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Plant hormone crosstalk mediated by humic acids

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Abstract

Background: The reliance on chemical inputs to support high yields is the Achilles' heel of modern crop production. The soil organic matter management is as old as agriculture itself. Recently, the use of soluble humic substances as plant growth promoters has been brought to attention due to their effects on nutrient uptake and water use efficiency. Humic substances applied directly at low concentrations can trigger different molecular, biochemical, and physiological processes in plants. However, how humic substances exert this plethoric regulatory action remains unclear. The objective of this study was to evaluate changes in the transcription level of genes coding cell receptors, phosphatases, synthesis, and function of different plant hormones and transcription factors.

Materials and methods: After seven days of humic acid treatment, we used RNAseq in maize root seedlings. The level of gene transcription was compared with control plants.

Results: Plant kinase receptors and different phosphatases were regulated by humic acids. Likewise, genes related to plant hormones (auxin, gibberellin, ethylene, cytokinin, abscisic acid, brassinosteroids, jasmonic and salicylic acids) were transcript in differential levels in maize root seedlings as well as the expression of a hundred of transcription factors modifying the signal transduction pathway via alterations of the subsequent gene response.

Conclusion: We showed a general mechanism for simultaneously regulating the activity of several hormones where humic acids act as a key regulatory hub in plant responses integrating hormonal signalling and response pathways.

Keywords: Perception, Sensing, Cellular signalling, Hormonal response

Introduction

Soil organic matter has a central role in human civilization. The relationship among humus content, soil fertility, and social development is not a coincidence and was essential for the development of the food-producing economy of Neolithic farmers [1]. Today, the challenge is feeding an increasingly demanding and growing population, concomitant to reducing external inputs and minimization of environmental impacts, even under variable and enhanced weather conditions expected in the future [2]. Reliance on the use of chemical inputs to support high yields is perhaps the environmental drawback of modern crop production. The overuse of fertilizers does not correspond to a significant increase in yields and is responsible for polluting waters and soils [3]. Pesticides were detected in 97% of stream water samples in agricultural areas of the USA [4]. While different efforts to reduce excessive use of agricultural chemicals have been exerted [5], plant growth promoters, and plant biostimulants may have a relevant role in this strategy.

Humic substances (HSs) are the major component of organic matter from soils, waters, and sediments [6] and are regarded as a complex non-covalent supramolecular association of relative small heterogeneous

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molecules that survive microbial degradation of plant and animal tissues and are held together by weak dispersive forces, hydrogen bonds, and metal-bridged intermolecular electrostatic bonds [7]. Humic substances became the most common material employed in biostimulants [8]. Plant metabolism and morphology are influenced by humic substances that modulate various biochemical mechanisms and physiological processes, stimulating growth, and increasing nutrient uptake [9]. Moreover, the best performance of humic substances was observed under stress conditions [10]. It was previously reported that HS significantly improves plant resistance to abiotic stresses [11–15]. This HS ability can be attributed to the interaction of multiple effects among which biosynthesis and concentration of secondary metabolites directly involved in stress alleviation, like phenols [16], expression of genes involved in plant responses to abiotic stress [17–19], including protective enzymes include catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD), and polyphenol oxidase (PPO) [15, 20], and regulation of reactive oxygen species accumulation and metabolism [11–14]. Furthermore, it is well known that HS promotes several interconnected, hormone-mediated signalling pathways related to plant growth and defence [21]. Several plant hormones, such as ethylene [21–23], abscisic acid [24], salicylic acid [25], gibberellins [26], cytokinins [27], auxin [28], jasmonates [29], and brassinosteroids [30], were advocated to be involved in stress signalling. Furthermore, hormone-like activity is one of the humic substances' most suggested physiological effects [31]. Hormone-like responses of humic substances emulating auxins [32, 33], gibberellins [34, 35], cytokinins [36, 37], jasmonate [38, 39], alkamides [40], and nitric oxide [41] have been reported. However, how the plant cell perceives humic substances remains unclear.

According to Shah et al. [42], humic substances trigger various molecular processes in plant cells, manifesting their effects in cells through genetic, post-transcriptional, and post-translational modifications of signalling entities that promote different molecular, biochemical, and physiological processes. Nevertheless, how this happens remains again a matter of speculation. Our work hypothesizes that kinase receptors perceive humic substances that initialize a complex interactome via phosphorylation and downstream cascade by using hormone signalling pathways and regulation of transcription factors acting as key hub agents. In other words, plants perceive soluble humic substances as a typical environmental cue or stress agent that triggers hormonal crosstalk.

The objective of this work was to challenge this hypothesis by identifying the differential transcription level of hormonal signalling, transcription factors, and protein kinases codifying genes in the RNAseq for maize seedlings roots treated or untreated with humic acids.

Materials and methods

Humic acids

A solution of 0.5 M NaOH was added under shaking to earthworm compost (10:1, v/v) under N_2 atmosphere. After 12 h, the suspension was centrifuged at 5000 ×*g*, and the humic acids (HA) were precipitated by adding 6 M HCl until pH 1.5. After centrifugation (5000 ×*g*) for 15 min, the sample was repeatedly washed with water until chloride free. Subsequently, the sample was dialysed against deionized water using a 1000-Da cut-off membrane (Thomas Scientific, Swedesboro, NJ, USA) and lyophilized. The HA solution was prepared by solubilizing HA powder in 1 mL of 0.01 M NaOH, followed by pH adjustment to 6.5 with 0.1 M HCl. After freeze drying, the carbon content was analysed by dry combustion (CHN analyser Perkin Elmer series 2400, Norwalk, CT, USA). The molecular composition of HA was evaluated by Cross-polarization magic-angle spinning (CP/MAS) ^{13}C nuclear magnetic resonance (^{13}C-NMR). The spectrum was acquired from the solid sample with a Bruker Avance 500 MHz (Bruker, Karlsruhe, Germany), equipped with a 4-mm-wide bore MAS probe, operating at a ^{13}C-resonating frequency of 75.47 MHz. The spectra were integrated over the chemical shift (ppm) resonance intervals of 0 to 46 ppm (alkyl C, mainly CH_2 and CH_3 sp^3 carbons), 46 to 65 ppm (methoxy and N alkyl C from OCH_3, C–N, and complex aliphatic carbons), 65 to 90 ppm (O-alkyl C, such as alcohols and ethers), 90 to 108 ppm (anomeric carbons in carbohydrate-like structures), 108 to 145 ppm (phenolic carbons), 145 to 160 ppm (aromatic and olefinic sp^2 carbons), 160 to 185 ppm (carboxyl, amides, and esters), and 185 to 225 ppm (carbonyls).

Plant treatment

Maize seeds (*Zea mays* L., var. Dekalb 177) were surface-sterilized by soaking in 0.5% NaClO for 30 min, rinsing, and then soaking in water for 6 h. Then, the seeds were sown in 2.0-L pots filled with washed and sterilized sand wetted with 1/3 strength Furlani nutrient solution [43] (μmol L^{-1}: 3.527 Ca; 2.310 K; 855 Mg; 45 P; 587 S; 25 B; 77 Fe; 9.1 Mn; 0.63 Cu; 0.83 Mo; 2.29 Zn; 1.74 Na; and 75 EDTA), with the N content adjusted to a low concentration (100 μmol L^{-1} $NO_3 + NH_4$). Six replicates were used in a randomized statistical design. After 1 week, the solution was changed for one-half of the ionic force. At 7 days after planting, the maize seedlings were treated with solutions containing humic acids diluted with low N Furlani nutrient solution at 0 and 4 mM C L^{-1}: Seven days after treatment, imposing three plants per pot were

collected, and root tissues were analysed individually for RNAseq, and the mean value was considered. Three replicates were used, with a total of nine plants analysed. The experiment was entirely repeated twice.

Transcriptional analysis of humic acid-treated maize root plants

For RNA extraction, 100 mg of control roots and HA-treated roots, using the best dose for root growth at 4 mM C HA L^{-1}, was macerated in liquid nitrogen. According to the manufacturer's instructions, the total RNA of the samples (3 biological replicates per treatment) was extracted with the RNeasy Plant Mini Kit (Qiagen). Total RNA was quantified using the Nanodrop 1000 spectrophotometer. The RNA was eluted in DEPC-treated water (total amount of 4–10 µg RNA) digested with DNAse and depleted ribosomal RNA using the GOTAQ® 1-STEP RT-QPCR (PROMEGA). Subsequently, a 1% free RNAse agarose gel was made to analyse the RNA extracted. According to the manufacturer's protocol, sequencing libraries were prepared using the Whole Transcriptome Analysis kit (Applied Biosystems). Libraries were sequenced on the Illumina platform by LacTad company—Brazil. The reads obtained from the RNAseq technology were analysed to identify ribosomal RNA (rRNA) sequences in two steps: 1- rRNA sequences of *Zea mays* were downloaded from NCBI, and an index file of rRNA was created using Novoalign v3.06.05. (http://www.novocraft.com/products/novoalign/). Then reads were mapped on index file using Novoalign; 2- All fastq files were converted into Fasta, and BLASTN analysis was performed against downloaded rRNA sequences. Identified rRNA sequences were removed, and reads were cleaned. Further, the quality of all reads was accessed by running the FastQC software [64], and high-quality cleaned reads were aligned on *Z. mays* genome using Novoalign. Gene expression levels were normalized as reads per kilobase of transcript per million mapped reads (RPKM). The differential gene expression between control and HA-treated samples was determined by using Cuffdiff v2.2.1. The genes with differences of at least one-fold change along with adjusted p-value (FDR) ≤ 0.05 were considered to be significantly differentially expressed. Functional classification analysis was executed with MapMan version 3.6.0RC1 (https://mapman.gabipd.org/).

Results

Receptor protein kinases (RPKs) are a diverse group of single-pass transmembrane proteins with extracellular and cytosolic domains that allow cells to recognize and respond to their extracellular environment. Table 1 shows that 261 related kinases receptors, located at the cell wall, membranes, and cytosol, were differentially expressed in maize root seedlings when treated with HA. The majority RPKs found in a higher transcript level contained leucine-rich repeat (LRR) sequences implicated in protein–protein interactions. In addition, another group was found to contain an S-domain, and several others contained unique features, such as tyrosine and chitin kinase.

Following RPKs modulation by HA, we expected that also protein phosphorylation might be induced since protein phosphorylation is the most common mechanism for regulating and controlling protein activity and function [44]. Table 2 shows the main phosphatases up and down-regulated by HA grouped by the functional category.

Phosphatases and kinases control multiple cellular events, including cell proliferation, differentiation, and stress responses, by regulating reversible protein phosphorylation, the most important post-translational modification. The activation of genes codified different cell wall acid phosphatases, representing a group of essential proteins capable of hydrolysing and solubilizing organic soil phosphate independently of soil microbial activity [45]. Purple acid phosphatases (PAPs) belong to the family of binuclear metallohydrolases, which catalyse a wide range of phosphomonoesters and are involved in plants' phosphate uptake [46]. Nine PAPs were found in a larger significant transcription level than for control, seven of which were inhibited and two up-regulated (Table 2). Other phosphatases with metal ion binding functions were found, including different inorganic pyrophosphatases. The cell wall is characterized by a significantly significant presence of a stem 28 kDa glycoprotein [47] that was down-regulated by HA treatment in root seedlings. Several cytosolic phosphatases with differential transcription were found, including those linked to carbohydrate metabolism. Putative ribose-5-phosphate isomerase and similar proteins were both down- and up-regulated by HA. These enzymes catalyse the reversible conversion between ribose-5-phosphate and ribulose-5-phosphate in the pentose phosphate pathway [48]. Glucose-6-phosphate dehydrogenase (G6PDH) provides nicotinamide adenine dinucleotide phosphate (NADPH) and intermediate metabolites for the biosynthesis of several compounds that control the flux through this non-reversible branch of the oxidative pentose phosphate pathway [49] and were found in differential transcription level in maize root seedlings treated with HA (Table 2). The 2-hydroxy-3-oxopropionate reductase (garR) is involved in galactarate degradation and catalyses the reduction of tatronate semialdehyde to D-glycerate and is involved in the carbohydrate acid metabolism. This enzyme was also found down- and up-regulated in

Table 1 Number of related kinases receptors found at significant ($p < 0.001$) differential transcription level in root maize seedlings in response to HA exposition

Receptor protein kinases (RPKs)	Up-regulated	Down-regulated
Serine/threonine protein kinase receptor	16	28
Protein of the protein kinase superfamily	8	19
Protein kinase similar to the leucine-rich repeat receptor	22	15
Uncharacterized	23	12
Cysteine-rich receptor-like protein kinase	9	5
Receptor Kinases, in general	9	4
Protein from the putative DUF26 domain protein kinase family	1	4
Signalling receptor kinases misc	0	4
Protein kinase similar to putative inactive repeat receptor rich in leucine	0	4
Receptor kinases, wall-associated kinase	3	3
PR5-like receptor	0	3
Protein kinase similar to FERONIA receptor	0	2
Putative L-type lectin domain containing the S.5 kinase receptor	0	2
Protein from the putative S-locus receptor protein kinase family	0	2
Protein from the putative CRINKLY4 receptor protein kinase family	0	2
Protein kinase similar to a proline-rich receptor PERK1	0	2
Signalling receptor kinases leucine-rich repeat	0	2
Protein MALE DISCOVERER 2	0	2
Tyrosine-sulfated glycopeptide receptor 1	2	1
XC3 tyrosine protein kinase similar to the repeating receptor rich in leucine	1	1
Calcium/calmodulin regulated by receptor-like kinase 2	1	1
Protein from the protein kinase family similar to the putative lysM domain receptor	0	1
Protein kinase similar to HERK receptor 1	0	1
Protein kinase similar to the WAK80-OsWAK receptor	0	1
Protein from the D-mannose-binding lectin family	0	1
GUB_WAK_bind protein containing domain	0	1
Sister of liguleless narrow 1	0	1
Chitin kinase eliciting receptor 1	0	1
Protein similar to OSJNBb0022F16.11	0	1
Protein kinase APK1A	0	1
Putative receptor protein kinase ZmPK1	0	1
Gene 20 associated with senescence	0	1
Protein containing forkhead-associated domain/protein containing FHA domain	0	1
Rust sheet 10 resistance to locus disease resistant to protein kinase-like 1.1	0	1
Repeating secretory protein rich in cysteine	0	1
PIRL- LRR protein 4 related to the vegetable intracellular Ras group	0	1
Protein-type hydrolase	0	1
Protein of the phosphoglucosamine mutase family	0	1
Leucine-rich transmembrane repeat protein kinase	6	0
Protein NSP-INTERACTION	4	0
Protein kinase receptor family STRUBBELIG putative	3	0
Protein from the putative phytosulfocin receptor family (protein kinase containing LRR repeat)	1	0
LRR serine/threonine protein kinase receptor similar to FLS2	1	0
LRR serine/threonine protein kinase receptor similar to EFR	1	0
ATP binding protein	1	0
LRR receptor kinase brassinosteroid	1	0
Protein from the phosphoglycerate mutase family	1	0
Protein of the ubiquitin carboxyl-terminal hydrolase family	1	0

Table 1 (continued)

Receptor protein kinases (RPKs)	Up-regulated	Down-regulated
Helicase Protein MOM1	1	0
Ear Fasciada 3	1	0
Receptor kinase 1 associated with INSENSITIVE BRASSINOSTEROID 1	1	0
2 organizing protein Hsp70-Hsp90	1	0
HSL1 receptor-like protein kinase	1	0
Protein containing BTP domain Protein	1	0
Protein of the/oxidoreductase superfamily	1	0
Sub-unit of interaction with ALA	1	0
Disease resistance protein RPM1	1	0
Brick3	1	0
Resistance kinase rust Lr	1	0
Total	126	135

respect to control. Different DNA-binding phosphatases were found for the HA treatment, including glyoxylate reductase (NADP) activity (GLYRs) as glyoxylate/hydroxypyruvate reductase (HPR3), glyoxylate/succinic semialdehyde reductase 2 chloroplastic, glyoxylate reductase, and 2-hydroxy-3-oxopropionate reductase. These enzymes catalyse the reduction of glyoxylate to glycolate by using the NADH or NADPH cofactor and were involved in detoxifying aldehydes during stress and contributing to the redox balance [50].

Our experiment found two dose-dependent cell cycle regulator 2 (Dcr2) phosphatases up-regulated by the HA treatment (Table 2). These enzymes played a positive role in cell cycle progression and stress response, acting as an antagonist of unfolded protein response (UPR) [51]. Sit4 was down-regulated by HA, implying a phosphatase's involvement in various processes, such as transcription, translation, bud formation, glycogen metabolism, monovalent ion homeostasis, and H + transport, including telomere function being functionally linked to the ubiquitin–proteasome system. Lipid phosphatases were found in differential transcription levels, including lipid phosphate phosphatase 2, which was involved in abscisic acid signalling by dephosphorylation of diacylglycerol pyrophosphate (DGPP) and phosphatidic acid (PA), which are known secondary messengers [52]. Phosphatidylinositol was found in different transcription levels and is responsible for transferring the phosphorylinositol group from phosphatidylinositol (PI) to phytoceramide, an essential step in sphingolipid biosynthesis, and is believed to be associated with self-defence through the promotion of sphingolipid metabolism and regulation of ceramide accumulation. Different inositol phosphate synthases were coupled to lipid metabolism and plant stress response by using lipids phosphorylation [53]. Myo-inositol 1-phosphate synthase and Myo-inositol-3-phosphate were implied in high transcription levels. They were involved in various biochemical and physiological processes, such as intracellular signal transduction, membrane construction and trafficking, membrane-related protein anchoring, and cell wall construction [54]. Mitogen-activated protein kinase (MAPK) cascades acted as signal transduction pathways to translate external stimuli into cellular responses, including hormones responses. Table 3 shows the MAPK enzymes found in significantly differential transcription levels in respect to control, most of which were inhibited by HA treatment.

Auxins showed the highest number of genes up- and down-regulated for root tissues exposed to HA (Table 4). Sixty-three genes related to metabolism and auxin synthesis were found at a high transcription level, with 26 down-regulated and 37 up-regulated control.

The second plant hormone with a transcriptional level modified by HA was ethylene (50 genes), of which 28 was down- and 22 up-regulated. ABA [39] was another plant hormone with more genes [25] down-regulated than up-regulated [14] by HA treatment in respect to control. There were 22 genes involved in gibberellin synthesis, signalling and metabolism with high transcript levels, compared to the control, with 13 down- and 9 up-regulated. We found 18 proteins related to brassinosteroids (BR) and jasmonates (JA) biosynthesis and metabolic processes. However, in BR, most genes were up-regulated, while in JA, the HA treatment displayed more genes down-regulated in respect to control. Finally, 14 genes were found to be related to CK and 4 linked to SA synthesis and metabolism. It is the first time evidence has been presented on humic matter capacity to modify gene responses linked to BR signalling in plants.

Table 2 Functional categories of genes codifying phosphatases proteins significantly ($p < 0.001$) up (+) and down (−) regulated by humic acids in maize roots seedlings

Functional categories	Gene name	±[a]	Phosphatases proteins
Metal ion binding, acid phosphatase activity	Grmzm5g836174_t01	− 8.91	Inorganic pyrophosphatase 1
	Grmzm2g015908_t01	− 2.41	Inorganic pyrophosphatase 1
	Grmzm5g868679_t01	− 1.63	Purple acid phosphatase
	Grmzm2g093101_t01	− 1.57	Purple acid phosphatase
	Grmzm2g111425_t01	− 3.80	Purple acid phosphatase
	Grmzm2g136453_t01	− 2.36	Purple acid phosphatase
	Grmzm2g315848_t01	− 1.84	Purple acid phosphatase
	Grmzm2g157027_t01	− 1.23	Purple acid phosphatase
	Grmzm2g152477_t01	1.30	Purple acid phosphatase
	Grmzm2g152447_t01	1.55	Purple acid phosphatase
	Grmzm2g014193_t01	1.84	Purple acid phosphatase
	Grmzm2g021106_t01	2.03	Inorganic pyrophosphatase 1
	Grmzm2g371793_t01	− 2.23	Stem 28 kDa glycoprotein
	Grmzm5g839794_t01	− 2.54	Acid phosphatase 1
Isomerase activity	Grmzm5g891282_t01	− 6.73	Putative ribose-5-phosphate isomerase 2
Lipid phosphatase activity, phospholipid metabolic process	Grmzm2g024144_t01	− 6.02	Lipid phosphate phosphatase 2
	Grmzm2g077187_t01	− 2.10	Lipid phosphate phosphatase 2
	Grmzm2g024615_t01	+ 1.33	Lipid phosphate phosphatase 2
	Grmzm2g447433_t01	+ 1.38	Lipid phosphate phosphatase 2
	Grmzm2g050658_t01	+ 2.11	Lipid phosphate phosphatase 2
	Grmzm2g174511_t01	+ 2.36	Putative lipid phosphate phosphatase beta
Ceramide cholinephosphotransferase activity, inositol phosphoceramide synthase activity, sphingomyelin synthase activity	Grmzm2g045976_t01	− 5.12	Phosphatidylinositol:ceramide inositolphosphotransferase 1
	Grmzm2g147724_t01	− 2.46	Phosphatidylinositol:ceramide inositolphosphotransferase 1
	Grmzm2g004949_t01	− 2.09	Phosphatidylinositol:ceramide inositolphosphotransferase 1
	Rmzm2g169293_t01	− 1.65	Phosphatidylinositol:ceramide inositolphosphotransferase 1
Phosphoprotein phosphatase activity	Grmzm2g068690_t01	− 2.37	CTD-phosphatase-like protein
Inositol-3-phosphate synthase activity, inositol biosynthetic process, phospholipid biosynthetic process	Grmzm2g155242_t01	− 2.46	Inositol-3-phosphate synthase
	Grmzm2g004528_t01	− 1.70	Myo-inositol 1-phosphate synthase (MIPS2)
Catalyses the oxidative decarboxylation of 6-phosphogluconate to ribulose 5-phosphate and CO_2, with concomitant reduction of NADP to NADPH	Grmzm2g127798_t01	− 2.26	6-phosphogluconate dehydrogenase, decarboxylating
Catalyses the rate-limiting step of the oxidative pentose-phosphate pathway, representing a route for the dissimilation of carbohydrates besides glycolysis	Grmzm2g426964_t01	1.33	Glucose-6-phosphate 1-dehydrogenase
	Grmzm2g130230_t01	− 2.12	Glucose-6-phosphate 1-dehydrogenase
	Grmzm2g031107_t01	− 2.04	Glucose-6-phosphate 1-dehydrogenase
	Grmzm2g177077_t01	− 1.55	Glucose-6-phosphate 1-dehydrogenase
Ribose-5-phosphate isomerase activity, pentose-phosphate shunt, non-oxidative branch	Grmzm2g035599_t01	− 1.78	Ribose-5-phosphate isomerase
	Grmzm5g874903_t01	2.90	Ribose-5-phosphate isomerase
	Grmzm2g122126_t01	− 2.05	Probable 6-phosphogluconolactonase
	Grmzm2g136918_t01	− 1.33	Probable 6-phosphogluconolactonase
Catalyses the oxidative decarboxylation of 6-phosphogluconate to ribulose 5-phosphate and CO2, with concomitant reduction of NADP to NADPH	Grmzm2g145715_t01	− 1.71	6-phosphogluconate dehydrogenase, decarboxylating
	Grmzm2g440208_t01	− 1.46	6-phosphogluconate dehydrogenase, decarboxylating
Pentose-phosphate pathway: intermediate pathway synthesizes D-glyceraldehyde 3-phosphate and beta-D-fructose 6-phosphate from D-ribose 5-phosphate and D-xylulose 5-phosphate (non-oxidative stage)	Grmzm2g134256_t01	− 1.82	Transaldolase

Table 2 (continued)

Functional categories	Gene name	± [a]	Phosphatases proteins
Diacylglycerol cholinephosphotransferase activity	Grmzm2g015040_t01	− 1.82	Phosphatidylcholine:diacylglycerol cholinephosphotransferase 1
Glyoxylate reductase (NADP) activity, hydroxyphenylpyruvate reductase activity, hydroxypyruvate reductase activity, NAD binding, oxidative photosynthetic carbon pathway	Grmzm2g159587_t01	− 1.50	Glyoxylate reductase
	Grmzm5g848696_t01	+ 1.57	Glyoxylate/succinic semialdehyde reductase 2 chloroplastic
	Grmzm2g136072_t01	+ 1.73	Glyoxylate reductase
	Grmzm2g072388_t01	+ 2.77	Glyoxylate/hydroxypyruvate reductase (HPR3)
3-hydroxybutyrate dehydrogenase activity, NAD binding, ADP binding, oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor, response to oxidative stress	Grmzm2g153984_t01	− 1.42	2-hydroxy-3-oxopropionate reductase
Protein phosphatase binding, regulation of phosphoprotein phosphatase activity	Grmzm5g806347_t01	− 1.45	SIT4 phosphatase-associated family protein
5′-nucleotidase activity, metal ion binding, nucleotide binding	Grmzm2g102404_t01	− 1.44	5′-nucleotidase
Hydrolase activity, acting on ester bonds	Grmzm2g366607_t01	+ 1.83	Putative inactive purple acid phosphatase 16
	Grmzm2g109405_t01	+ 1.29	Phosphatase DCR2
	Grmzm2g096363_t01	+ 1.38	Phosphatase DCR2
Uncharacterized	Grmzm5g828312_t01	+ 1.32	Uncharacterized
	Grmzm2g396477_t01	+ 2.62	Uncharacterized

[a] + equal up-regulated gene,− equal

Table 3 Mitogen-Activated Protein kinases (MAPK) significantly ($p < 0.001$) up- (+) and down- (−) regulated by humic acids in maize roots seedlings related to the control treatment

Gene ID	Mitogen-Activated Protein kinases (MAP)	±
grmzm2g030305_t01	Uncharacterized	− 8.37
grmzm5g878379_t01	Map kinase kinase 4 (MAPKK4)	− 5.12
grmzm2g165679_t01	Protein kinase superfamily protein	− 5.11
grmzm2g017792_t01	Mitogen-activated protein kinase (MPK)	− 5.00
grmzm2g163861_t01	Mitogen-activated protein kinase (MPK)	− 4.02
grmzm2g053987_t01	Mitogen-activated protein kinase (MPK)	− 3.16
grmzm2g048455_t01	Mitogen-activated protein kinase (MPK4)	− 2.90
grmzm2g344388_t01	Mitogen-activated protein kinase kinase 9 (MPKK9)	− 2.75
grmzm2g078650_t01	Phospholipase C	− 2.30
grmzm2g135904_t01	Mitogen-activated protein kinase (MPK)	− 2.28
grmzm2g163709_t01	MAP3K-like protein kinase	− 2.04
grmzm2g127141_t01	Mitogen-activated protein kinase (MPK)	− 1.97
grmzm2g167856_t01	Mitogen-activated protein kinase kinase 1 (MPKK1)	− 1.43
grmzm2g400470_t01	Mitogen-activated protein kinase kinase (MPKK)	− 1.33
grmzm2g374088_t01	Mitogen-activated protein kinase (MPK)	− 1.21
grmzm2g007848_t01	Mitogen-activated protein kinase (MPK)	+ 1.39
grmzm2g122335_t01	Mitogen-activated protein kinase (MPK)	+ 1.51
grmzm2g008854_t01	Protein phosphatase 2C family protein	+ 1.89

Table 5 shows the relationship among genes linked to plant hormones in significant transcriptional levels, considering positive or inhibitory effects. Although the classical literature about the hormone-like activity of humic substances report each specific and separately hormone-like activity, it is evident that HA is promoting changes in the overall hormone balance (Table 5).

Extensive interactions among genes in both synergist and inhibitory pathways were observed for different plant hormones. Genes related to auxin synthesis, metabolism,

Table 4 The number of genes encoding proteins related to different hormones perception, metabolism, and synthesis degradation and signalling significantly ($p < 0.001$) regulated (±) in maize root seedlings treated with humic acids

Hormone	Total	Up (+) regulated	Down (−) regulated
Abscisic acid (ABA)	39	14	25
Auxins (AUX)	63	37	26
Brassinosteroids (BR)	18	13	5
Cytokinins (CK)	14	8	6
Ethylene (ET)	50	22	28
Gibberellins (GA)	22	9	13
Jasmonates (JA)	18	1	17
Salicylic acid (SA)	4	3	1

and action interplaying with other plant hormones are well explored in the literature (Table 5). However, it is also clear that HA induces changes in all plant hormone responses. The ultimate interaction of cell receptors, phosphatases, MAPK, and hormonal signalling is the activation of transcription factors (TF) and induction of gene responses. It was observed that 948 different TFs were transcripted in different levels in response to HA, with 330 up-regulated and 518 down-regulated. Figure 1 summarizes the main families of TFs induced by HA treatments, while Table 6 reports TFs up and down-regulated related to plant hormone gene response. HA inhibited most TF, but a wider redundancy indicates that almost all TF are inhibited at a significant and positive expression.

Discussion

Due to the significant molecular heterogeneity and superstructural complexity of HA, it is unlikely that specific plant cell receptors may be specifically elicited. It would be more reasonable to imagine general cell pseudo-receptors being sensitized. One of the central and well-known wide energy cell sensors is the sucrose non-fermenting-1 (SNF-1) kinases family. In fact, differential expression of the SnRK2.2 protein kinase genes (serine/threonine kinase related to non-fermentative sucrose SNF) induced by HA was previously observed by RTq-PCR [73]. Other general pseudo-receptors are proteins linked to H^+-ATPase that change plasma membrane potential after being challenged by various types of signals to initiate a Ca^{2+}-mediated signal transduction cascade [74]. The effect of HA on H^+-ATPase activity is one of the most studied physiological effects [9]. However, more than 250 RPKs were elicited by HA, including cell wall, transmembrane, and cytosolic receptors (Table 1), which revealed a putative network dedicated to perceiving and transmitting the HA cue. This humic effect is reported here for the first time, opening a new perspective on how plants perceive biostimulants based on HA applied at low concentrations.

The subsequent event after cell perception is the amplification of the signal, which is manifested by the specific signal transduction cascade pathways. Post-translational modification of Ser, Thr, and Tyr residues by protein kinases and phosphatases is a major transduction route for many signals [75]. According to Trevawas [76], protein phosphorylation is a common means of manipulating connection strength. There are about 1000 protein kinases and hundreds of protein phosphatases in plants with differing degrees of specificity and control for constructing a phosphoproteome. Almost a quarter of these proteins were differentially transcribed in the HA-treated maize root seedlings showing a strong effect on signal transduction (Tables 1 and 2). In a previous proteomic study with maize seedlings treated with HA isolated from vermicompost, 24 up-regulated proteins linked to protein modifications were found, but few were phosphatases [77]. One of the most important groups of protein kinases comprises the MAPK cascade involved in transduction signals triggered by plant hormones. Interestingly, the treatment inhibited most MAPK and MAPKK (Table 3). However, the genes codifying proteins related to synthesis, functioning, and regulation of plant hormones were found to be both up- and down-regulated by HA (Table 4).

Indole-3-acetic acid-amido synthetase (GH3) was both up- and down-regulated by the HA treatment. The conjugation of indole-3-acetic acid (IAA) to amino acids by GH3 is an essential part of regulation for auxin level, thus providing a mechanism for the plant to cope with excess auxin. According to Ding et al. [78], GH3 activates disease resistance in salicylic acid signalling and jasmonic acid signalling-independent pathways. The IAA-amino acid hydrolase ILR1-like 4 is a family of hydrolase genes initially isolated in *Arabidopsis thaliana* and involved in regulating auxin levels [79] and hydrolysing amino acids acid conjugates with IAA, including IAA-Ala, IAA-Asn, IAA-Cys, IAA-Glu, IAA-Met, IAA-Ser, and IAA-Gly [79]. ILR1 hydrolyses amino acids that conjugate with jasmonic and hydroxy jasmonic acids and is also induced by jasmonic acid (JA).

The small Auxin Up-regulated RNA (SAUR) family is controlled by HA and consists of early auxin-responsive genes with an overall effect on auxin signalling-regulated plant growth and development. These genes can be readily induced by exogenous auxin [80] and are involved in plant growth by inhibiting PP2CD phosphatases, which activate plasma membrane (PM) H^+-ATPases promoting cell expansion [80]. The effect

Table 5 Genes encoding proteins related to synergist (✓) and antagonist (✗) interaction among different plant hormones significantly regulated in maize root seedlings treated with humic acids

Gene notation	ABA	AUX	BR	CK	ET	GA	JA	SA	Reference
AAMT1								✓	[54]
ABI3	✓	✓							[56]
ACS4		✓		✓	✓				[57]
ACS5		✓		✓	✓				[57]
AHK	✗			✓					[58]
AHP6		✓		✗			✓		[59, 60]
ARF	✓	✓	✓						[61, 62]
ARR1				✓		✗			[58]
ARR5				✓	✓				[59]
AUX/IAA		✗							[61]
BIN2		✓	✓						[63]
BZR1	✗	✓	✓			✓	✓		[62, 64]
CKX	✗	✓		✓					[57, 65]
CPK4	✓				✓				[66]
DELLA						✗	✓		[67]
EIN3				✗	✓				[69]
ERF	✗				✓		✓		[24]
ETR1	✗			✗	✓				[66]
FLS		✓			✓				[68]
GA20ox		✓				✓			[69, 70]
IAA19		✓	✓						[70]
IAA5		✓	✓						[70]
IAR3		✓					✓		[70]
IPT	✗	✓		✓					[65, 70]
JAZ9						✓			[60, 67]
LOX						✗	✗		[67]
MYC2		✗		✗		✓	✓		[60]
PIN		✓			✓				[58, s69]
PLS		✓			✗				[58]
PLT1/2		✓				✗			[60]
PP2C	✗	✗					✓		[71]
PR1				✓			✓		[72]
SHY2		✓		✗					[59]
TIR1		✓			✓				[68]

ABA abscisic acid, *AUX* auxins, *BR* Brassinosteroids, *CK* Cytokinin, *ET* ethylene, *GA* gibberellic acid, *JA* jasmonate, *AS* salicylic acid *AAMT1* Anthranilate O-methyltransferase, *ABI3* Abscisic acid insensitive 3, *ACS4* 1-aminocyclopropane-1-Carboxylic acid synthase 4, *ACS5* 1-aminocyclopropane-1-Carboxylic acid synthase 5, *AHK* Histidine kinases, *AHP6* Histidine phosphotransfer protein 6, *ARF* Auxin response factor, *ARR1* Two-component response regulator/ARR1 (Arabidopsis Response Regulator), *ARR5* Two-component response regulator/ARR5 (Arabidopsis Response Regulator), *AUX/IAA* Auxin/indole-3-acetic acid, *BIN2* Brassinosteroid insensitive 2, *BZR1* Brassinazole resistant 1, *CKX* Cytokinin oxidases/dehydrogenases, *CPK4* Calcium-dependent Protein Kinase 4, *DELLA* DELLA protein Family, *EIN3* Ethylene insensitive 3, *ERF* Ethylene response factor, *ETR1* Ethylene response1, *FLS* Flavonol synthase, *GA20ox* Gibberellin 3-oxidase 1, *IAA19* Auxin-responsive protein IAA19, *IAA5* Auxin-responsive protein IAA5, *IAR3* Iaa-alanine resistant 3, *IPT* Adenylate isopentenyltransferase, *JAZ9* Jasmonate zim domain 9, *LOX* Lipoxygenase, *MYC2 MYC2* Proto-Oncogene, *PIN* Auxin efflux carrier component 1, *PLS* Polaris, *PLT1/2* AP2-like ethylene-responsive transcription factor (plethoras), *PP2C* 2C protein phosphatases, *PR1* Pathogenesis-related 1, *SHY2* Auxin-responsive protein, *TIR1* Transport inhibitor response

of HA on PM H^+-ATPase activity was previously reported [70–72]. Besides auxin, hormonal regulation of SAUR expression was observed with other hormones, such as brassinosteroids (BR), gibberellins (GA), jasmonate (AJ), and abscisic acid (ABA) [84, 85]. HA treatment increased the differential transcription level of specific SAUR11, SAUR32, and SAUR36 compared to control.

Parker et al. [86] have identified two recessive allelic mutants in Arabidopsis, designated as a continuous vascular ring (cov1), that display a dramatic increase in vascular tissue development and were induced by auxin. A

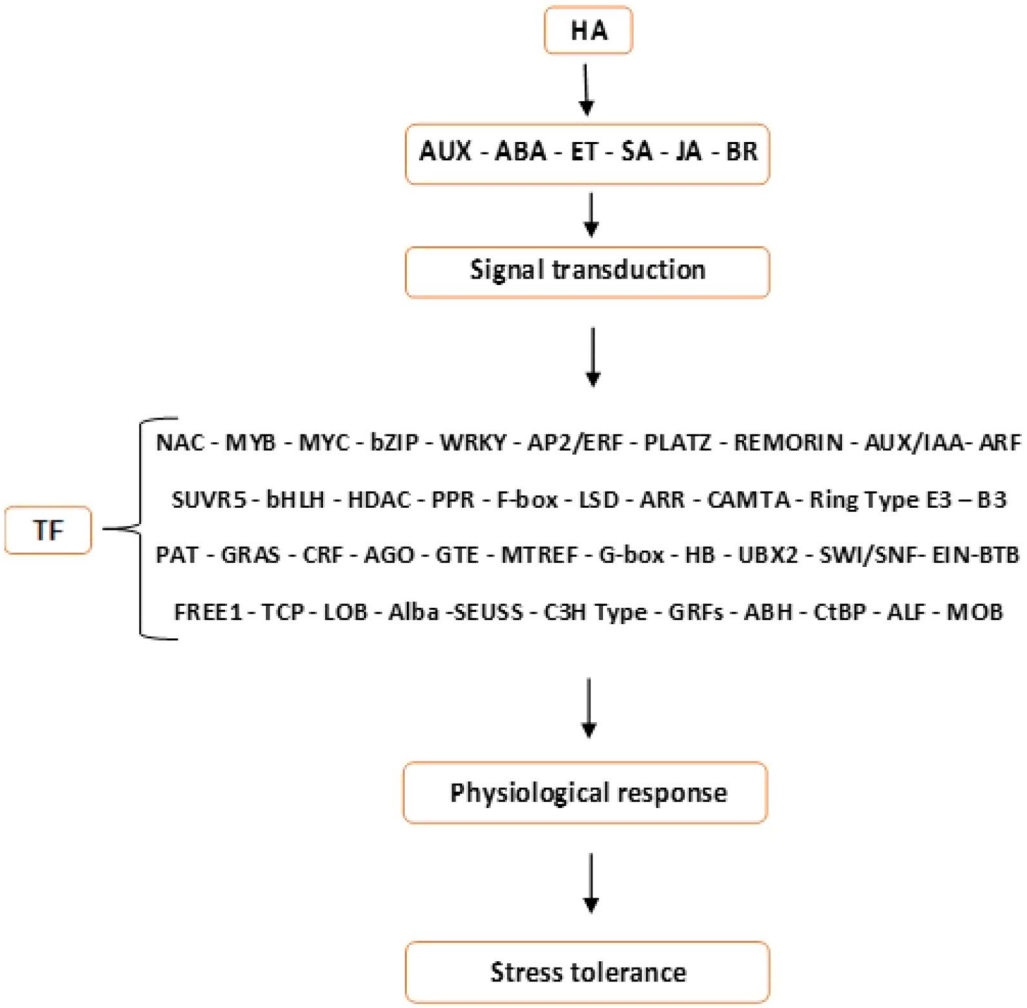

Fig. 1 Hormonal Transcription factor and regulatory genes integration network induced by humic acid (HA) treatment. Abbreviations: NAC: NAC (NAM, ATAF1,2, and CUC2; MYB: MYB Proto-Oncogene; MYC Proto-Oncogene; bZIP: Basic leucine zípper; WRKY: WRKY Family; AP2/ERF: APETALA2/Ethylene-responsive element-binding protein family; B3: B3 domain-containing protein; ARR: Two-component response regulator; REMORIN: Remorin family protein; Aux/IAA: Auxin/indole-3-acetic acid protein Family; ARF: Auxin Response Factor Family; SUVR5: Histone-lysine N-methyltransferase; bHLH: Basic Helix-Loop-Helix Family; HDAC: Histone deacetylase; PPR: Putative pentatricopeptide repeat-containing protein; F-box: F-box protein; LSD: Lysine-specific demethylase; PLATZ: Plant AT-rich sequence and zinc-binding proteins; CAMTA: Calmodulin-binding transcription activators; Ring-type E3: RING-type E3 ubiquitin transferase; PAT: S-acyltransferase; GRAS: GRAS transcription factor family (Scarecrow); CRF: Chromatin Remodelling Factors; AGO: Argonaute; GTE: Transcription factor group E; MTREF: Mitochondrial transcription termination factor family protein; G-box: G-box protein; MOB: MOB kinase activator like; UBX2: Ubiquitin regulatory X domain-containing protein 2; SWI/SNF: SWItch/Sucrose Non-Fermentable; EIN3: EIN3-like (EIL) transcription factor Family; FREE1: fyve domain protein required for endosomal sorting 1; TCP: TCP protein domain; LOB: LOB domain-containing protein; Alba: Alba DNA/RNA-binding protein; SEUSS: Transcriptional corepressor SEUSS; C3H Type: C3H-type transcription factor; GRFs: GRF transcription factor; ABH: Alpha/beta hydrolase; CtBP: C-terminal-binding protein; ALF: Alfin-like transcription factor; HB: Homeobox transcription factor Family

high auxin level induces polar auxin transport, resulting in vascular differentiation. PIN1 protein is essential to auxin transport and has a high transcription level in HA-treated seedlings. PIN proteins induce polar auxin transport because of their asymmetric subcellular localizations [87]. Moreover, it was found that genes differentially expressed coding proteins linked to a ubiquitination process that is directed by auxins, such as F-box FBX14, BIG (BIG) binding/ubiquitin-protein ligase/zinc ion binding, auxin signalling F-BOX 3, DCN1-like protein 2 (Defective in cullin neddylation protein), and TIR1 (TRANSPORT INHIBITOR RESPONSE 1). Ubiquitination of

Table 6 Related Transcription Factors (TFs) and Regulatory Genes (RG) that were found at significant ($p < 0.001$) differential transcription level due to HA exposition

Transcription factors	Description	UP	DOWN
MYB transcription factor	DNA-binding domains	43	66
Zinc finger family	Metal-binding, Zinc	37	58
WRKY domain transcription factor family	DNA-binding transcription factor activity, sequence-specific DNA binding	10	49
AP2/EREBP, APETALA2/Ethylene-responsive element-binding protein family	DNA-binding, transcription factor activity	12	42
Basic Helix-Loop-Helix family (bHLH)	DNA-binding, protein dimerization activity	20	32
NAC domain transcription factor family	DNA binding, regulation of transcription, DNA templated	8	31
Homeobox transcription factor family	DNA-binding transcription factor activity, sequence-specific DNA binding, RNA polymerase II-specific, positive regulation of transcription, DNA templated	24	17
bZIP transcription factor	DNA binding, DNA-binding transcription factor activity	20	17
Auxin Response Factor family (ARF)	Auxin-activated signalling pathway, regulation of transcription, DNA templated	9	12
Chromatin Remodelling Factors	Uncharacterized	0	12
GRAS transcription factor family (Scarecrow)	DNA binding, DNA-binding transcription factor activity, transferase activity, transferring acyl groups other than amino-acyl groups	14	11
Remorin family protein	Abscisic acid-activated signalling pathway. Negative regulation of brassinosteroid-mediated signalling pathway	5	8
Triple-Helix transcription factor family	DNA-binding transcription factor activity, sequence-specific DNA binding	1	8
Aux/IAA family	Auxin-activated signalling pathway, regulation of transcription, DNA-templated. Repressor	0	8
MADS transcription factor	Protein dimerization activity, DNA-binding transcription factor activity. RNA polymerase II transcription regulatory region sequence-specific DNA binding	0	8
Argonaute	Argonaute protein, nucleic acid binding, gene silencing by RNA	1	7
Eukaryotic aspartyl protease family protein	Aspartic-type endopeptidase activity	7	6
Histone-lysine N-methyltransferase	Histone binding, metal ion binding, methyltransferase activity, methylation. Negative regulation of transcription, DNA template, and positive regulation of transcription by RNA polymerase II	6	6
B3 domain-containing protein	Plant-specific B3 DNA-binding domain, PHD finger	3	6
G2-like transcription factor family	Transcription regulation	2	6
Nuclear transcription factor Y subunit	DNA-binding transcription factor activity, RNA polymerase II-specific, regulation of transcription, protein heterodimerization activity, RNA polymerase II cis-regulatory region sequence-specific DNA binding	9	5
Two-component response regulator (ARR)	DNA-binding transcription factor activity, sequence-specific DNA binding, phosphorelay signal transduction system. Transcriptional activator that binds specific DNA sequence	6	5
Aspartic proteinase nepenthesin-1	Aspartic-type endopeptidase activity	0	5
Transcription factor GTE	Bromodomain, chromatin-associated proteins and in nuclear histone acetyltransferases. They interact specifically with acetylated lysine	3	4
DNA-binding bromodomain-containing protein	DNA binding	3	4
Histone deacetylase	NAD-dependent histone deacetylase activity (H3-K14 specific)	1	4
Putative pentatricopeptide repeat-containing protein	DNA binding, zinc ion binding, RNA modification	9	3
S-acyltransferase	Protein-cysteine S-palmitoyltransferase activity, peptidyl-L-cysteine S-palmitoylation, protein targeting to the membrane	9	3
Agamous-like MADS-box protein	Protein dimerization activity, DNA-binding transcription factor activity. RNA polymerase II transcription regulatory region sequence-specific DNA binding	7	3
Lysine-specific demethylase	Histone demethylase activity, histone demethylase activity (H3-trimethyl-K4 specific), methyltransferase activity, chromatin remodelling, methylation	2	3
PLATZ transcription factor (PLT)	Leaf development and leaf senescence	2	3

Table 6 (continued)

Transcription factors	Description	UP	DOWN
Calmodulin-binding transcription	DNA binding, calmodulin binding	1	3
DUF domain family protein	DNA-binding transcription factor activity, sequence-specific DNA binding	1	3
Exocyst family protein	Exocytosis, protein transport	1	3
Charged multivesicular body protein	Late endosome to vacuole transport via multivesicular body sorting pathway, vesicle budding from the membrane	1	3
RING-type E3 ubiquitin transferase	This protein is involved in the pathway protein ubiquitination, which is part of Protein modification	0	3
GTPase activating protein	GTPase activator activity	0	3
WD-40 repeat family protein	Regulatory modules in signal transduction, pre-mRNA processing and cytoskeleton assembly	0	3
Dehydration-responsive element-binding protein (DREB)	DNA-binding transcription factor activity. DNA-binding	0	3
AT-hook motif nuclear-localized protein	DNA-binding transcription factor activity, the minor groove of adenine–thymine-rich DNA binding	5	2
Nucleic acid-binding protein	DNA binding, RNA binding, metal ion binding	4	2
PHD finger protein	Metal ion binding	3	2
Putative mediator of RNA polymerase II transcription subunit 26b	DNA binding	2	2
Mitochondrial transcription termination factor family protein	Double-stranded DNA binding, DNA-templated transcription, regulation of transcription, DNA templated	2	2
G-box binding factor	DNA-binding transcription factor activity, sequence-specific DNA binding, regulation of transcription	1	2
EIN3-like (EIL) transcription factor family	DNA binding, DNA-binding transcription factor activity, ethylene-activated signalling pathway	0	2
Hydroxyproline-rich glycoprotein family protein	Structural constituent of the cell wall	0	2
MOB kinase activator-like	Kinase activity	0	2
UBX domain-containing protein 2	Defence response to fungus	0	2
SWI/SNF complex	Endosome transport via multivesicular body sorting pathway, late endosome to vacuole transport, protein transport	0	2
Protein FREE1	Metal ion binding	0	2
TCP transcription factor family	DNA-binding transcription factor activity, sequence-specific DNA binding	6	1
LOB domain-containing protein	Multicellular organism development, regulation of gene expression	4	1
Polyadenylate-binding protein	RNA binding, regulation of mRNA splicing, via spliceosome	3	1
NIN-like bZIP-related family	DNA binding, DNA-binding transcription factor activity	2	1
BTB domain-containing protein	May act as a substrate-specific adapter of an E3 ubiquitin-protein ligase complex (CUL3-RBX1-BTB) which mediates the ubiquitination and subsequent proteasomal degradation of target proteins	2	1
Transcription elongation factor	Structural constituent of ribosome, translation elongation factor activity, regulation of DNA-templated transcription, elongation, regulation of transcription by RNA polymerase II	2	1
Alba DNA/RNA-binding protein	RNA binding	2	1
Transcriptional corepressor SEUSS	Negative regulation of transcription, DNA-templated, negative regulation of transcription by RNA polymerase II, positive regulation of transcription by RNA polymerase II, RNA polymerase II activating transcription factor binding	2	1
Transcription initiation factor	Initiation factor, translation initiation factor activity	2	1
Forkhead-associated (FHA) domain-containing protein	G-quadruplex RNA binding	2	1
JUMONJI family/Lysine-specific demethylase	Methyltransferase activity	1	1
C3H-type transcription factor	DNA binding, metal ion binding	1	1
GRF transcription factor	ATP binding, developmental process, regulation of transcription, DNA-templated transcription, DNA-templated	1	1
Rubisco methyltransferase family protein	Rubisco LSMT substrate-binding	1	1

Table 6 (continued)

Transcription factors	Description	UP	DOWN
Histone acetyltransferase	Chromatin DNA binding, histone acetyltransferase activity, RNA polymerase II transcription factor binding, transcription coactivator activity, zinc ion binding, histone acetylation, positive regulation of transcription by RNA polymerase II	1	1
AtSR Transcription Factor family	Hydrolase activity	0	1
C-terminal-binding protein	NAD binding, oxidation–reduction process	0	1
MYC transcription factor	DNA-binding transcription factor activity, sequence-specific DNA binding, protein dimerization activity	0	1
Alfin-like transcription factor (ALF2)	Histone binding, metal ion binding, regulation of transcription, DNA templated	2	0
F-box protein	DNA binding	2	0
Total		330	518

proteins is a widely employed mechanism for hormonal regulation. Ubiquitin is covalently attached to an internal lysine residue or the N terminus of a substrate protein, as those conjugated with auxins promoted by GH3 and ILR1. Additional ubiquitin moieties are attached to the initial ubiquitin, resulting in the formation of ubiquitin chains. In most cases, these chains are recognized by the 26S proteasome, which degrades the tagged substrates. It is possible that HA can regulate auxin signalling by an interaction between TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALINGF-BOX proteins (TIR1/AFBs) and AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) coreceptor proteins and the subsequent transcriptional regulation. The underlined mechanism was extensively studied, showing that the process of auxin perception by TIR1 (an F-box protein) forms an SKP1–Cullin–F-box (SCF) ubiquitin ligase complex inducing further complexation with the AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) transcription regulator and results in proteasomal degradation of AUX/IAA, thereby promoting the expression of auxin-inducible genes [88]. Cullin-based E3 ubiquitin ligases are activated by modifying the cullin subunit with the ubiquitin-like protein Nedd8. DCN1 regulates cullin neddylation and thus ubiquitin ligase activity [89] and was found in a high transcription level in root seedlings treated with HA. The inhibition of various auxin-responsive proteins (IAA4, IAA9, IAA13, IAA24) can be interpreted as one additional evidence of promoting the ubiquitination process induced by HA.

Degradation of the Aux/IAAs activates the auxin response factor (ARF) family of transcription factors, whose activities in regulating auxin-responsive genes are otherwise inhibited by the Aux/IAA proteins. This mechanism is mediated by the TIR1 ubiquitin ligase [90]. HA treatments also induced other genes related to mechanisms of auxin homeostasis regulation. Glycosylation is one of the general homeostatic mechanisms of metabolism of small molecules, and glycosyltransferases recognize a great diversity of substrates, including hormones (auxin and cytokinins), secondary metabolites, and xenobiotics as pesticides and herbicides [91]. In addition, O-fucosyltransferases are glycosyltransferase regulated by the HA treatment. Finally, we found a particular mechanism of cell homeostasis triggered by HA involving the redox process. It is well known that plants can detoxify reactive oxygen species and reactive aldehydes induced by stress, to less toxic compounds, such as alcohols or carboxylic acids, by up-regulating the expression of a few enzymes with oxidoreductase activity. Aldo–keto reductases (AKRs), belonging to the oxidoreductase superfamily, are stress-regulated genes and play an essential role in the cellular response to electrophilic, osmotic, and oxidative stress depending on the presence of coenzyme NAD (P)(H) (Nicotinamide Adenine Dinucleotide Phosphate). The presence of many auxin and abscisic acid response elements in the promoters of MtAKRs indicates potential functions in plant growth and responses to environmental stresses.

The MtAKR2 promoter contains both auxin and MeJA response elements [92]. Apoplastic reductants act as an antioxidant barrier and modulate oxidative signals, thus contributing to cell homeostasis. Ascorbate occurs at 10^{-4} to 10^{-3} M concentrations in the apoplast, representing the primary pool of low-molecular-mass antioxidants. Preger et al. [93] reported that the trans-PM electron transfer from cytosolic ascorbate to apoplastic MDA is affected by a cytochrome b561. The major ascorbate-reducible cytochrome b is associated with the PM of soybean codified by AIR12 (for auxin-induced in root cultures). A DOMON-plus-cytochrome b561 protein was found to be induced by HA treatment. In this respect, the increase in ascorbate concentration in plants treated with HA was previously reported [94].

We found 39 genes promoted by HA linked to ABA activity. Among them, 25 were negatively regulated, and 14 were positively regulated. ABA response element-binding factor has a DNA-binding transcription factor activity displaying abscisic acid-activated signalling pathway. Both are up- and down-regulated by HA. bZIP (basic zipper) leucine was up-regulated in the HA treatment. The primary target of the bZIP class of transcription factors is the ABRE motif, an important one for its proper regulation by ABA. ABF4 (ABRE BINDING FACTOR 4) was found in a high level of transcription regarding control. At the same time, ABA INSENSITIVE(ABI)5/ABA Response Element (ABRE)-binding factor (ABF/AREB) is another clade of basic leucine zipper (bZIP) transcription factors that are generally induced by ABA in response to dehydrating stresses promoted by the HA treatment. Lynch et al. [95] reported that these bZIPs must be phosphorylated to be active and are inhibited by protein phosphatases and ubiquitin ligases, which affect their activity and stability, respectively. Putative GEM-like protein 8 belongs to GRAM domain-containing gene family has a multiple ABRE cis-element [96], which was termed the ABA-responsive protein (ABR) family and was up-regulated by HA. These domains are implicated in functional protein association networks. ABI1 was also shown to bind weakly with the Sucrose non-fermenting Related Kinase, SnRK3.1, that participates in global stress responses [97]. Protein serine/threonine phosphatase was up-regulated by HA and are receptors that start ABA signalling cascade by the phosphorylation of the C-terminus of the H^+-ATPase to release the protein from autoinhibition, thus allowing access by 14–3-3 proteins to maintain the proton pump in the active conformation [98]. ABA counteracts the blue-light-dependent activity of the H^+-ATPase and locks the membrane potential to depolarize state, thereby allowing the continuous effluxes of cations and anions [99, 100].

A critical rate-limiting step in ABA synthesis is the cleavage of the carotenoids 9-cisneoxanthin and 9-cis-violaxanthin in the chloroplast to yield xanthoxin, which is exported to the cytoplasm and metabolized to ABA [101]. This carotenoid cleavage reaction is catalysed by the 9-cisepoxycartenoid dioxygenases (NCEDs) strongly down-regulated by the HA treatment. Furthermore, AAO (Arabidopsis Aldehyde Oxidase) promotes the last step of ABA biosynthesis, catalysing the oxidation of abscisic aldehyde [102], and was also down-regulated by HA. Besides NCED, others called carotenoid cleavage dioxygenases (CCD) genes down-regulated by HA and may be involved in another hormone biosynthesis. Indole-3-acetaldehyde oxidase is an enzyme involved in the redox process and the abscisic acid and auxin biosynthetic process. This enzyme belongs to the family of oxidoreductases, specifically acting on the aldehyde or oxo group of donors with oxygen as acceptors. The oxidation of abscisic aldehyde in the last step of ABA biosynthesis is catalysed by aldehyde oxidase (EC 1.2.3.1) [103], which is also involved in tryptophan synthesis [104]. Abscisic acid-induced-like protein (HVA22) was both up- and down-regulated by HA and involved ABA perception. Polyadenylation is an alternative post-transcriptional regulatory mechanism that is important in generating new transcript isoforms and, therefore, new gene functions. Polyadenylation is a highly regulated process that involves the activity of FIP1, which provides a bridge between poly(A) polymerase (PAP) and other subunits of the cleavage and polyadenylation factors [94]. Genes possessing polyadenylation sites within protein-coding regions or introns are significantly associated with stress responses. According to Tellez-Robledo et al. [105], FIP1 is needed for the correct polyadenylation of transcripts that display ABA signalling responses and stresses, such as salt or cadmium genes. In addition, Wang et al. [106] revealed that FIP1 negatively regulated the expression of CIPK8 and CIPK23, two protein kinases involved in nitrate signalling. FIP1 was both up- and down-regulated by the HA treatment.

The homeobox gene GLABRA2 (GL2) has been considered a key component in the root-hair pattern determined by a regulatory circuit composed of transcription factor genes. GL2 exerts its regulatory effect on root-hair development by modulating phospholipid signalling [107]. ABA INSENSITIVE 8 (ABI8]/ELONGATION EFFECTIVE 1 (ELD1]/KOBITO1 (KOB1] promoted the expression of genes involved in cell elongation and cellulose synthesis in addition to the traditional light response, encoding a glycosyltransferase-like protein in *Arabidopsis* previously that is implicated in cellulose biosynthesis and hypocotyl elongation [108].

Gibberellin 2-oxidases (GA2oxs) are a group of 2-oxoglutarate-dependent dioxygenases that catalyse the deactivation of bioactive GA or its precursors through the 2β-hydroxylation reaction [109]. We found seven genes linked to this family, with six down-regulated and one up-regulated by the HA treatment. Gibberellins (GAs) are a large group of diterpenoid natural products that regulate various developmental and growth processes and impact crop yields. When gibberellin synthesis is inhibited, more precursors in the terpenoid pathway accumulate, potentially increasing the production of other molecules, such as abscisic acid. Some plant growth regulators used in agriculture, such as Paclobutrazol, inhibit the GA synthesis inducing anti-stress response. The main target of this kind of plant growth regulator is the inhibition of the Cyt P450 necessary for the kaurene oxidase activity. Ent-kaurene oxidase catalyses three steps of GA biosynthesis

[110], and both ent-kaurene oxidase and kaurene oxidase 2 were down-regulated by HA. However, the intermediate steps that oxidize ent-kaurene to GA12 are catalysed by several Cyt P450 monooxygenases found at high transcriptional levels in roots seedlings treated with HA.

Furthermore, ent-copalyl diphosphate synthases also have a distinct ent-kaurene synthase activity associated with the same protein molecule. The suppression of ent-kaurene synthase activity of the protein leads to the build-up of ent-copalyl pyrophosphate found in high transcript levels by the HA treatment. In addition, ent-copalyl as precursors for several classes of phytoalexins involved in stress response besides GAs [111]. Phytoalexins are low-molecular-weight compounds produced after exposure to microorganism attacks and other elicitors, and it has been suggested that they serve as plant antibiotics. GA signalling is mediated by GIBBERELLIN-INSENSITIVE DWARF1 (GID1) and DELLA proteins in collaboration with a GA-specific F-box protein. The DELLA protein belongs to the GRAS superfamily of putative transcription factors, a well-characterized factor involved in the GA signalling pathway. The DELLA protein functions as a negative regulator of GA signalling and is rapidly degraded when plants are treated with GA [112]. These authors also reported that GIBBERELLIN-INSENSITIVE DWARF2(GID2) and SLEEPY1 (SLY1), candidate F-box components of Skp1-Cullin-F box protein (SCF) E3 ubiquitin ligases, are responsible for targeting DELLA proteins to the proteasome. It is found that HA down-regulated genes that encode DELLA and SLEEPPY proteins. The binding of GA to its receptor GA INSENSITIVE DWARF1 (GID1) enhances the GID1–DELLA interaction, which, in turn, leads to the rapid proteolysis of DELLA through the ubiquitin–proteasome pathway and allows transcriptional reprogramming of GA-responsive genes. The mechanism of this regulation is not clear, although SCARECROW (SCR) appeared to be implicated as a regulatory protein [113]. GASA (gibberellic acid stimulated in *Arabidopsis*) proteins are small peptides localized in the apoplast or cell wall, and it is found that HA up-regulated their expression. The role of GASA in the interaction between different hormonal signalling pathways in redox regulation and several aspects of plant biology was reported [114, 115].

Ethylene is an essential regulator of many developmental and physiological processes and plays a vital role in the plant's defence against biotic and abiotic stress factors [116]. The biosynthetic pathway of the ethylene is well characterized, and two enzymes involved in the pathway, ACC synthase (EC 4.4.1.14) and ACC oxidase (EC 1.4.3), have been identified [117]. Furthermore, the genes capable of codifying both proteins were down-regulated by HA treatment. Ethylene response factors (ERFs) are integral to the ethylene signalling and response pathway. ERFs are transcription factors that can bind to cis-acting elements, such as GCC-box motifs and dehydration-responsive elements (DREs) [118]. Ethylene is sensed by receptors localized at the endoplasmic reticulum membrane, including Ethylene Response1 [ETR], and results to be down- [ETR40] and up- [ETR1] regulated by HA. ERFs are key regulators in abiotic stress tolerance, such as drought, salinity, light stress, and cold and heat treatments. ERF3 was up-regulated, while ERF8 and ERF1B were down-regulated. The APETALA2/ethylene-responsive element-binding protein (AP2/EREBP) superfamily is one of the largest groups of plant-specific transcription factors. They play vital roles in plant growth, development, and response to abiotic and biotic stresses. As ERF, AP2/EREB was found to be implicated in various hormone-related signal transduction pathways, including ABA, CK, and JA [119]. Another candidate to make a bridge between stress and transcription factors are the Multiprotein Bridging Factor 1 [MBF1) family of proteins [120]. According to these authors, some genes related to abiotic and biotic stress response regulated by ET (and other plant hormones, like JA and SA) were up-regulated in the MBF1a overexpression in plants, suggesting that MBF1a could be protecting the plant against pathogen attack via ethylene and jasmonic acid-dependent pathways. All three MBF1 forms were found negatively regulated by the HA treatment. The *Arabidopsis thaliana* zinc finger transcription factor (ZF-TF), S-nitrosothiol (SNO) Regulated 1 (SRG1), is a central target of NO bioactivity during plant immunity. SRG1 appears to act as a transcriptional repressor utilizing its putative ERF-associated amphiphilic repression (EAR) domain to recruit the corepressor TOPLESS, thus contributing to plant defence responses [121]. HA was found to down-regulate SRG1. Finally, DeSI-like protein belongs to an ethylene-responsive element-binding protein with ubiquitinyl hydrolase activity. Its function on hormone homeostasis is linked to protein modification by small protein removal. These proteins were found to be regulated by the HA treatment.

Brassinosteroids (BRs) are a group of plant steroidal hormones ubiquitous in the plant kingdom and regulate various physiological responses. The BRASSINAZOLE-RESISTANT 1 (BZR1) transcription factor family is essential in plant brassinosteroid (BR) signalling. The well-known negative regulation of abscisic acid inhibits root growth by brassinosteroids and is partially mediated via direct suppression of ABSCISIC ACID INSENSITIVE 5 expression by BRASSINAZOLE RESISTANT 1 [122]. The transcription level of BZR1 was significantly down-regulated in the roots treated with HA. BRs are perceived at the cell surface, and their biosynthesis occurs on the endoplasmic reticulum (ER) using different

pathways. [123]. Two different BRs receptors were down-regulated by HA (Brassinazole-resistant 1 protein and Brassinosteroid-responsive/RING-H2). However, several proteins involved in plant steroid biosynthesis were up-regulated by HA, including Delta (24)-sterol reductase, Steroid 5-alpha-reductase (DET2), Polyprenol reductase, Squalene monooxygenase, Sterol 14-demethylase, and 3-Epi-6-deoxocathasterone 23-monooxygenase (cytochrome P450 90C1/CYP90D1), among others.

Cytokinin (CK) plays an important role in regulating shoot and root growth, leaf senescence, chloroplast development, stress response, and pathogen resistance [124]. Isopentenyl transferase1 (IPT1) catalysed the first step in the Ck biosynthesis and was up-regulated by HA. Moreover, Ck oxidases/dehydrogenase catalyses the irreversible degradation of Ck. CKX4 are strongly inhibited by the HA treatment, while CKX6 was up-regulated. The Ck signal is perceived in *Arabidopsis* by histidine kinase receptors that were up- and down-regulated by HA addition to maize root seedlings. The common Ck signalling system has a membrane-bound receptor histidine kinase, which senses the signal and autophosphorylates. A response regulator activates the transcription of its target genes upon phosphorylation by the receptor kinase or initiates another output reaction [125]. The histidine kinase was found to be regulated by the HA treatment (Table 1).

JAs (jasmonates) are cyclopentanone compounds derived from linolenic acid via an octadecanoid pathway consisting of several enzymatic steps. The early steps of this pathway occur in chloroplasts, where linolenic acid is converted to 12-oxo-phytodienoic acid (OPDA) using three enzymes, lipoxygenase, allene oxide synthase (AOS), and allene oxide cyclase, and is subsequently reduced in a cyclopentenone ring by a peroxisome-localized enzyme, 12-oxophytodienoic acid reductase 3 [126]. All these proteins involved in JA biosynthesis were negatively regulated in the HA treatment, except for lipoxygenase (LOX11).

The integration of different plant hormones is a well-known process in system biology studies, and its magnitude is often dependent on plant development and physiological processes. For example, auxins are a pivotal plant hormone that commands rooting [127], and the auxin-like activity is the most reported effect of HA [21]. Here, we integrated the HA and plant hormone network using transcriptomic data.

The keystone of environmental and hormonal effects in plants is the signal perception and transduction cascade. Our results showed that HA alters genes' expression, codifying proteins related to the perception, metabolism, and signalling of ABA, CK, AUX, BR, CH, ET, GA, JA, and SA, thereby changing the hormonal crosstalk. These genes have multiple regulations and are both inhibited or promoted by hormones indicating putative complex crosstalk. Table 5 shows the main known hormonal crosstalk previously defined in the scientific literature involving the genes significantly regulated by HA. Genes stimulated by HA include regulatory proteins that further alter gene expression and possibly function in hormonal crosstalk response. They comprise several transcription factors (TFs), emphasizing the role of various transcriptional regulatory mechanisms in the hormone signal transduction pathways. The TFs interact with *cis*-elements in the promoter regions of several genes and thus up-regulate the expression of many downstream genes, resulting in imparting hormone response. Table 6 reports the relationship among TFs found in differential transcription level and plant hormone crosstalk based on literature, while Table 7 reports the interaction between TFs and plant hormones.

The phytohormone auxin is the central regulator of root architecture, particularly lateral root emergence in plants, and coordinates a complex gene regulatory network enabling plants to cope with decreased water and nutrient availability. HA was found to change root development and its response to environmental cues. These responses include changes in transcript levels of different plant hormones (Table 2). Since auxin is a master regulator of root traits, we put this hormone in focus in this discussion. Genes for various transcription factors containing typical DNA-binding motifs, such as MYB, bZIP, MYC, ERF/AP2, and Zinc fingers, have been inducible by HA (Fig. 1).

MYB, Zinc finger family, WRKY, AP2/EREB, and NAC were the TFs that held more differential transcription levels induced by HA treatment that, in turn, promoted both up- and down-regulation, with the majority being negatively regulated. Myeloblastosis viral oncogene homolog (MYB) transcription factors are active players in abiotic stress signalling [177] and correspond to one of the most prominent TF families in plants. MYB is induced by drought and other abiotic stresses and positively controls the drought resistance by inducing stress and auxin-responsive genes [167]. MYB TFs also affect signal transduction and phytohormone biosynthesis, including auxin, gibberellic acid, methyl jasmonate, and ABA [179], becoming a strong candidate for a mediator of the integration among different hormonal signals. The role of MYBs in root development and differentiation has been previously reported [180]. It involves the control of cell cycle progression at the root tips [129] and the expression of auxin-inducible genes regulating lateral root formation [181]. In addition, it was observed that MYB TF can regulate lateral root meristem activation under drought conditions via ABA–auxin signalling

Table 7 Transcription factors were found at a significant level ($P < 0.001$) in root maize seedlings treated with humic acids encoding proteins related to synergist (✓) and antagonist (✗) interaction among different plant hormones

Gene notation	ABA	AUX	BR	CK	ET	GA	JA	SA	References
MYB	✓	✗				✓	✓		[128]
HB	✓	✓							[129–131]
bHLH	✓				✓	✓			[132]
bZIP	✓				✓		✓	✓	[133, 134]
GRAS	✓	✓			✓	✗	✓		[135–137]
AP2/ERF	✓ ✗	✓	✓		✓	✗	✓	✓	[132, 138]
WRKY	✓		✓			✗	✓	✓	[134, 139, 140]
ARF	✓	✓					✓	✓	[141]
PPR	✓								[142]
PATs	✗	✗		✗				✗	[143]
NAC	✓				✓	✗	✓		[144]
SUVR5	✓								[145]
ARR				✓	✓				[146]
TCP				✓	✓				[146]
Remorin	✓						✓		[147]
LOB			✗	✓					[148]
B3	✓	✓	✓			✓			[149]
GTE	✗								[150]
LSD	✗	✓							[151]
PLATZ	✓					✓	✓	✓	[152]
MTERFs	✓						✓	✓	[153]
BTB	✗	✓							[154]
Alba	✓						✓	✓	[155]
SEUSS		✓							[156]
ALF2	✓								[157]
F-box		✓			✗	✓	✓		[158]
AGO		✓					✓	✓	[159, 160]
HDAC	✗						✓		[161]
CAMTA	✓	✓					✓	✓	[162]
G-box	✓		✓	✓			✓		[163]
C3H type	✗					✗			[164]
GRF	✓					✓	✓		[165]
CRF	✗	✓		✗	✗		✓		[166, 167]
Aux/IAA	✓	✗✓	✓	✓			✓		[168]
RING-type E3	✗	✓		✗	✗		✓		[166, 167]
EIN3					✗				[169]
MOB		✓							[170]
UBX2		✓							[171]
SWI/SNF	✓				✗	✗	✗		[172]
FREE1	✓								[173]
ABH						✗			[174]
CtBP					✗		✗	✗	[175]
MYC							✓		[176]

Transcription factors NAC: NAC (NAM, ATAF1,2, and CUC2, *MYB* MYB Proto-Oncogene, *MYC* Proto-Oncogene, *bZIP* Basic leucine zipper, *WRKY* WRKY Family, *AP2/ERF* APETALA2/Ethylene-responsive element-binding protein family, *B3* B3 domain-containing protein, *ARR* Two-component response regulator, *REMORIN* Remorin family protein, *Aux/IAA* Auxin/indole-3-acetic acid protein Family, *ARF* Auxin Response Factor Family, *SUVR5* Histone-lysine N-methyltransferase, *bHLH* Basic Helix-Loop-Helix Family, *HDAC* Histone deacetylase, *PPR* Putative pentatricopeptide repeat-containing protein, *F-box* F-box protein, *LSD* Lysine-specific demethylase, *PLATZ* Plant AT-rich sequence and zinc-binding proteins, *BTB* BTB domain-containing protein, *CAMTA* Calmodulin-binding transcription activators, *Ring-type E3* RING-type E3 ubiquitin transferase, *PAT* S-acyltransferase, *GRAS* GRAS transcription factor family (Scarecrow), *CRF* Chromatin Remodelling Factors, *AGO* Argonaute, *GTE* Transcription factor group E, *MTREF* Mitochondrial transcription termination factor family protein, *G-box* G-box protein, *MOB* MOB kinase activator-like, *UBX2* Ubiquitin regulatory X domain-containing protein 2, *SWI/SNF* SWItch/Sucrose Non-Fermentable, *EIN3* EIN3-like (EIL) transcription factor Family, *FREE1* fyve domain protein required for endosomal sorting 1, *TCP* TCP protein domain, *LOB* LOB domain-containing protein, *Alba* Alba DNA/RNA-binding protein, *SEUSS* Transcriptional corepressor SEUSS, *C3H Type* C3H-type transcription factor, *GRFs* GRF transcription factor, *ABH* Alpha/beta hydrolase, *CtBP* C-terminal-binding protein, *ALF* Alfin-like transcription factor, *HB* Homeobox transcription factor Family

crosstalk [182]. MYB are also critical regulators of the synthesis of phenylpropanoid-derived compounds [183]. Plant phenylpropanoid-derived compounds are a large family of phenylalanine-derived secondary metabolites, including monolignols, flavonoids (anthocyanins, proanthocyanidins, flavonols), flavones, flavanones, isoflavonoids, and phlobaphenes), various phenolic acids, and stilbenes. Humic substances can induce the first step of synthesizing phenylpropanoid-derived compounds catalysed by phenylalanine ammonia lyase [184]. MYB proteins can act as transcriptional activators and repressors that control the synthesis of the phenylpropanoid-derived compounds.

WRKY TFs have been reported to play pivotal roles in defence signalling and in regulating plants' growth and developmental processes [185]. For example, Yang et al. [186] have shown that rice ABI5-Like1 (ABL1) regulates ABA and auxin responses through ABRE-containing WRKY genes, suppressing auxin signalling while enhancing ABA signalling, thereby providing insights into ABA and auxin crosstalk. Furthermore, according to Tripathi et al. [187], the interaction of bZIP transcription factors with promoters from some WRKY gene promoters is crucial for ABA and auxin signalling.

The NAC [for NAM-ATAF1/2-CUC2) transcription factor constitutes one of the most prominent transcription factor families in plant genomes. Roles of many NAC transcription factors have been demonstrated in diverse processes, such as cell cycle control [188] and AtNAC2 functioning in root development [18]. According to Park et al. [189], some NAC proteins are membrane associated, and controlled proteolytic activation of the membrane-bound NAC transcription factors has been proposed to serve as an adaptive strategy that ensures rapid transcriptional responses to abrupt environmental changes. Xie et al. [190] demonstrated that NAC1 is induced by auxin and mediates auxin signalling to promote lateral root development. That is the other typical effect of HA on root traits [70]. Moreover, NAC2 integrates auxin and cytokinin pathways to modulate root development in rice [191], binding of promoters of IAA inactivation-related genes (GH3.6 and GH3.8), IAA signalling-related gene (ARF25), and a cytokinin oxidase gene (CKX4). Phytohormones influence signalling responses by acting in conjunction with or in opposition to each other to maintain cellular homeostasis. Nuruzzaman et al. [192] showed that besides the response to biotic and abiotic stress NAC TFs are also crucial in the phytohormone signalling pathway and reported the NAC involvement on ABA and JA regulation, ABA, and JA/ET and in the ABA-independent pathway of abiotic stress and in regulating biotic stress via an antagonistic JA and SA pathway and can regulate GA3-mediated salt signalling in seed germination [192, 193].

Hormonal crosstalk occurs between Auxin/GA/Cytokinin/ABA/Ethylene/SA through TFs. Both ABA-independent and ABA-dependent signal transduction pathways convert the initial HA signalling to cellular responses. The TF family members involved in both ABA-independent (AP2/ERF, bHLH, and NAC) and ABA-dependent (MYB, bZIP, and MYC) pathways were up-and down-regulated in maize root seedlings by HA, showing high and wide redundancy. A way to interpret and unify this vast redundancy of both genes encoding proteins related to the various hormones and TFs is to consider HA per se as one agent of abiotic stress when applied directly to plant surface [62, 183]. For different reasons, such as the presence of aromatic compounds, dissociation of organic acids, compounds with structures, like plant hormones, and general changes in membrane potential, HA can boost the plant signalling, which finally leads to enhanced growth and crop yield. Srivastava et al. [195] proposed a unified mechanism of action of different plant bioregulators that includes plant redox homeostasis control, which regulates root growth to improve plant water/nutrient status, photosynthetic efficiency, and source–sink homeostasis for enhanced crop yield and metabolism for an overall improvement of plant growth.

The potential role of HA in preventing and restoring the cytosolic redox homeostasis was described by García et al. [12, 14]. After exposure of HA, the general mechanism is triggered to prevent cellular damage by reactive oxygen species (ROS) that induces the synthesis of antioxidative enzymes, such as superoxide dismutase, peroxidases, catalase, and ascorbate peroxidase, and that of non-enzymatic production of scavenging compounds, like ascorbate, tocopherol, and phenolics, are induced. In addition, compatible solutes, such as proline, are also produced to protect cells against ROS accumulation. Lamar [196] elegantly showed the electron shuttling capacity of HA. The HA interacts with plant response via redox activity by relating their electron-donating capacities with processes, such as stimulation of H^+-ATPase activity, increase in $[Ca^{2+}]$cyt, and polarization-dependent Ca^{2+} channels and membrane depolarization. Finally, Monda et al. [197] also observed that the HA redox effect was determinant for maintaining cell homeostasis in tomatoes under nutritional stress and improving nutrient use efficiency.

Conclusion

Here we proposed a unified mechanism to plant stimulation showing that HA can act as a key regulatory hub in plant hormone crosstalk by modifying plant receptors,

phosphatases activity, and changing expression of different TFs. Among the hormones most affected by HA, auxins showed the highest number of up-regulated genes, corroborating previous studies demonstrating the auxin activity of HA. On the other hand, ethylene, ABA, and jasmonic acid, which are hormones commonly involved in stress response, displayed the highest number of down-regulated genes, suggesting a role of HA as a plant growth stimulant instead of an integrator of stress responses. Several specific and non-specific (proton pumps) membrane receptors were sensitized by HA, and different phosphatases were found in the differential transcription level. In addition, most of the synthesis and metabolism of genes encoding hormones have also been elicited by HA, and an expected response on the main Transcription Factors (TFs) and other regulatory genes was observed. Therefore, HA, acting as external cues, mediates plant hormone response using intricately interconnected signalling pathways and facilitates the generation of gene responses regulating plant hormones crosstalk.

Abbreviations

AAMT1: Anthranilate O-methyltransferase; ABA: Abscisic acid; ABI3: Abscisic acid insensitive 3; ACS4: 1-Aminocyclopropane-1-Carboxylic acid synthase 4; ACS5: 1-Aminocyclopropane-1-Carboxylic acid synthase 5; AGO: Argonaute; GTE: Transcription factor group E; AHK: Histidine kinases; AHP6: Histidine phosphotransfer protein 6; Alba: Alba DNA/RNA-binding protein; ALF: Alfin-like transcription factor; AP2/ERF: APETALA2/Ethylene-responsive element-binding protein family; ARF: Auxin Response Factor Family; ARF: Auxin response factor; ARR: Two-component response regulator; ARR1: Two-component response regulator/ARR1 (Arabidopsis Response Regulator); ARR5: Two-component response regulator/ARR5 (Arabidopsis Response Regulator); AS: Salicylic acid; Aux/IAA: Auxin/indole-3-acetic acid protein Family; AUX/IAA: Auxin/indole-3-acetic acid; AUX: Auxins; B3: B3 domain-containing protein; bHLH: Basic Helix-Loop-Helix Family; BIN2: Brassinosteroid insensitive 2; BR: Brassinosteroids; BTB: BTB domain-containing protein; bZIP: Basic leucine zípper; BZR1: Brassinazole-resistant 1; C3H Type: C3H-type transcription factor; CAMTA: Calmodulin-binding transcription activators; CK: Cytokinin; CKX: Cytokinin oxidases/dehydrogenases; CPK4: Calcium-dependent Protein Kinase 4; CRF: Chromatin Remodelling Factors; CtBP: C-terminal-binding protein; ALF: Alfin-like transcription factor; DELLA: DELLA protein Family; EIN3: EIN3-like (EIL) transcription factor Family; EIN3: Ethylene insensitive 3; ERF: Ethylene response factor; ET: Ethylene; ETR1: Ethylene response1; F-box: F-box protein; FLS: Flavonol synthase; FREE1: Fyve domain protein required for endosomal sorting 1; GA: Gibberellic acid; GA20ox: Gibberellin 3-oxidase 1; GRAS: GRAS transcription factor family (Scarecrow); GRFs: GRF transcription factor; ABH: Alpha/beta hydrolase; HA: Humic acids; HB: Homeobox transcription factor Family; HDAC: Histone deacetylase; IAA19: Auxin-responsive protein IAA19; IAA5: Auxin-responsive protein IAA5; IAR3: Iaa-alanine resistant 3; IPT: Adenylate isopentenyl transferase; JA: Jasmonate; JAZ9: Jasmonate zim-domain 9; LOB: LOB domain-containing protein; LOB: LOB domain-containing protein; Alba: Alba DNA/RNA-binding protein; LOX: Lipoxygenase; LSD: Lysine-specific demethylase; MOB: MOB kinase activator-like; MTREF: Mitochondrial transcription termination factor family protein; G-box: G-box protein; MYC2: MYC2 Proto-Oncogene; NAC: NAC (NAM, ATAF1,2, and CUC2; MYB: MYB Proto-Oncogene; PAT: S-acyltransferase; PIN: Auxin efflux carrier component 1; PLATZ: Plant AT-rich sequence and zinc-binding proteins; PLS: Polaris; PLT1/2: AP2-like ethylene-responsive transcription factor (plethoras); PP2C: 2C protein phosphatases; PPR: Putative pentatricopeptide repeat-containing protein; PR1: Pathogenesis-related 1; REMORIN: Remorin family protein; Ring type E3: RING-type E3 ubiquitin transferase; SEUSS: Transcriptional corepressor; SEUSS; C3H Type: C3H-type transcription factor; SHY2: Auxin-responsive protein; SUVR5: Histone-lysine N-methyltransferase; SWI/SNF: SWItch/Sucrose Non-Fermentable; TCP: TCP protein domain; TCP: TCP protein domain; TIR1: Transport inhibitor response; UBX2: Ubiquitin regulatory X domain-containing protein 2; WRKY: WRKY Family.

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Author contributions

LPC, FLO, AP, and LEPP conceived the experiments. ACS carried out the experiments. FLO supervised the RNAseq experiment. LPC wrote the first version of the manuscript. All the authors read and approved the final manuscript.

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Ethics approval and consent to participate

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Consent for publication

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Competing interests

The authors declare that they have no competing interests.

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