were exclusively represented in the transcriptomics data set while majority of the pathways exclusively represented by the proteomic data set were related to primary and energy metabolism, xenobiotics biodegradation among others (Figure S4B, Supporting Information).

A strikingly higher number of pathways regulated both at transcriptome and proteome levels was noted with the full dose APR treatment. Notable common pathways were collectively related to primary metabolism, MAPK—signaling pathway and biosynthesis of phenylpropanoids (Figure S5A, Supporting Information). Biosynthetic pathways of carotenoids, flavonoids and BR, and plant hormone signal transduction were exclusively represented by transcriptomic data set while

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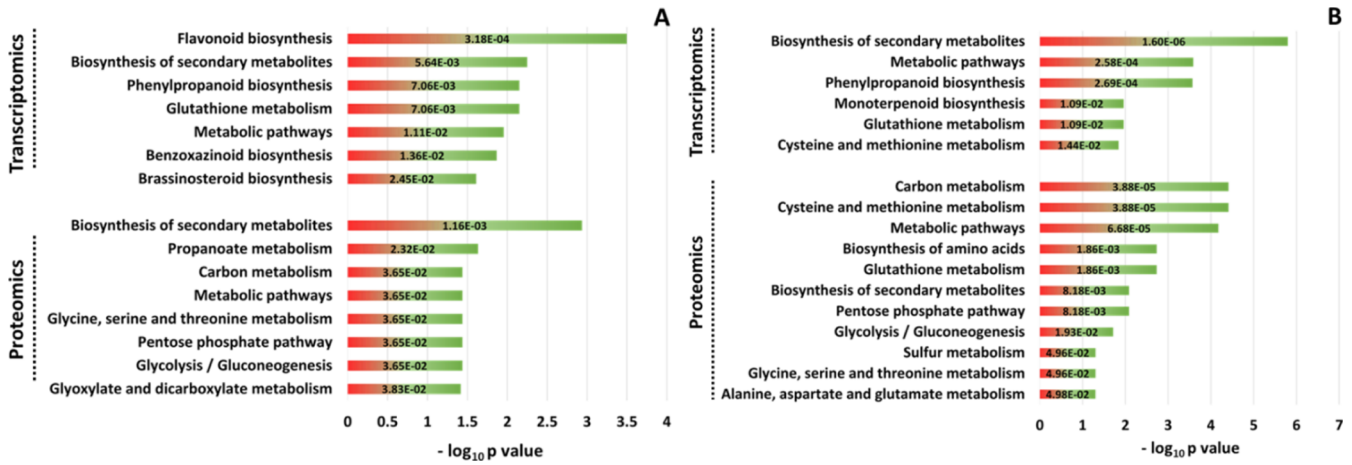

Figure 6. Pathway enrichment analysis of transcriptomics and proteomics data sets of A1/2 (A) and A1 (B) APR treatment. *p* values are denoted adjacent to the pathways.

metabolic pathways related to fructose, mannose, fatty acid, ascorbate, and aldarate, proteolysis and xenobiotics biodegradation were exclusively represented in the proteomic data set (Figure S5B, Supporting Information).

Pathway enrichment analysis indicated that biosynthesis of secondary metabolites was significantly enriched in transcriptomics and proteomics data sets of both the doses. Biosynthetic pathways of flavonoids, phenylpropanoids, benzoxazinoids, BRs, and glutathione were found to be enriched within the transcriptomics data set of A1/2 APR treatment (Figure 6A), while glycolysis/gluconeogenesis, pentose phosphate pathways (PPPs), and metabolic pathways of propanoate, amino acids, glyoxylate, and dicarboxylate, were enriched within the proteomic analysis (Figure 6A).

Metabolic pathways of glutathione, cysteine, and methionine were enriched in both transcriptomics and proteomics data set of A1 dosage. Along with the pathways enriched within the A1/2 data set, biosynthetic and metabolic pathways of monoterpenoids, and various amino acids were enriched within the transcriptomic data set of A1 APR treatment, while pathways related to biosynthesis and metabolism of various amino acids and sulfur were enriched in the proteomic data set (Figure 6B).

Molecular Mechanisms Responsive to APR Treatment. Metabolism-Related Genes and Proteins. Among the 6 DEGs related to amino acid metabolism with A1/2 transcriptomic data set, the highest FC was observed with a gene (Zm00001d048709) (FC: 4.6) involved in tryptophan biosynthesis and 2 other genes associated with amino acid biosynthesis were also upregulated. With the A1 treatment, there were 10 other genes related to amino acid metabolism that were upregulated of which several were synthetases and aminotransferases.

Regarding the proteomic analysis, only abundance of proteins associated with metabolism of arginine (A0A1D6FXP9) and glycine (K7TIN2) was increased. Five DAPs that were in common between the treatments had a similar trend in abundance. Abundance of a protein related to lysine biosynthesis (B4FT32) was increased, while the abundance of eight other proteins related to metabolism of cysteine, glutamine, and serine was reduced in response to A1 dosage.

Among the genes related to carbohydrate metabolism identified with A1/2 treatment, the highest FC was observed with a multifunctional fusion protein (Zm00001d028270, FC: 4.36) related to the glycolytic process. Upregulation of three glycosyl hydrolase family genes and two ribose-5-phosphate isomerases was observed. Six common genes between the treatments showed similar trend in their expression. Of the 10 DEGs related to carbohydrate metabolism with A1/2, seven were upregulated while only five were upregulated among the 19 DEGs with A1.

Abundance of 13 and 26 proteins related to carbohydrate metabolism was altered in response to A1/2 and A1 treatments, respectively. Proteins related to glycolysis (A0A1D6Q8I9, B4FQM2, B8A312, and A0A1D6L6F3) and tricarboxylic acid cycle (TCA) (A0A1D6J321, A0A1D6I1V3) were increased in abundance with A1/2 dosage. In A1 treatment, abundance of eight proteins associated with glycolysis, three related to TCA and four related to PPP were increased. There were seven DAPs common between A1/2 and A1 treatments, of which abundance of six proteins had increased.

Transcripts related to lipid metabolism especially GDSL esterase/lipases and phospholipases had increased in expression in both treatments. Further, abundance of two proteins related to fatty acid biosynthesis and metabolism was increased in response to both treatments.

Secondary Metabolite-Related Genes and Proteins. Eight genes related to phenylpropanoid metabolism, five genes specifically associated with flavonoid metabolism, and eight genes related to various other secondary metabolite biosynthesis were upregulated in response to A1/2 treatment. Similarly, genes related to the metabolism of phenylpropanoids (3), flavonoids (2), terpenoids (4), and other secondary metabolites (9) were upregulated with A1 treatment. In total, there were nine common genes, of which eight were upregulated in both treatments.

Abundance of two proteins related to phenylpropanoid metabolism including a phenylalanine ammonia-lyase was increased in both the treatments. Abundance of proteins related to the biosynthesis of isoprenoid (A0A1D6MNJ0), chorismate (A0A1D6IK09), and taxol (B6TJ78, B4G144) also increased in response to both APR treatments.

Interestingly, there were seven and nine cytochrome P450 family genes upregulated in response to A1/2 and A1, respectively; three of them were upregulated in both the conditions, whereas three DAPs related to cytochrome P450

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family identified in the proteomic analyses had lower FC and/or statistical significance than the set threshold. Six genes associated with nicotianamine metabolism were upregulated in both the treatments in the transcriptomic data sets, while only three related proteins showed increased abundance with A1 dosage (A0A1D6QNK5, A0A1D6K0A7, K7VEJ7). Furthermore, genes related to UDP-glycosyltransferases were upregulated in A1/2 (3) and A1 (6) treatments, while one associated protein (B4G072) found with A1 had increased in abundance.

Stress-Related and Responsive Genes and Proteins. Stress-related and responsive genes including several classes of heat shock proteins (HSPs), chaperonins, chitinases, pathogenesis-related proteins (PRs), dehydrins, late embryogenesis abundant protein (LEA), DUF—domain-containing proteins, germin—like proteins, osmotin—like proteins were highly represented in the transcriptome analyses. Among which, transcripts of LEA-protein, HSP 90 kDa, DUF674 family protein and a disease resistance-associated protein were more than 2.5 fold upregulated with A1/2 treatment. Notably, gene expression of all the identified LEA proteins, 3 PRs and 2 chitinases, was upregulated, while all the dehydrins were downregulated.

In A1-treated roots, transcripts of DUF3511 domain protein, PR1, senescence-specific cysteine protease SAG12, and a LEA protein were 2 folds upregulated compared to control. Gene expression of all the identified chitinases, dehydrins, and seven HSPs and PRs (PR1, PR3) was downregulated with A1 treatment.

HSPs represented most of the stress-related DAPs. Two HSPs had a similar trend in response to both the APR treatments. Four other HSPs were altered exclusively with A1 treatment, two of which (A0A1D6ES19, C1K9J1) increased in abundance. Consistent with the transcriptomic analyses of A1 dosage, the abundance of chitinase, PR1s, and a thaumatin-like protein was reduced.

Redox Homeostasis-Related Genes and Proteins. All the DEGs of glutathione S-transferases (GSTs) identified in the transcriptomic analyses were upregulated; in particular, five and seven DEGs were identified with A1/2, and A1 treatments, respectively. In plants treated with A1/2, all the 5 DEGs of peroxidases (PODs) were upregulated, while 13 DEGs of PODs were upregulated in A1-treated compared to control. Two POD genes (POD 64 and 52) were upregulated in response to both the APR treatments so was a NADP-dependent alkenyl double bond reductase P2 (Zm00001d007285). Of the 2 nucleoredoxins (NRXs) found among DEGs, NRX 1 (Zm00001d029459) was upregulated 2 folds in response to A1 dosage.

In the proteomic analyses, increased abundance was observed only with an acyl-coenzyme A oxidase (A0A1D6NUS9) and a catalase (B6UHU1). Similarly, the abundance of all 10 PODs including an ascorbate peroxidase (APX1) (B6U9S6) decreased, while in contrast, the abundance of the six identified GSTs increased with A1 dosage.

Hormone-Related and Responsive Genes and Proteins. In response to APR treatments, several genes related to IAA, abscisic acid (ABA), SL, jasmonic acid (JA), CK, ethylene (ET), and phytosulfokines were upregulated, whereas in the proteomic analyses, an increased abundance of carotenoid cleavage dioxygenase (C4PJN4) related to SL biosynthesis was noticed with both the APR treatments. With A1/2 treatment, three DAPs related to JA and one related to CK decreased in abundance and so were the DAPs related to JA (G9I6E1, B6U2I9) and IAA (C0PD61, A0A1D6GY58, C0P506) with A1 dosage.

Signaling-Related Genes and Proteins. Except for a calcium-binding EF-hand family protein, all the other genes related to signaling were upregulated in response to A1/2 treatment. Especially, eight leucine-rich repeat (LRR) family genes, three zinc finger family (Znf) genes, and two lectin domain-containing receptor kinases (LecRK) were upregulated more than 1.6 fold. Highest FC was found with nicastrin (FC: 5.09)—a gene in notch-receptor signaling cascade, a BR LRR receptor kinase (FC: 3.35), and putative calcium-dependent protein kinase family gene (FC: 2.35), while their expression was induced to 6.17, 6.45, and 5.89 fold, respectively, in response to A1 dosage. In addition, two other (LRR) family genes (Zm00001d035756, Zm00001d020185) and two LecRKs (Zm00001d019334, Zm00001d043256) were upregulated with the A1 dosage. Other DEGs with more than 2-fold upregulation include a Ser/Thr-rich protein T10 (Zm00001d014852), a putative MAPKKK family protein kinase (Zm00001d038884), a calmodulin-binding protein (Zm00001d006101), and a NEP-interacting protein 1 (Zm00001d048644).

There were nine proteins related to signaling that had changed in abundance with A1/2. The highest increase in abundance was observed for RAN GTPase -activating protein 1 (A0A1D6HD80) followed by a TolB protein-related protein (A0A1D6IKV5) and a transducin/WD40 repeat-like superfamily protein (A0A1D6P2Q6), while the abundance of 3 RAS-related proteins (RAP) exclusively identified in response to A1/2 treatment was reduced. Four DAPs identified with A1 were common with A1/2 and had a similar trend in abundance.

Transport-Related Genes and Proteins. Twenty genes related to transport were upregulated in response to A1/2, of which 10 were also upregulated in A1 dose. Several genes including ABC transporter family, aquaporins, transporters of various ions and proton, nitrate, phosphate, zinc, copper, malate, and iron-phytosiderophores were upregulated in response to A1/2 treatment. Of the 60 genes associated with transport, 32 genes were upregulated in the A1 dosage. In addition to transport—related genes responsive to A1/2, amino acid permeases, transporters of calcium, iron, polyol, amino acids, sugar, silicon, and several other ion channels and transmembrane transporters were found to be upregulated in response to A1 treatment.

In the proteomic analyses, abundance of 6 and 11 proteins related to transport increased in response to A1/2 and A1, respectively. Specifically, two proteins essential for retrograde vesicle-mediated transport of proteins (A0A1D6MGB9, A0A1D6F8N4), an aquaporin (A0A1D6HDN1), and a proton pump (A0A1D6NTY4) showed increased abundance with A1/2. An aquaporin PIP2-1 (A0A1D6HYR6) exclusively identified with A1 dosage and six other proteins related to vesicle-mediated transport, and proton transport increased in abundance. Of these, four proteins also increased in abundance in response to A1/2 treatment.

Cytoskeleton-Related Genes and Proteins. There were 16 DEGs related to cytoskeletal reorganization that were upregulated with A1/2, while of the 29 DEGs in response to A1 treatment, only 15 were upregulated. There were four common genes that showed differential expression in both the treatments. Notably, genes of fasciclin-like arabinogalactan proteins, root cap periphery genes, polygalacturonases, wax

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synthase, cellulose synthase, and pistil-specific extensin-like proteins were upregulated in response to A1/2 treatment, while with A1 dosage, differential expression of multiple isoforms of expansins, glycine-rich cell wall structural proteins, villin, WAT1-related proteins, and xyloglucan endotransglucosylase was observed. Abundance of six proteins increased with A1/2 and of the 11, while only five were observed with increased abundance with A1 treatment. Abundance of all the four DAPs common between the treatments increased. Notably, different chains of tubulin, villins, cell division control protein, expansins, UDP-arabinopyranose mutase, and cell wall integrity protein showed differential abundance in response to APR treatment.

Transcription, Translation, Protein Folding, and Proteolysis-Related Genes and Proteins. Major classes of transcription factors that were differentially expressed in the transcriptomic analyses were phytohormone responsive transcription factors (6), zinc finger (6), MYB (5), bZIP (2), NAC-domain containing factors (2) and 24 genes that are transcription factors or related to transcription. In total, there were 38 DEGs collectively with APR treatment that were related to translation and proteolysis and several of which were classes of proteases and genes related to protein ubiquitination, and proteasome machinery. In the proteomic analyses, 77 DAPs were related with translation, protein folding, and proteolysis; collectively, six proteins that were either translation initiation or elongation factors increased and so were all the identified tRNA ligases and synthetases, six proteases—like proteins, and two proteins associated with ubiquitination and proteasome, whereas abundance of nine ribosomal proteins decreased.

Cross-Validation of Protein Abundance with the Gene Expression Level. Several of the identified DEGs did not have stable gene accessions and hence could not be mapped to their corresponding protein accessions. Also, a proportion of gene accessions had multiple corresponding protein accessions. Despite these discrepancies, direct accession—based comparison between the transcriptomics and proteomics data sets of A1/2 and A1 treatments resulted in only 5 and 12 matches, respectively. To expand the comparison between the data sets, a BLAST-based sequence-level comparison between the transcriptomics and proteomics data sets of A1/2 and A1 was carried out with *E*-value 0.0001 as the cut-off. The statistically significant matches were further filtered with % identity (>70%) as threshold, and the match between the description of genes and proteins was also manually verified (Table S13, Supporting Information).

For the A1/2 data set, BLAST with the sequence of 128 DAPs against the transcriptome data set (amino acid sequence of 262 DEGs) resulted in 5 statistically significant hits excluding the ones that were obtained by direct comparison of accessions (Figure 7, Table S13, Supporting Information). Spearman correlation coefficient was −0.516 between the FC of 10 DEGs and DAPs with the A1/2 data sets. Interestingly with A1/2 data sets, an aquaporin PIP2-4 (Zm00001d017288; FC: 1.6) and carotenoid cleavage dioxygenase (Zm00001d043442; FC: 1.6) were upregulated in the transcriptomics analyses and its corresponding DAPs—A0A1D6HDN1 (FC: 1.8) and C4PJN4 (FC: 2.7) also increased in abundance, while the other eight genes and proteins showed a contrasting trend in their regulation.

In addition to the 12 DEGs and DAPs identified by direct accession comparison with the A1 data sets, 16 DAPs had

Figure 7. Fold change of DEGs and its corresponding DAPs with A1/2 and A1 data sets. Color key indicates log2 fold change. * before the UniProt accessions indicate matches identified by BLAST.

statistically significant matches with 22 DEGs (Figure 7, Table S13, Supporting Information). Consistent with A1/2 data sets, there was no correlation between the transcriptomics and proteomics data sets as indicated by Spearman correlation (0.330).

With the A1 data sets, there were 15 DEGs whose corresponding or associated proteins showed a similar trend in abundance. Remarkably, genes and proteins of carotenoid cleavage dioxygenase (Zm00001d043442; FC: 1.6 and C4PJN4; FC: 2.9), GST 4 (Zm00001d024839; FC: 1.6 and P46420; FC: 1.9), and GST 1 (Zm00001d027557; FC: 1.5 and A0A1D6JMZ7: FC: 1.7) were upregulated. Other notable genes and proteins which showed similar upregulation trend were cotianamine aminotransferase, nicotianamine synthase 1 and 9, and bifunctional methylthioribulose-1-phosphate, whereas beta-expansin 1a (Zm00001d029913; FC: −2.1 and Q94KS8; FC: −1.6), chitinase B1 (Zm00001d025753; FC: −6.1 and B8QUT5; FC: −1.7) POD 52 (Zm00001d046184; FC: −2.6 and A0A1D6H658; FC: −2.3), and POD 5 (Zm00001d037550; FC: −1.7 and A0A1D6FHV4; FC: −2.3) were downregulated. In spite of the observed cross-validation of the protein abundance with gene expression in our analyses, correlation coefficients suggest that there is no correlation between transcriptomics and proteomics at the FC level.

DISCUSSION

APR—a biostimulant derived from collagen was previously reported to stimulate root biomass in maize plantlets, induce

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alterations in the root transcriptome,[19] and to improve the maize tolerance to abiotic stress.[23] To the best of our knowledge, the effects of a collagen derived-PH on the proteome of maize roots have not been investigated yet. In the present study, the effects of APR on the proteome of maize roots were examined and the previously published root transcriptomics data were reanalyzed, compared and integrated. The information from the two different approaches was collated to glean comprehensive insights on the molecular mechanisms triggered by APR in maize roots. This study also is the first of its kind to integrate transcriptomic data with proteomics results to understand the responses triggered by a biostimulant at the molecular level. Previously, several researchers have carried out comparative studies integrating their results with previously published data. On such exemplary research was an in depth investigation of soybean seed-filling proteome which was compared with a parallel study on rapeseed.[24]

Results from our study suggest that APR treatment induced global changes in the transcriptome and proteome of maize roots. A comparatively higher number of DEGs and DAPs were responsive to the full dose treatment (Figure 1, Figure S1, Supporting Information) suggesting that the impact of APR at the molecular level could be dose-dependent. On the contrary, the increase in root biomass was comparatively higher with A1/2 dose (20%) than A1 (12%) treatment.[19] This implies that a higher impact at the molecular level by a biostimulant treatment does not necessarily correspond to higher stimulatory effects on plants. On the contrary, the magnitude of the molecular responses could be attributed to the adaptive mechanisms of the plants to doses exceeding the optimal concentration. Phytotoxicity effects including suppression of growth were reported earlier in other biostimulants repeatedly applied at high doses.[25]

Interestingly, in response to APR treatment, several DEGs and DAPs including GSTs, glutathione peroxidases (GPxs), NRXs, thioredoxins (Trxs), and dihydrolipoamide dehydrogenases (DLDs) that are unequivocally related to redox homeostasis were identified. This implies that there could be a direct or indirect triggering of ROS in maize roots treated with APR, as also reported previously by Trevisan et al.[19] 2017. Hence, we consider this triggering of ROS—processing or antioxidative system to be one of the vital upstream events that could be regulating a cascade of other downstream processes. A microarray-based study showed that an alfalfa-based PH induced several redox homeostasis- and antioxidative system-related genes in *Solanum lycopersicon* L.[26] Other classes of biostimulants including HS,[27] seaweed extracts,[15] EXPANDO,[28] and Erger[29] have been reported to induce the expression of genes related to redox homeostasis in various plant species. Similarly, biostimulants have also been reported to alter the abundance of redox homeostasis related proteins such as malate dehydrogenase[30] and superoxide dismutases (SOD).[31] Roomi et al.[18] 2018 have reported alteration in the abundance of POD and multiple GSTs in response to HS treatment, and increased abundance of 2-Cys peroxiredoxin and GPx was reported in maize roots treated with HS.[17]

ROS, besides being traditionally considered as cellular damaging agents leading to oxidative stress, also function as a unique class of signaling molecule and as core regulators of various biological processes such as growth, development, and responses to stress and stimuli in plants.[32] Plants rely on their anti-oxidative systems or ROS-processing systems to maintain ROS at a basal non-toxic level, and any deviation from this fine-tuned balance could be used for ROS signaling reactions as oxidative signals.[32,33] Global changes in gene expression and consequently alteration in protein abundance are known to occur in response to redox-signals.[33−35] In previous reports, an AH−root interaction has been suggested to trigger changes in membrane functionality which is recognized by plants as a state of mild physiological stress.[36] Plants sense and transduce oxidative signals by means of redox-sensitive proteins (RSPs) undergo reversible oxidation/reduction depending on the cellular redox state.[37] Several RSPs notably Nrx and Trx and multiple other genes and proteins related to ROS-processing and/or antioxidative systems were differentially regulated in response to APR in our study. Induced oxidative stress responsive enzymes might prime and fortify the plant for future stress.[36] Present and previous results taken together suggest that APR and other classes of biostimulants evidently trigger the redox-circuitry of the plants, and this could be one of the conserved mechanisms by which biostimulants act on plants.

Several stress-related and responsive DEGs and DAPs were differentially regulated with APR treatment consistent with the different PHs previously reported.[16,26] Besides PHs, other classes of biostimulants have been demonstrated to induce stress-responsive and related mechanisms and to mitigate the negative impact of various stresses in different plants. This is not surprising, and as in addition to improving the productivity of crops, biostimulants are extensively applied to ameliorate the stress effects in plants.[25,38]

In our transcriptomics analyses, major classes of transcription factors including zinc finger and MYB were differentially expressed in response to APR treatment. As expected, no transcription factors or proteins related to transcription were identified in the proteomic analyses, owing to their low abundance. However, the induction of transcription was evidenced at the proteome level as a multitude of proteins related to translation and protein turnover processes were altered in abundance (21 and 56 DAPs in response to A1/2 and A1 treatment, respectively). It is well established that multiple downstream signaling pathways and phytohormone-mediated pathways are dependent on redox signals and the redox state of the cell.[39,40] Taking into consideration that the observed induction of transcription and translation-related processes in APR-treated maize roots may not be entirely redox-dependent, the possibility of redox homeostasis and redox signals steering or regulating these processes alongside other secondary signals cannot be ruled out.

Genes and proteins related to various signaling pathways were differentially regulated in response to APR treatment. These signaling pathways could have been triggered by redox signals or indirectly by other unknown signals. In addition, an alternative mechanism of amino acids and peptides functioning as signaling molecules in the regulation of plant growth and development could be occurring.[38] Plant growth and development, root growth, leaf-shape regulation, defense responses, and nodule development were reported to be affected by specific signaling peptides contained in a plant-derived PH.[41,42]

Furthermore, APR treatment induced changes in the expression and abundance of genes/proteins involved in the biosynthesis and metabolism of phythormones especially IAA, ABA, SL, JA, CK, BR, and ET. Some classes of biostimulants, especially HS, are known to contain hormone or hormone-like components and hence have effects similar to phytohormones

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in treated plants.[43,44] PHs have also been reported to trigger phytohormone-related processes in plants. Commercial biostimulants based on PHs have been shown to elicit hormone-like activities especially IAA and gibberellins.[45,46] Previous studies with a plant-derived PH was shown to induce biosynthetic genes of ET, JA, ABA, and salicylic acid.[26] PHs-based biostimulants essentially contain bioactive peptides, amino acids, and other trace components including glycosides, polysaccharides, and organic acids which may act as precursors or activators of endogenous plant hormones.[47] An alternate perspective is that the stimulation of phytohormones by PHs could occur indirectly by means of redox or other signals, as there is a substantial body of literature to support the interlink between ROS and phytohormones.[40]

Functional classification and GO enrichment analyses of the DEGs and DAPs indicated that metabolic processes were among the processes most impacted by the APR treatment in maize roots (Figure 2−5). Pathway enrichment analyses highlighted that several metabolic and biosynthetic pathways were significantly enriched in response to APR (Figure 6). In particular, pathways related to biosynthesis of secondary metabolites including flavonoid, phenylpropanoid, glutathione, and monoterpenoid were highly represented in the transcriptomics analyses, while pathways related to carbon, amino acid, propanoate, glyoxylate, glutathione, sulfur, including central pathways—glycolysis/gluconeogenesis and PPPs were enriched in the proteomic analyses.

Furthermore, it was evidenced in our results that APR treatment had an effect on the central metabolic pathways especially glycolysis, TCA, PPP, and several other related carbohydrate pathways and on amino acid, lipid, and fatty acid metabolism consistent with previous reports on PH.[6,16,26]

Our comparative study suggested that several pathways related to biosynthesis of various secondary metabolites were enriched and were regulated by both the APR dosages (Figures 6, S4, S5, Supporting Information). Secondary metabolites especially phenylpropanoids, which were significantly represented in our results, are involved in numerous physiological functions, that are essential for plant growth, development, and adaptation to stimuli and stress.[48]

Additionally, multiple genes related to cytochrome P450 family were found to be upregulated with APR treatment. Three cytochrome P450 family proteins identified in the both A1/2 and A1 proteomic data sets had FC and p value below the set cut-off. However, several DAPs associated with various metabolic pathways that are regulated by this multi-functional gene family were observed. Cytochrome P450 family enzymes catalyze a wide range of reactions and hence are involved in the biosynthesis of a myriad of primary and secondary metabolites in particular diterpenes.[49] Diterpenes are precursors for biosynthesis of several phytohormones, and indeed, P450 enzymes are established regulators of phytohormone biosynthesis and homeostasis, especially BR. Thus, cytochrome P450 family plays important roles in the regulation of plant growth, development, and stress adaptation.[50,51] Given the significance of secondary metabolites and the regulatory function of cytochrome P450 family in growth and stress tolerance, these could be potential targets of APR and other biostimulants. PHs and other classes of biostimulants have been shown earlier to induce genes and proteins related to the biosynthesis of secondary metabolites and cytochrome P450 family.[15,17,18,43]

The other major categories of biological processes that were significantly stimulated in response to APR treatment are transport and cytoskeleton reorganization. This included ABC transporter family, aquaporins, transporters of proton, nitrate, phosphate, zinc, copper, malate, iron-phytosiderophores, calcium, iron, polyol, amino acids, sugar, silicon, and various ion channels and transmembrane transporters. Furthermore, proteins related to retrograde vesicle-mediated transport of proteins, proton pump, and aquaporins were altered in abundance. Differential regulation of multiple transporters and transport associated genes and proteins could have impact on the efficiency of root transport mechanisms upon treatment with APR, thus substantiating the already reported improvement of nutrient uptake and plant growth observed in response to PHs[25] and confirming previous transcriptomics and proteomics-based studies.[15−18,26]

In the present study, DEGs (e.g., fasciclin-like arabinogalactan proteins, root cap periphery genes, and polygalacturonases) and DAPs (e.g., tubulin, cell division control protein, villins, and expansins) associated with cytoskeletal reorganization were highly represented which is also consistent with previous reports.[15−18,26] Comparing the two treatments, a higher percentage of DEGs and DAPs were upregulated in A1/2 (94%) than A1 (52%), thus supporting the root biomass data reported by Trevisan et al.[19] 2017.

Linking the inferences drawn from our transcriptomics and proteomics analyses with the established roles of redox homeostasis in regulating plant growth, development, and cross-tolerance, we propose a simplified view of one of the probable modes of action of APR. We hypothesize biostimulants, at least APR, could alter the basal level of ROS maintained by the plants to a beneficial level, thereby mimicking a sub-lethal stressful condition. This oxidative signal along with other complex signals are then processed by RSPs, other redox-dependent, or regulated players, leading to a cascade of downstream events involving multiple other signaling and hormonal pathways, with extensive cross-talks at multiple levels. These upstream events consequently would lead to the regulation of transcription and translation machineries resulting in an altered physiological and metabolic state which contribute to growth, development and stress adaptation. Thus, improved stress tolerance in maize upon APR treatment could probably be achieved through cross-tolerance because of prior priming with the biostimulant. It would be interesting to establish if the model hypothesized for APR is extensible to other PHs and biostimulants.

It is evident from our results (Figure 7) and numerous other reports that the gene expression level and protein abundance do not sufficiently correlate to orthogonally validate each other.[52−54] There was no correlation between the FC of DEGs and their corresponding DAPs in our study as the Spearman correlation coefficients were −0.516 and 0.330 for A1/2 and A1 data sets, respectively. Majority of this non-correlative discrepancies are a manifestation of the complexity of biological regulation by post-transcriptional and post-translational modifications and may not be attributed to platform-specific errors or measurement bias. In addition, differences in the abundance of transcripts and proteins could also be attributed to discrete genetic loci controlling the expression of genes and proteins.[55] Other confounding factors include the inherent variation in the synthesis and turnover rates of transcripts and proteins and oscillations in their relative timing and abundance. Hence, large overlaps between transcriptomics and proteomics should not be expected.[56]

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Interestingly, we observed a good correlation between the percentage of DEGs and DAPs classified based on their biological process level (Figure 2A,B). This suggests that the global changes occurring at the transcriptome and proteome level converge at the biological processes level ultimately enabling the growth and adaptation of the plant, although they do not correlate in terms of the magnitude (FC). This correlation of transcriptomics and proteomics data at the biological process level needs to be verified with other comparable steady-state data sets. This would have a broader significance and impact on the notion of relying on mRNA abundance to validate the proteomics data. Conclusively, these omics tools are undoubtedly robust in quantifying different biomolecules (genes and proteins), and albeit the limited overlap, multi-omics experiments are invaluable to obtain comprehensive information on the molecular mechanisms triggered by an external stimulus.

Conclusively, treatment with APR—a PH-based biostimulant induced global alterations in the transcriptome and proteome of maize roots. Our results indicated that APR treatment altered genes and proteins related to ROS-processing system, phytohormones, specific primary and secondary metabolic pathways, transport, and cytoskeletal reorganization. In particular, ROS scavenging system appears pivotal to regulate the responses to biostimulants, as evidenced also in previous reports. This observation points toward the possibility of identifying common mechanisms targeted by biostimulants, irrespective of their source or composition. On a futuristic note, novel biostimulants would be continually manufactured and so would be the constant demand to understand the molecular mechanisms triggered by them. Henceforth, high-throughput plant phenotyping data substantiated with molecular evidence obtained from multi-omics experiments would definitely be contributing and steering the venture to develop novel biostimulants with enhanced effects and extended applicability.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.0c01593.

List of DEGs identified with A1/2 dosage of APR, list of DEGs identified with A1 dosage of APR, list of DAPs identified with A1/2 dosage of APR, list of DAPs identified with A1 dosage of APR, blast2GO analysis: biological processes of DEGs identified with A1/2 dosage of APR, blast2GO analysis: biological processes of DEGs identified with A1 dosage of APR, blast2GO analysis: biological processes of DAPs identified with A1/2 dosage of APR, blast2GO analysis: biological processes of DAPs identified with A1 dosage of APR, GO enrichment analysis of the biological processes of DEGs identified with A1/2 dosage of APR, GO enrichment analysis of the biological processes of DEGs identified with A1 dosage of APR, GO enrichment analysis of the biological processes of DAPs identified with A1/2 dosage of APR, GO enrichment analysis of the biological processes of DAPs identified with A1 dosage of APR, BLAST-based comparison of transcriptomic and proteomic data sets of A1/2 and A1 APR treatments, list of all proteins and peptides identified in the TMT experiment, and all parameters useful to assess the identification and quantification confidence are reported (XLSX)

Number of up and down-regulated DEGs and DAPs identified with the transcriptomic and proteomic analyses, classification of DEGs and DAPs based on molecular functions, sub-cellular localization prediction of DEGs and DAPs common (A) and exclusive (B) pathways regulated at the transcriptome and proteome level with A1/2 APR treatment, and common (A) and exclusive (B) pathways regulated at the transcriptome and proteome levels with A1 APR treatment (PDF)

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Author Contributions

L.B.E. analyzed the data and wrote the manuscript with contribution from all co-authors. A.R.T. carried out the protein extraction. C.F. and G.A. carried out the proteomics experiments and statistical analysis. S.T. and S.Q. carried out the transcriptomic analyses and contributed to critical revision of

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the manuscript. P.C., G.K.A., G.A., R.R., and A.M. contributed to the interpretation of results and revision of the manuscript. A.M. conceived the original idea and supervised the project.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AACP, amino acid-containing products; ABA, abscisic acid; ACN, acetonitrile; APR, rhizosphere protein activator; BR, brassinosteroid; CK, cytokinin; COG, clusters of orthologous groups; DAPs, differentially abundant proteins; DEGs, differentially expressed genes; DLDs, dihydrolipoamide dehydrogenases; DTT, dithiothreitol; EBIC, European Biostimulant Industry Council; ET, ethylene; FC, fold change; FDR, false discovery rate; GO, gene ontology; GPxs, glutathione peroxidases; GSTs, glutathione S-transferases; HCP, hormone-containing products; HS, humic substances; HSP, heat shock protein; IAA, auxin; JA, jasmonic acid; LC−MS/MS, liquid chromatography coupled to tandem mass spectrometry; LEA, late embryogenesis abundant protein; LecRK, lectin domain-containing receptor kinases; LRR, leucine-rich repeat; NRXs, nucleoredoxins; PHs, protein hydrolysates; PODs, peroxidases; PPP, pentose phosphate pathway; PRs, pathogenesis-related proteins; ROS, reactive oxygen species; RSPs, redox-sensitive proteins; SCX, strong cation exchange; SL, strigolactone; SOD, superoxide dismutases; TCA, tricarboxylic acid cycle; TMT, tandem mass tag; Trxs, thioredoxins; Znf, zinc finger family

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