High-throughput transcriptomic analysis reveals new players in popcorn (*Zea mays*) aluminum resistance

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Abstract

Background

At the date, the investigation of genes involved in Al resistance has focused mainly in gene expression profiles based on microarray and in short periods of exposure to Al. Under a long term of Al exposure, such as 72 h, resistance maintenance mechanisms might be created in which several metabolic processes are working simultaneously. To investigate new players involved in defense response under Al stress, we tracked the expression profile of two Al-contrasting popcorn inbred lines during 72 h under Al-stress using Illumina high-throughput mRNA sequencing technology.

Results

A total of 1,121 differentially expressed genes (DEGs) were identified in the Al-sensitive line and 2,872 DEGs in the Al-resistant line, of which 384 were shared in both lines. Genes categorized in lipid metabolic process and oxidation-reduction were present in both lines, but lipid metabolic process was up-regulated in the Al-resistant line but down-regulated in the Al-sensitive line. The biological process category revealed that the down-regulated genes on the Al-resistant line were involved in signaling. The most significant GO modules were clustered in response to a stimulus. Differentially expressed transporters were also found in both lines, including ABC transporters, Nramp, aquaporins, SWEET transporters, Al-activated malate transporter (ALMT), and multidrug and toxic compound extrusion (MATE). Several transcription factors and genes involved in cell wall modifications were also detected.

Conclusions

The Al-resistant line presented genes that play a role in an efficient oxidative system against ROS, involved in cell wall stiffening and dynamic changes of the cell wall to prevent the Al ion transport via the symplast. Also, we detected transporters belonging to families already known to perform a role in Al-detoxification and organic acid exclusion, and we proposed a class of SWEET transporters that might be involved in regulation of vacuolar sugar storage under Al-stress. Although the exact functions of these genes remain to be investigated, these results provide a platform for functional analysis of the defense response against Al-stress in maize.

Background

Aluminum (Al) is the third most abundant element in the earth's crust. In acid soils, with pH values at or below 5, the phytotoxic species Al^{3+} is solubilized in soil solution and becomes one of the most important abiotic stresses limiting crop production. Al stress occurs in approximately 30% of world's arable soils and in more than 50% of potentially arable land. From this total, approximately 60% is located in tropical and subtropical regions, and negatively impacting the food supply chain. The phytotoxic form Al^{3+} inhibits root growth, thereby altering water and nutrients absorption and consequently reducing plant development [1–3].

Plants use multiple strategies against Al-stress, and two types of mechanisms are described: 1) Al-exclusion, preventing the Al entrance in the root apex, and 2) the tolerant mechanism, where Al enters the plant and is detoxified and sequestered [3]. The well-characterized exclusion mechanism is dependent on organic acid (OA) exudation from the root apex [4, 5]. Although the root citrate release plays an important mechanism against the Al-stress in maize, as identified from the citrate transporter ZmMATE1 [6], this mechanism is not well correlated with Al-resistance, suggesting that others Al resistance mechanisms are operating in maize roots [7]. Organic acids such as malate, citrate, and oxalate can chelate Al and attenuate Al toxicity [3]. The Al exposure induced malate secretion in wheat (*Triticum aestivum*) [8], *Arabidopsis thaliana* [9], rape (*Brassica napus*) [10], citrate secretion in sorghum (*Sorghum bicolor*) [11], barley (*Hordeum vulgare*) [12], rice bean (*Vigna umbellata*) [13], rice (*Oryza sativa*) [14], wheat [15, 16] and maize (*Zea mays*) [17], and oxalate secretion in buckwheat (*Fagopyrum esculentum*) [18], spinach (*Spinacia oleracea*) [19] and tomato [20].

RNA sequencing technology (RNA-Seq) has been revolutionary in the study of transcript expression. To understand the elements involved in response to biotic and abiotic stresses, the whole transcriptome has been used to identify new plant defense responses. Using RNA-Seq technology, H Zhu, H Wang, Y Zhu, J Zou, F-J Zhao and C-F Huang [21] have identified genes involved in the cell wall toxicity and oxidative stress during 6 h of Al-stress in buckwheat. In roots and leaves of *Hydrangea macrophylla*, were found many transporters involved in the Al-citrate complex transporting during 4 h of Al-stress [22]. In *Stylosanthes* sp., C Jiang, L Liu, X Li, R Han, Y Wei and Y Yu [23] reported that Al-resistance involves multiple strategies and enhancement of citrate anabolism during 24 h of exposure. During 48 h of exposure of two contrasting soybean lines under Al-stress, RNA-Seq analysis revealed that citrate metabolism and secretion are preferentially expressed in the root of Al-resistance soybean, and the jasmonate biosynthesis and signaling are induced in leaves of Al-sensitive soybean [24]. Y Li, J Huang, X Song, Z Zhang, Y Jiang, Y Zhu, H Zhao and D Ni [25], following a gradient Al-level exposure, found ideal candidates involved in the Al resistance or accumulation in tea plant tracked by RNA-Seq.

In maize, no RNA-seq studies tracking the expression profile of Al resistance responsive genes have been performed yet. LG Maron, M Kirst, C Mao, MJ Milner, M Menossi and LV Kochian [26] performed the first microarray study of global transcriptional regulation under Al-stress in maize in hydroponic system in a short term of Al exposure. Likewise, L Mattiello, M Kirst, FR da Silva, RA Jorge and M Menossi [27] characterized the gene expression profile of maize roots grown in acid soil. This results also provided the integration of microarray data and previously published Al tolerance maize QTLs, allowing the identification of potential Al-resistance genes [28].

As previously observed, the investigation of Al-responsive genes in maize was focused mainly in an early response. We believe that in a long term of Al exposure, such as 72 h, a robust maintenance mechanism is activated in which several metabolic processes are working at the same time. We investigated new players involved in defense response under Al stress tracked by the expression profile of two popcorn lines (one resistant to and the other sensitive to Al stress) during 72 h under stress by looking for genes that are important for the resistance, and not only signaling. Here we present the first high-throughput

RNA sequencing in popcorn and new components regulating the resistance mechanism that will expand knowledge about this trait and might help to identify new markers of Al-resistance in crops.

Results

We generated a range of 38.5 ~ 46.5 million clean reads after the quality process for each sample and obtained around 52.87% of GC content. An average of 80.10% of the reads was mapped, and from this total, only 6.33% presented multiple alignments to the B73 reference genome using the default parameters (Additional file: Table S1).

We observed that the control and +Al treatment were clustered together for both lines, but the lines were grouped into separated clusters, revealing differences on the genetic background between both lines (Fig. 1) as previously reported by [F Rahim, VC Almeida, JMS Viana, C Ribeiro, LA Risso and MP Ribeiro \[29\]](#). We detected a total of 1,121 differentially expressed genes (DEGs) for the Al-sensitive line (527 were down-regulated, and 594 were up-regulated) and 2,872 DEGs in the Al-resistant line (1,469 down-regulated and 1,403 up-regulated) at an FDR of $q < 0.01$ (Fig 2; Additional file: Figure S1; Additional file: Table S2). The different number of expressed genes in both lines reveal that the Al-resistant line has a broader response to Al stress, regulating multiple secondary pathways against the Al damage (Fig. 2).

Furthermore, we found 384 common expressed genes in both lines under Al-stress (Fig. 2; Additional file: Figure S2). Among those, 57 were up-regulated while 51 were down-regulated in both lines. Many of the genes with the same differential expression pattern are involved in response to a stimulus, playing an important role in the defense system. Besides, 145 up-regulated genes in the Al-sensitive line were down-regulated in the Al-resistant line, and many of them are related to “oxidation-reduction” class. Meanwhile, 131 genes had the opposite behavior: down-regulated in the sensitive line and up-regulated in the Al-resistant line, and many of them are involved in lipid metabolic process, catalytic activity, metal binding, and oxidoreductase activity (Fig. 2; Additional file: Table S3).

The Gene Ontology (GO) analysis categorized DEGs into different groups. For both of the tested lines, many of the differentially expressed genes were classed as belonging to the “metabolic process” category (Fig. 3; Fig. 4). In the biological process category, “response to stimulus” and “oxidation-reduction” were also found highly enriched, “membrane” was enriched in the cellular component category, and “catalytic activity” and “metal ion binding” in molecular function category (Additional files: Figures S3 and S4).

The GO enrichment analysis revealed that genes categorized in lipid metabolic process, oxidation-reduction, oxidoreductase activity, peroxidase activity, and antioxidant activity were present in both lines (Fig. 3; Fig. 4), but the lipid metabolic process was up-regulated in the Al-resistant line but down-regulated in the Al-sensitive line. In the Al-resistant line, we only found significantly up-regulated genes categorized as involved in glutathione transferase (GST) activity (Table 1). Additionally, peroxidase (POD), catalase (CAT), and some reductase (RE) playing a role in reactive oxygen species (ROS) scavenging were detected only in the Al-resistant line, but cytochrome P450 genes with oxidoreductase activity were found

in both lines (Additional files: Table S4; Table S5). Among the GST genes expressed in both lines, 11 were up-regulated in the Al-resistant line, contrasting with only three in the Al-sensitive line (Table 1). Down-regulated GST genes were detected: GST34 (Zm00001d029696) and GST U16 (Zm00001d029702) in 11–133 and 11–60, respectively.

The biological process category revealed that the down-regulated genes on the Al-resistant line were involved in signaling. The most significant GO modules were clustered in response to a stimulus. We also detected genes involved in phosphorylation, oxidation-reduction, metabolic sugar process (hexose, fructose, glucose), organic and carboxylic acid metabolic processes, and carbohydrate and lipid metabolic process. However, the up-regulated genes were involved in cellulose, glucan, and suberin biosynthetic processes. We observed only up-regulated genes on cell wall categories in the Al-resistant line, which was consistent with the pattern of enriched terms on biological process network analysis conducted in Cytoscape (Additional file: Figure S5). Significant terms found on this analysis were cell wall organization, primary cell wall biogenesis, and polysaccharides responsible for cell wall stiffening (Additional file: Figure S5). On the other hand, GO analysis in the Al-sensitive line identified 30 cell wall enriched genes (Additional file: Figure S3), and no significant enriched terms were reported on protein-protein interaction network, contrasting with 106 genes in the Al-resistant line (Additional file: Figure S5).

The predicted metabolic pathway analysis revealed that the metabolic routes in the Al-resistant line may highly modified under aluminum stress in comparison with the Al-sensitive line (Additional files: Figure S6; Figure S7). After 72 h of Al exposure, this prediction indicated that the brassinosteroid biosynthesis route was induced in the Al-resistant line and deactivated in the sensitive line (Additional files: Figure S6; Figure S7).

Likewise, part of the tricarboxylic acid (TCA) cycle may have been induced in the resistant line. In this predicted map, we visualized a deactivation in 2-oxoglutarate production, and consequently, a reduction in the amino acid metabolism route dependent on this compound. We also observed an exclusive induction of oxaloacetate production in the Al-resistant line. However, in the Al-sensitive line, isocitrate on the TCA cycle was used to produce glyoxylate and induce the glyoxylate cycle. It is noteworthy that the lines showed an opposite pattern when comparing glycine, serine, and threonine metabolism. These pathways were up-regulated in the Al-resistant line and down-regulated in the Al-sensitive line (Additional files: Figure S6; Figure S7).

The fatty acid biosynthesis and degradation route may have been induced only in the Al-resistant line (Additional files: Figure S6; Figure S8). We found a curious pattern of alternation in fatty acid biosynthesis between the seven main enzymes. Beta-ketoacyl synthase I, beta-ketoacyl synthase II, fatty acid synthase, fatty acyl-CoA synthase, and 3-hydroxyacyl dehydratase were up-regulated while 3-oxoacyl reductase, fatty acid synthase, fatty acyl-CoA synthase and enoyl reductase (NADPH Si-specific, NADPH and NADH) were down-regulated. However, the final step possibly induced the production of octanoic acid, decanoic acid, dodecanoic acid, tetradecanoic acid and, mainly, hexadecanoic acid. This compound is responsible for inducing cutin, suberine and wax biosynthesis route and might play an important role in

cell wall stiffening. In Al-sensitive line, was predicted just an inactivation at the end of the lipid biosynthesis route, probably reducing the production of hexadecanoic acid (Additional files: Figure S7). We also observed that the sphingolipid, linoleic acid, arachidonic acid, and glycerolipid metabolism may have been altered in Al-resistant line, showing an opposite way on the Al-sensitive line.

Differentially expressed transporters were also found in both lines (Table 2; Table 3), including ABC transporters, Nramp, aquaporins, SWEET transporters, Al-activated malate transporter (ALMT), and multidrug and toxic compound extrusion (MATE). The majority of transporters in both lines were ABC transporters, of both the G and B families. The most expressive in the Al-resistant line was the ABC G member 29 and ABC B member 19. Nramp1 was up-regulated, while Nramp6 were down-regulated in the Al-sensitive line. We also found four genes encoding aquaporin proteins only in the Al-resistant line. PIP2-2 (Zm00001d005410 and Zm00001d014285) was up-regulated and TIP3-1 (Zm00001d048520), and an ortholog PIP2-2 (Zm00001d022608), was down-regulated. We also identified seven SWEET transporters, of which one was down-regulated in the Al-resistant line, and three were up-regulated in the Al-sensitive line.

Several transcription factors (TFs) were also detected among our differentially expressed genes. The number of TFs in the Al-sensitive line were 32 and 23 down- and up-regulated, respectively, and the Al-resistant line presented 89 and 38, also down- and up-regulated, respectively (Additional files: Table S6; Table S7). Among these, were found TFs belonged to the families AP2/EREBP, MYB, bHLH and WRKY that were already described in the regulation of gene expression of responsive genes in plants under Al-stress. We found TFs exclusively overexpressed in the Al-resistant line, including ZF-HD, CO-like, ARF, and E2F/DP families.

Four transposable elements were found in both lines (Additional file: Table S2). In Al-sensitive line was found a gene that encodes a retrotransposon protein (Zm00001d029444) and in Al-resistant line was found a gene that encodes a transposon protein CACTA%2C En/Spm sub-class (Zm00001d039524). Moreover, a transposon protein (Zm00001d041987) was found down-regulated in Al-sensitive, but up-regulated in Al-resistant.

To validate the RNA-seq results, thirteen differentially expressed genes were selected and real-time qPCR was performed (Fig. 5). This validation was done with two independent biological replicates (different from the replicates used for the RNA-seq experiment). A total of 84.62% of these selected genes was validated by RT-qPCR.

Discussion

Aluminum toxicity is one of the main factors limiting crop cultivation on acidic soils [30]. For more than 50 years, breeders have explored the genetic diversity to improve Al-resistance in several crops, especially in tropical breeding programs [3]. Some critical Al toxicity events are initiated at the transcriptional, biochemical, and physiological levels [31]. To date, several Al-tolerance mechanisms have been described, but much more needs to be discovered to reveal the complex response to Al-stress. Here, we

provided a comprehensive RNA-sequencing analysis revealing new important players involved with Al-resistance in a high concentration of Al^{3+} (160 μM) under a long period (72 h).

Our RNA-Seq data revealed a large number of differentially expressed genes regulated by Al-stress in both genotypes. Also, the number of genes differentially expressed in the Al-resistant line was higher than in the Al-sensitive line, suggesting several maintenance responses playing a tolerance role in the Al-resistant genotype. Moreover, under normal conditions, the Al-resistant popcorn has an expression pattern that is clearly distinct from the sensitive genotype, and the basal separation between both popcorn lines by PCA analyses indicates that the resistance is clearly intrinsic to the Al-resistant genotype.

Transporters are one of the most important players in plant Al-resistance, having a role in the plasma membrane and the tonoplast, acting in the exclusion and tolerance mechanisms [3]. The aluminum-activated malate transporter 10 (ALMT10) was up-regulated in the Al-sensitive (Zm00001d026102) and Al-resistant (Zm00001d046029) lines, but a putative MATE efflux family protein (Zm00001d009494) was down-regulated in the Al-resistant line.

Although it is clear that Al-resistance is related to higher MATE1 gene copy in maize [17], at the time point of our analysis, it is not possible to track the response of this gene, possibly because it was already activated during initial steps of Al-stress or perhaps a different allele is present in our Al-resistant genotype. Although there are multigenes members of ALMT in maize genome, the only functional study has been made on the ZmALMT, showing that this member is not play a role in malate exudation in maize [32]. Besides that, ALMTs genes are involved in diverse physiological processes not linked only with Al resistance [33, 34]. Sequencing alignment of these transporters with genes from MATE [35] and ALMT [33, 34] families with functions already described indicates that these genes showed other functions not yet characterized. Based on this, the ALMT and MATE gene response between the genotypes may acts in other response not exactly in the organic acid production which needs further functional investigation.

The predicted metabolic pathway analysis in Al-resistant line, has been showed a probable deactivation of 2-oxoglutarate production from isocitrate in the TCA cycle and induction of oxaloacetate production from the malate (Additional files: Figure S6). This pattern might be due to the release of citrate from maize roots or the production of malate acting as an exclusion mechanism against Al. Citrate release might affect the availability of substrate to increase levels of isocitrate and consequently 2-oxoglutarate. However, further metabolic analysis need to be made to quantify these compounds in popcorn roots. The organization of carboxylic acid metabolism in plants is highly dependent on the metabolic and physiological demands of the cell [36] and the Al-stress condition might induce this non-cyclic flux.

The in silico analysis showed that possibly were no responses detected in the TCA cycle in the Al-sensitive line. However, we observed a supposed partial activation in part of the glyoxylate cycle. Since isocitrate serves as a substrate for activating this cycle (Additional files: Figure S5), this may work as an extra production of succinate and posterior conversion to malate to supply the TCA cycle with

metabolites or a new energy source to the cell from acetyl-CoA. To date, no evidence has been presented demonstrating glyoxylate cycle change in plants under Al-stress, and this hypothesis needs further investigation.

Other transporters play an important role in the tolerance mechanism by detoxifying the Al ion inside the cells. The ABC transporters families found in both lines suggest that these transporters work together with other detoxifying systems to increase the tolerance response in Al-resistant line. Nr1 (Nramp aluminum transporter 1) is a specific Al transporter identified in rice that uptakes Al to cells for sequestration to vacuoles, and it is required for the initial steps of internal Al-detoxification [37]. The best candidate gene as homolog of OsNr1, showed an up-regulation pattern in Al-sensitive line. We proposed that, although our sensitive plant can respond with multiple mechanisms against the absorption of Al ion in popcorn roots, this is not sufficient to support this stress due to the metabolic unbalance caused by the Al toxicity condition. Aquaporins are a group of highly conserved membrane proteins that facilitate water transport across biological membranes [38] and, in our study, our observations suggest that tonoplast aquaporins are likely involved in the Al-tolerance response, but it remains unclear whether PIP proteins might play an important role in response to Al-stress in popcorn.

Cellular efflux of sugar plays important roles in the maintenance of sugar efflux in phloem loading, nectar secretion, for the supply such as mycorrhiza, and as maternal efflux for filial tissue development [39]. Plants need to maintain a rigid regulation in the storage and transport of vacuolar sugar to deal with environmental adverse conditions [40]. The role of SWEET in Al-tolerance responses remains unclear, but it plays an important role in adverse conditions, such as tolerance to osmotic stress, including cold, high salinity and drought [41] and a mechanism to accumulate sugars in vacuoles as a tolerance mechanism to freezing stress has been described [42]. Based on this, we suggest that these SWEET transporters play a role in maintaining the tight regulation on vacuolar sugar storage.

Plants under aluminum stress might produce ROS in mitochondria, chloroplast, and peroxisome [43] and to defend themselves, they might control the ROS levels by deploying a complex antioxidant defense system comprised of enzymes and non-enzymatic metabolites that might accumulate in various stresses conditions and scavenging the ROS [44].

Testing two contrasting maize lines under different concentration of ion aluminum, [A Giannakoula, M Moustakas, T Syros and T Yupsanis \[45\]](#) showed that the anionic POD isoforms and superoxide dismutase (SOD) isoforms increased with increasing of Al stress in the tolerant line, and that this antioxidant system up-regulation might provide considerable protection to roots against oxidative damage. In the same way, the CAT enzyme acts as an auxiliary antioxidant working selectively either with SOD or POD during the peroxidation caused by Al stress, being the major enzyme responsible for root growth [46], thereby corroborating with our results.

Also acting as detoxification enzymes, GSTs are positioned in the central glutathione network, playing a role against plant oxidative damage [47]. Overexpressing the tobacco par B, which encodes an antioxidant phi class GST enzyme, in *A. thaliana* demonstrated that the transgenic plants were more

tolerant than the wild-type under aluminum and copper stress, and able to significantly decrease the lipid peroxidation [48, 49]. GM Cançado, VE De Rosa, JH Fernandez, LG Maron, RA Jorge and M Menossi [50] described how an up-regulation of GST27.2 might play a role in maize roots alleviation against Al toxicity. These results support the observations that GSTs might reduce the oxidative damage caused by Al stress in our Al-resistant genotype.

The function of cytochromes P450 involved in heavy metal stress is not totally clear. Cytochrome P450 might act as monooxygenases in the biosynthetic pathways of lignin, defense compounds, hormones, pigments, fatty acids, and signaling molecules. Moreover, there is a function in the detoxification pathway to catalyzes several endogenous and exogenous toxic compounds in both the cytoplasm and the endoplasmic reticulum. A decrease in ROS production has been reported following the interaction between NADPH-dependent cytochrome P450 oxidoreductase with human Bax inhibitor-1, and this results in the electron uncoupling between this P450 reductase and cytochrome P450 2E1, reducing the source of ROS at the endoplasmic reticulum membrane [51]. The differential expression of several genes that encode cytochrome P450 proteins in both lines might indicate an important adaptive mechanism, which relieves the endoplasmic reticulum through a decrement of oxidative load under Al-stress.

In maize, the ROS production might trigger a signal transduction cascade which induces callose production and inhibits the Al ion migration into the cortex via symplast and, consequently, the apoptosis of the epidermal layers [52]. Moreover, the cell wall represents a physical barrier against the entry into the symplastic compartment, and plants might remove Al ion from the protoplasts by sequestration and compartmentalize in the cell wall [53, 54].

Cell wall modification response under abiotic stresses might involve maintenance of cell wall plasticity due to an increased level in xyloglucan endotransglucosylase/hydrolase and expansin proteins associated with an increase in the degree of rhamnogalacturonan I or by the increase of hemicellulose and lignin deposition contributing to the reinforcement of secondary wall and, consequently, cell wall thickening [55].

D Eticha, A Stass and WJ Horst [56] demonstrated that Al accumulates primarily in the maize root apoplast, where Al^{3+} can bind to the negatively-charged bindings sites provided by non-methylesterified pectin in the cell wall, and the degree of pectin methylation might contribute to genotypic differences in aluminum tolerance in maize. In rye, it was reported that the pectin biosynthesis induced by Al is directly related tolerance, which is related to the relative pectin methylesterase (PME) expression levels [57]. In *Medicago truncatula*, pectin esterase and pectin esterase precursors were up-regulated following treatment with Al, while the silencing of genes that encode pectin acetylesterase and annexin increased the sensitivity of the plant to Al [58]. Unlike these authors, in our RNA-seq analysis, we found only one gene encoding a PME that was down-regulated in the resistant genotype, as well as putative pectin-esterases and pectin-lyases in both genotypes, but without a regulation pattern, probably due to the relatively long exposure time of our experiment.

Expansins modify the cellulose and non-cellulosic components of the cell wall, thereby loosening and modifying the plant cell wall during growth and adaptation to biotic and abiotic stress [59], but the role of expansins in Al-stress remains unclear. R Tenhaken [60] proposed that expansin and xyloglucan endotransglucosylases/hydrolases frequently show differential expression under abiotic stress conditions and consequently increasing presence of ROS, ultimately pausing the plant growth. In our study, the differentially expressed expansins were EXPANSIN A11 (EXPA11) and EXPANSIN B4 (EXPB4), up-regulated in the Al-resistant line, but with opposite regulation in the Al-sensitive line, suggesting that these specific expansins play an important role in the cell wall modification under Al-stress.

With a role in the construction of barriers to the environment, as components of cellular membranes, suberin and cutin waxes [61], fatty acids are among the main components responsible for membrane integrity and function, which is determined by structure and fluidity [62]. The changes induced by abiotic stress in the fatty acid composition of plant membrane lipids occur due to the regulated activities of fatty acid desaturases and the ability to adjust membrane lipid fluidity by changing the level of unsaturated fatty acids [63].

Growing in nutritive solution, seedlings of Al-tolerant sorghum cultivar demonstrated an increase of linolenic and palmitic acids in the plasma membrane under Al-stress, but the concentration of these fatty acids decreased in the Al-sensitive line, and these fatty acids might be useful to indicate Al tolerance in sorghum [64].

In predicted metabolic pathway maps, we reported the induction of fatty acid biosynthesis that may have been produced hexadecanoic acid activating the cutin, suberin, wax biosynthesis route, and down-regulation in part of the fatty acid degradation route in Al-resistant (Supplemental Figure 6). However, the Al-sensitive line demonstrated a predicted down-regulation in the fatty acid biosynthesis pathways (Supplemental Figure 7), demonstrating that fatty acids might contribute to membrane integrity under Al-stress.

Root growth and development response, which are also involved with cell enlargement, were up-regulated in the Al-resistant line. Our results indicate that the majority of transcripts associated with hormones were related to cytokinin, auxin (IAA), and abscisic acid (ABA). Absciscic acid might regulate Al resistance in soybean through the possible involvement with citrate release [65]. In Arabidopsis, the AtALMT1 expression levels are up-regulated by ABA, suggesting that this hormone might activate ALMT1 expression as malate transport activity and then inducing organic acid expression [66]. Furthermore, using a microarray approach, Al-stress was shown to induce the expression of ABA-related genes (DREB1A and DREB1A) in *A. thaliana* [67].

The transcription of Al-tolerance genes might also be activated by IAA [66]. In Arabidopsis mutants with internal Al-detoxification mechanisms, it was verified that endogenous IAA suppress the transport of symplastic Al³⁺ to the vacuole, thereby negatively regulating Al-tolerance [68], but exogenous IAA induced high expression levels in AtALMT1, as well as a slight increase in AtMATE expression levels [66].

We also found a class of steroid hormones, the brassinosteroids (BRs), that might minimize the toxic effects caused by aluminum and other heavy metals, thereby reducing the accumulation of these elements due to their capacity to regulate the uptake of ions inside plant cells and inhibit the degradation of lipids resulting from the overproduction of ROS. Furthermore, BRs trigger the accumulation of apoplastic H₂O₂, which up-regulates the antioxidant system, thereby increasing the stress tolerance [69–71].

Little is known about the effect of BRs in Al stressed plants, but most evidence suggests that BRs improve the response of the antioxidant system. Treating mung bean seedlings with exogenous BRs, 28-homobrassinolide or 24-epibrassinolide (EBL), [B Ali, S Hasan, S Hayat, Q Hayat, S Yadav, Q Fariduddin and A Ahmad \[72\]](#) evidenced an increase in proline content and the activities of SOD, CAT, and guaiacol peroxidase in response to Al stress, indirectly contributing to the improvement of plant growth and photosynthesis. Additionally, EBL significantly increased the chlorophyll content and fresh masses of shoots and roots.

In our study, we reported a possible differential response in BRs biosynthesis route between the contrasting lines under aluminum stress for 72 h through in silico metabolic analysis (Supplemental Figure 6; Supplemental Figure 7). Two reaction paths may have been induced in the Al-resistant line in the formation of 3-dehydro–6-deoxoteasterone and typhasterol, this one closer to the final path in the production of brassinolide. On the other hand, this same route had four paths that may have been disabled related to the production of 6-deoxocathasterone and 3-dehydroteasterone in the Al-sensitive line, which supported these previous observations that BRs might induce a response against Al-stress. These components might be involved in a defense mechanism carried out by brassinosteroids in our Al-resistant line.

The importance of TFs under Al-stress have been investigated. [M Kumari, GJ Taylor and MK Deyholos \[73\]](#) identified AP2/EREBP, MYB, and bHLH as predominant families of TFs responsive to Al-stress in *Arabidopsis thaliana* and [JM Xu, W Fan, JF Jin, HQ Lou, WW Chen, JL Yang and SJ Zheng \[31\]](#) found an increased expression of 27 TFs in buckwheat, where most were categorized in the NAC family, but only three down-regulated (bHLH1, OVATE family protein 17, and MYB19). In rice, some authors reported ASR (Absciscic Acid, Stress and Ripening) as involved in Al and other stresses [74]. MYB proteins were found to be regulated under Al-stress in common bean [75] and playing a role in the cleavage mediated by miRNA, essential for responding to Al-stress, in wild soybean [76]. Transcriptional factors belonged to these families were found with different expression regulation in both inbred lines and may be involved in regulation of downstream expression of genes involved in Al stress.

Members of the WRKY family might develop various responses under Al-stress. [ZJ Ding, JY Yan, XY Xu, GX Li and SJ Zheng \[77\]](#) reported that WRKY46 acts as a repressor of expression of AtALMT1 in *Arabidopsis*. WRKY22 has a positive role in increasing the expression of OsFRDL4, a gene that encodes a citrate transporter, enhancing the Al-tolerance in rice by citrate secretion [78]. In our work were found

several members of WRKY family in both inbred lines, but no one showed high similarity with previous WRKY described regulating the expression of genes under Al stress.

The zinc finger-associated to a homeodomain (ZF-HD) family regulates diverse plant-biological processes, such as development and responses to phytohormones and abiotic stresses [79]. An increase in the expression response of BraZF-HD under heat, cold, and salt stress was found in Chinese cabbage [79]. CONSTANS-like (CO-like) has been found to respond by the overexpression in ABA and salt stress as a positive regulator in Arabidopsis [80]. Q-Y Zeng, C-Y Yang, Q-B Ma, X-P Li, W-W Dong and H Nian [76] reported an important role for miR160 in the cleavage of Auxin Response Factor (ARF) transcripts in response to auxin, which might regulate the inhibition of root development under Al-stress. Little is known about the role of E2F/DP in abiotic stress but acts as a sub-category in response to heat stress in Arabidopsis and rice [81]. The relationships between ZF-HD, CO-like, and E2F/DP and aluminum stress are still poorly understood, although it is believed that this TFs might play diverse roles in transcriptional regulation of key genes in Al-stress response.

Some important Aluminium Resistance Transcription Factors that regulate the expression of genes related to Al tolerance have been identified in previous works. ART1 encodes a C2H2-type zinc finger TF required for Al resistance in rice [82], ART2 an ART1 homologs [83] and STOP1 that is involved in signal transduction pathways regulating aluminum responsive gene expression [84]. However, these TFs were not found in our analysis probably due the long term of exposure of Al toxicity levels. These TFs were induced in a short period of Al-exposure, regulating the expression of initial responsive genes in previous work. For these reasons, we believe that these TFs have been already expressed in initial steps in popcorn roots under Al-stress not being detected by the RNA-seq analysis.

Transposable elements (TE) are a source of spontaneous mutations [85–87] and play an important role in responding to environmental adverse conditions [88]. The beneficial mutations derivate by TEs may enhance plant adaptation to acid soils increasing the expression of Al-responsive genes or of the gene copy number [89]. On the other hand, TEs may reduce the expression of key genes affecting splicing patterns or altering protein function [89]. The TEs found in our data may contribute to activate or inactivate important genes that may confer tolerance to Al toxicity. However, further investigation has been made to characterize these TEs expressed under Al-stress.

Moreover, up-regulated genes expressed only in Al-resistant lines might be useful as a marker to screening Al-resistance in maize, which might help identify key players in future studies. Transporters that presented positive and high fold change values, such as ABC transporter B family member 9 (Zm00001d044564 and Zm00001d043766) and member 19 (Zm00001d024600), ABC transporter G family 29 (Zm00001d036986), SWEET 2 (Zm00001d009365), SWEET 4A (Zm00001d015905), SWEET 13C (Zm00001d041067), SWEET 13A (Zm00001d023677), SWEET 14B (Zm00001d049252), Aluminum-activated malate transporter (Zm00001d046029), and aquaporin PIP2–2 (Zm00001d005410), must be involved in specific mechanisms of defense response against Al ion to our Al-resistant line. Once the transporters have an important role in Al-tolerance, these genes must be potential markers linked to Al

tolerance candidate genes, and future studies should be performed to investigate the segregation of these genes.

Conclusions

We identified new players involved in Al-resistance response in popcorn using RNA-sequencing technology that were not found in previous studies using hydroponic experiments, thereby increasing our understanding of responses to Al-stress. Here, we found that the Al-resistant inbred line needs more than one effective mechanism to alleviate the damages caused by Al-stress and that the combination of several mechanisms cause Al resistance in popcorn. The Al-resistant line presented a hormonal balance suitable to plant development and favorable energy production. The up-regulation of genes involved in cell wall and fatty acid biosynthesis (important for the dynamic changes of the cell wall and membrane integrity) and an efficient oxidative system that increase the defense machinery against ROS might be performing an autophagy role to alleviate the damages and inducing a cell wall stiffening, thereby preventing Al ion transport via the symplast. Moreover, the presented transporters family are already known to perform a role in Al-detoxification and organic acid exclusion, and we proposed a class of SWEET transporters that might be involved in regulation of vacuolar sugar storage under Al-stress. These results open new avenues to further investigate the specific function of these genes, associating them with maize QTLs correlated with aluminum resistance and comparing these data with Al-resistance genes already identified as candidates for Al-resistance in popcorn.

Methods

Plant materials and growth conditions

The two contrasting popcorn inbred lines were developed by Popcorn Breeding Program of the Universidade Federal de Viçosa. From the screening of 18 inbred lines evaluated for relative root growth (RRG), hematoxylin staining, Al content, and external morphology of roots, [F Rahim](#), [VC Almeida](#), [JMS Viana](#), [C Ribeiro](#), [LA Risso](#) and [MP Ribeiro](#) [29] identified that the inbred line 11–133 was the most Al-resistant presenting statistical significance exhibiting greatest RRG (0.15 to 0.37), no damages on root apices, lower hematoxylin staining score and low Al accumulation (926.4 g/g). The inbred line 11–60 was the most Al-sensitive presenting lowest RRG (0.02 to 0.06), strong hematoxylin staining and epidermal degradation and high Al accumulation (1,660.3 g/g).

Initially, we treated seeds with fungicide (Captan–400®) and germinated at 25°C ± 1°C in a growth chamber for 7 days. Seedlings with uniform growth were picked randomly and transferred to a nutritive solution with constant aeration to acclimated for 24 h. The nutrient solution composition was: 1 mM KCl, 1.5 mM NH₄NO₃, 1 mM CaCl₂, 45 µM KH₂PO₄, 200 µM MgSO₄, 500 µM Mg(NO₃)₂, 155 µM MgCl₂, 11.8 µM MnCl₂·4H₂O, 33 µM H₃BO₃, 3.06 µM ZnSO₄·7H₂O, 0.8 µM CuSO₄·5H₂O, 1.07 µM Na₂MoO₄·H₂O, and 77 µM Fe-EDTA [90, 91]. Then, the treatment group was submitted to aluminum stress with 540 µM of

AlCl₃ (160 µM Al³⁺) at pH 4.5 for 72 h. The seedlings were maintained in a growth chamber at 25°C with 12/12 h light/dark cycle. Total roots from three biological replicates were collected and immediately frozen in liquid nitrogen.

RNA isolation and transcriptome sequencing

The total root RNA was isolated with Trizol LS reagent (Invitrogen, USA) according to the manufacturer's protocol. The RNA was treated with DNase I Amp Grade (Thermo Scientific) to remove contaminated DNA, and then quantified by spectrophotometry (NanoDrop 2000c, Thermo Scientific). The RNA integrity was verified by electrophoresis on 1.6% agarose gel in the presence of ethidium bromide. After quantification, the RNA samples were sent to MacroGen Inc. (Seoul, South Korea) where transcriptome sequencing library was generated using the TruSeq Stranded mRNA kit and sequenced using the Illumina HiSeq 2500 platform.

Reads preprocessing and differential expression analysis

Quality control was initially performed using the FastQC program (version 0.11.8) [92] to check the sequencing quality and identify reads with adaptors contamination. Then, the raw reads were trimmed, filtered, and adapters were removed using Trimmomatic (version 0.38) [93]. The clean reads of all twelve samples were aligned to the maize reference genome (B73 RefGenv4) using Bowtie2 (version 2.3.3.1) [94] and TopHat (version 2.1.1) [95], with default settings for all parameters. The Cuffdiff (v2.2.1) program [96] was used with default parameters to calculate gene expression levels and to identify differentially expressed genes (DEGs) in terms of fragments per kilobase per million mapped reads (FPKM). We considered DEGs those showing a FDR < 0.01 (false discovery rate) and a log₂ fold change value (treated/control) > 1 to up-regulated genes and < -1 to down-regulated genes. Aiming to assess the line groups, a principal component analysis (PCA) was conducted using stats (version 3.4.4) R-package and plotted in Prism 5 (GraphPad).

Gene function annotation and metabolic pathway analysis

Functional enrichment of all DEGs in both lines was conducted using agriGO (<http://systemsbiology.cau.edu.cn/agriGOv2>) [97]. Singular Enrichment Analysis (SEA) with *Zea mays* AGPv3.30 as the reference genome background was performed and the significant enriched GO terms were selected (q < 0.05). Protein sequences from each DEGs were submitted to a similarity search against the UniRef Enriched KEGG Orthology (UEKO) database (<http://maxixe.icb.ufmg.br/ueko/>) using BLAST

(version 2.7.1) [98], and a script was developed to parse the output and return the KO (KEGG Ontology) ID from each corresponding gene in both lines. The Interactive Pathways (ipath) analysis was carried out via Interactive Pathways Explorer (version 3) (<https://pathways.embl.de/>) using the KO ID.

Biological network analysis

The DEGs from each line were used to construct an interaction network. The first-degree of interaction was retrieved from STRING (version 10.5) (<https://string-db.org>). The resulting protein-protein interaction network was used as an input for downstream analysis in Cytoscape (version 3.4.0) [99], and the Moduland (version 2.0.2) and BiNGO (version 3.0.3) plugins were used to evaluate the significant overrepresentation genes involved in biological processes [100].

Real time qPCR

To validate the RNA-seq results, RNA from three independent replicates was treated with DNase I Amplification Grade (Invitrogen, USA) and the cDNA was synthesized from 2 µg of RNA using the SuperScript Reverse Transcriptase kit (Invitrogen, USA). Real-time qPCR for thirteen genes identified as differentially expressed at least one of the inbred lines was performed with an ABI 7500 (Applied Biosystems, USA). The primers were designed using Primer Express software (Applied Biosystems, USA), and the specificity confirmed by BLAST in the Phytozome database (Additional file: Table S8). The real-time qPCR reactions were performed using 1 µL of 1:10 diluted cDNA, 5 µL of primer Forward and Reverse mixed at 1.5 µM (each primer) and 6 µL of SYBR Green PCR Master Mix. The experiment was conducted using three biological replicates for each genotype and two technical replicates. The maize 18S rRNA was used as an endogenous control: 18S-Fw: GACTACGTCCCTGCCCTTTG and Rev-18S: TCACCGGACCATTCAATCG. To evaluate the relative expression was used the analysis described in J Hellemans, G Mortier, A De Paepe, F Speleman and J Vandesompele [101]. The results and the statistical analysis were plotted using GraphPad Prism.

Abbreviations

ABA—Abscisic acid

Al—Aluminum

ALMT—Al-activated malate transporter

BRs—Brassinosteroids

CAT—Catalase

DEGs—Differentially expressed genes

EBL—24-epibrassinolide

FPKM—Fragments per kilobase per million mapped reads

GO—Gene Ontology

GST—Glutathione transferase

IAA—Auxin

MATE—Multidrug and toxic compound extrusion

Nrat1—Nramp aluminum transporter 1

OA—Organic acid

PME—Pectin methylesterase

POD—Peroxidase

RE—Reductase

RNA-Seq—RNA sequencing technology

ROS—Reactive oxygen species

RRG—Relative root growth

SOD—Superoxide dismutase

TCA—Tricarboxylic acid

TEs—Transposable elements

TFs—Transcription factors

ZF-HD—Zinc finger-associated to a homeodomain

Declarations

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Authors' contribution: JMSV and MDB conceived the study and supervised the experiments. VBP conducted the experiments. VBP, PGF, PMPV and TAOM performed data analysis. VBP and MDB designed the primers and performed real-time qPCR. VBP wrote the manuscript; JMSV, MDB and PMPV critically reviewed the manuscript. All authors read and approved the manuscript.

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Tables

Table 1 Glutathione transferases proteins identified in RNA sequencing of Al-sensitive and Al-tolerant popcorn lines under 72 hours of Al-stress.

Gene ID	GST protein	Fold change
Al-sensitive		
Zm00001d029706	<i>gst39</i>	2.3630
Zm00001d029696	<i>gst34</i>	1.4779
Zm00001d029702	Glutathione S-transferase U16	-1.2121
Zm00001d048558	<i>gst25</i>	1.2982
Al-tolerant		
Zm00001d048353	<i>gst13</i>	2.0340
Zm00001d029707	<i>gst38</i>	1.8619
Zm00001d042104	<i>gst7</i>	1.6635
Zm00001d027539	<i>gst11</i>	1.3049
Zm00001d029699	<i>gst42</i>	1.2658
Zm00001d029801	<i>gst14</i>	1.2620
Zm00001d048354	<i>gst9</i>	1.1963
Zm00001d027540	<i>gst12</i>	1.0799
Zm00001d038192	<i>gst41</i>	1.0730
Zm00001d028692	Glutathione S-transferase L2 chloroplastic	1.0698
Zm00001d047765	Glutathione S-transferase L2 chloroplastic	1.0270
Zm00001d029696	<i>gst34</i>	-1.1529

Table 2 Aluminum responsive transporters related in Al-sensitive popcorn.

Class	Gene ID	Transporter	Fold change
ABC transporter	Zm00001d043598	ABC transporter G family member 29	-2.4384
	Zm00001d032279	ABC2 homolog 15	-1.4484
	Zm00001d025703	ABC transporter B family member 15	-1.3762
	Zm00001d011315	ABC transporter G family member 40	1.8044
	Zm00001d026041	ABC transporter C family member 9	1.7880
	Zm00001d021647	ABC transporter G family member 34	1.0676
SWEET	Zm00001d050577	SWEET 15a	2.2081
	Zm00001d015914	SWEET 4b	2.1945
	Zm00001d010440	SWEET 3a	1.0330
Nramp	Zm00001d019327	Metal transporter Nramp6	-1.0948
	Zm00001d014391	<i>Nrat1</i>	1.7826
ALMT	Zm00001d026102	Aluminum-activated malate transporter 10	1.9831

Table 3 Aluminum responsive transporters related in Al-resistant popcorn.

Class	Gene ID	Transporter	Fold change
ABC transporter	Zm00001d011315	ABC transporter G family member 40	-2.8548
	Zm00001d044442	ABC transporter G family member 40	-2.1104
	Zm00001d028870	ABC transporter family protein	-1.6613
	Zm00001d046225	ABC transporter family protein	-1.4889
	Zm00001d046226	<i>mrpa1</i> (ABC) transporter	-1.3632
	Zm00001d021647	ABC transporter G family member 34	-1.3278
	Zm00001d004361	ABC transporter C family member 4	-1.0053
	Zm00001d043598	ABC transporter G family member 29	2.5981
	Zm00001d024600	ABC transporter B family member 19	2.5639
	Zm00001d043766	ABC transporter B family member 9	1.8129
	Zm00001d036986	ABC transporter G family member 29	1.3984
	Zm00001d044564	ABC transporter B family member 9	1.1888
	Zm00001d042953	ABC transporter G family member 6	1.1345
SWEET	Zm00001d011299	SWEET 6b	-3.5525
	Zm00001d029135	SWEET 12a	3.4175
	Zm00001d023673	SWEET 13b	2.7135
	Zm00001d015905	SWEET 4a	1.5050
	Zm00001d041067	SWEET 13c	1.4285
	Zm00001d010440	SWEET 3a	1.0593
	Zm00001d015914	SWEET 4b	1.0114
Aquaporin	Zm00001d022608	Aquaporin PIP2-2	-2.0717
	Zm00001d048520	Aquaporin TIP3.1	-1.8133
	Zm00001d005410	Aquaporin PIP2-2	1.5432
	Zm00001d014285	Aquaporin PIP2-2	1.1855
ALMT	Zm00001d046029	Aluminum-activated malate transporter 10	1.0719
Heavy metal transporters		Heavy metal transport/detoxification	
	Zm00001d002496	superfamily protein	1.6792
	Zm00001d026298	Putative heavy metal transport/detoxification protein	1.2951
MATE	Zm00001d009494	Putative MATE efflux family protein	-2.9624

Figures

Figure 1

PCA plot analysis using FPKM values and performed in stats R-package and plotted with Prism 5 (●: sensitive control; ■: sensitive treatment; △: tolerant control; ◇: tolerant treatment).

Figure 2

Venn diagram of DEGs under Al-stress (UP_TT: up-regulated genes in Al-resistant; DOWN_TT: down-regulated genes in Al-resistant; UP_ST: up-regulated genes expressed in Al-sensitive; DOWN_ST: down-regulated genes expressed in Al-sensitive).

Figure 3

Gene ontology (GO) enrichment of biological process in Al-resistant popcorn line.

Figure 4

Gene ontology (GO) enrichment of biological process in Al-sensitive popcorn line.

Figure 5

Expression of selected genes. Data show the relative expression (in fold change) of the treatment with Al versus control condition. The results are from three independent biological replicates.

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