Stacked genetically modified soybean harboring herbicide resistance and insecticide rCry1Ac shows strong defense and redox homeostasis disturbance after glyphosate application

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Caroline Bedin Zanatta  
Universidade Federal de Santa Catarina

Rafael Fonseca Benevenuto  
Universidade Federal de Santa Catarina

Rubens Onofre Nodari  
Universidade Federal de Santa Catarina

Sarah Zanon Agapito-Tenfen  
Genok - Centre for Biosafety  
sarah.agapito@genok.no

**Corresponding Author**  
ORCiD: https://orcid.org/0000-0002-9773-0856

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Abstract

Background

World agricultural production of genetically modified (GM) products in particular, the combination of different traits/genes in the same plant has been intense over the last decade. The stacking of herbicide and insect-resistant transgenic genes can result in fitness costs that rely on the type and strength of the selection pressure exerted by the environment. Here we report the results of transcriptomic analysis comparing the effect of glyphosate on various biological processes, metabolic pathways, and main shikimic enzymes in stacked versus single soybean resistant varieties.

Results

Gene expression data were grouped according to treatment, ie the herbicidal factor strongly influenced. Common physiological results between the single and established varieties were mainly in Redox metabolism, energy, and metabolism. Photosynthesis was only found negatively affected in the single variety. The defense components, although present in both varieties, show a more intense presence in staked pathways, that demonstrated pathways related to up-regulated secondary metabolites biosynthesis, a known response when plants are under various stress conditions. RT-PCR results confirmed that native EPSPS expression was up-regulated at the same level for single and stacked events. However, metabolic differences in expression were observed, suggesting a distinct cascade effect between simple and stacked, triggered by glyphosate application.

Conclusion

Changes in plant metabolism by glyphosate application have been observed in several pathways, particularly the shiquimate pathway, suggesting that event staking may promote a more intense defensive genetic response. Omics profiling techniques, such as transcriptome, can be considered tools to support risk assessment based on detecting unwanted effects, both on plant physiological changes and on the safety of foods and products from new genetic editing technologies.

Background

The combination of different traits or genes in genetically modified (GM) plants has rapidly emerged in worldwide crop production. In recent years, an increasing number of GM plants with stacked traits
reached about 81 million hectares equivalent to 42% of the total 191.7 million hectares planted with transgenic crops worldwide in 2018 [1]. The predominant trait, for both single and stacked crop varieties is herbicide resistance and it is estimated to remain so in the near future [2].

According to the current regulatory practice within the European Union (EU), stacked events are considered new GM organisms, requiring similar risk assessment procedures to those from single events [3]. Whereas in other countries, such as Brazil, stacked events are also considered new GMOs but require simplified risk assessments upon approval of single parental events (CTNBio, 2009).

Previous studies have shown that stacking herbicide and insect-resistant transgenes can result in fitness costs that are dependent on the type and strength of selection pressure, and could also contribute to changes in plant communities through hitchhiking of unselected traits [4]. In that particular study, one of the tested selective pressure was the spray of glyphosate, which has been shown to adversely affect plant uptake and transport of micronutrients (e.g. Mn, Fe, Cu, and Zn) whose undersupply can reduce disease resistance and plant growth [5] [6].

Glyphosate manufacturers claim that it works by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which catalyzes the penultimate step of the shikimate pathway leading the conversion of shikimic acid to chorismate, the precursor for aromatic amino acids (tyrosine, phenylalanine, and tryptophan) and other secondary plant metabolites. Glyphosate competes with phosphoenolpyruvate (PEP), a substrate for the EPSPS enzyme, to form a very stable enzyme-herbicide complex that inhibits the product-formation reaction [7].

Despite glyphosate widespread use in global crop production, its precise mode(s)-of-action and cascade metabolic effects in plants remain unclear. After inhibition of EPSPS, many physiological processes were observed to be affected by glyphosate and these could also be associated with glyphosate toxicity [8]. It has been also shown that carbon and nitrogen metabolism are affected within hours after glyphosate treatment (Gruys and Sikorski, 1999) i.e., total free amino acid content increases, soluble protein content decreases [9] and carbohydrate content accumulates [10].

Application of transcriptomic and proteomic methods have helped to identify the common causes/mechanisms of the effect of glyphosate on the metabolism of resistant plants. The
accumulation of glyphosate in single-event transgenic soybean enhanced cellular oxidation, possibly through mechanisms involving stimulation of the photorespiratory pathway [11]. It also indicated that most of the glyphosate-induced genes are homologous to the known expression sequence tags - ESTs induced by abiotic stress factors [12]. *In silico* and *in vivo* studies also showed stress response, i.e. glutathione redox metabolism alteration, in glyphosate-treated resistant soybeans (Ayyadurai et al., 2011). The photosynthesis metabolism has also been affected by herbicide application in glyphosate resistant GM maize varieties [13].

Although the use of these approaches provides a robust understanding of the genetic regulation of the response of resistant genotypes, a comprehensive picture of the metabolic manifestations of resistant genotypes conferring multiple transgenic traits is still lacking. This is partly because previous untargeted omics approaches were limited to the analysis of single transgene resistant plants [12][11][14][13][15].

In order to gain new insights into the response of glyphosate application in soybean harboring two or more transgenic events, the current study compared the effect of glyphosate on several biological processes, metabolic pathways and key enzymes of the shikimate and phenylpropanoid pathway and other cascade pathways in stacked INTACTA RR2 PRO soybean varieties. We hypothesized that transgenic plants with a combination of transgenes respond differently to glyphosate accumulation due to (1) the cost of expressing more than one heterologous protein and (2) synergistic and antagonistic interactions of each transgene cascade pathways to glyphosate direct and indirect target pathways. This study was undertaken in order to characterize the interactions between the shikimate pathway and other unsupervised side-affected pathways as determined by changes in the transcriptome of glyphosate-treated stacked transgenic soybean variety, which express the *Agrobacterium tumefaciens* strain CP4-EPSPS enzyme that is resistant to inhibition by glyphosate. We undertook this work on soybean because it is a major crop species: genetically modified lines currently occupy more than 50% of the acreage planted globally. The data presented here provide new knowledge concerning the influence of recombinant Cry1Ac (rCry1ac) transgene cassette on the defense response and glutathione metabolism, the abundance of beta-glucosidase and
oxidoreductase enzymes when glyphosate is applied.

Methods

Plant material and herbicide treatment

Two soybean (Glycine max) varieties were used in this transcriptomic profiling study: NA 5909 RG and BRS 1001 iPRO. The first one, NA 5909 RG, is a single-event variety containing one transgenic event (GTS-40-3-2; unique identifier MON-Ø4Ø32-6) which confers herbicide tolerance (Roundup Ready technology, Monsanto do Brasil S.A.). The second variety, BRS 1001, is considered as stacked-event variety because it contains two combined transgenic events (MON87701 and MON89788; unique identifier MON-877Ø1-2 x MON-89788-1) conferring herbicide tolerance and resistance to lepidopteran species (Intacta RR2 PRO technology, Monsanto do Brasil S.A). Both commercial varieties are widely grown in Santa Catarina state and commonly found in the seed market in southern Brazil.

The experiment was conducted in a full-factorial no-choice experiment in block design with two factors: soybean variety and herbicide treatment. Seeds of each soybean variety were grown in greenhouse under two treatment conditions: herbicide spray application (treated group); and no herbicide application (control group). Seeds were grown in 14 L plastic pots filled with a substrate (1/3 clay soil; 1/3 cellulose residue and 1/3 poultry organic residue) with pH corrected to 6.0. There were three plants per pot, and three pots per treatment, disposed in three random blocks. The experiment was watered daily. All plants were kept under the same conditions until they reach V2 stage, approximately 34 days after emergence, when the herbicide treatment was applied to a subset of plants (treated group). Glyphosate treatment was conducted through spray application using glyphosate-based herbicide formulation Roundup Transorb® (Monsanto company) under the general maximum dosage informed by the leaflet (4.5 L/ha; 2.2 kg a.i./ha) for soybean crops [16]. To minimize spray contamination and drift, all plants were placed outside the greenhouse and separate into plastic barriers. Within each block (three) plants were randomized and border protected. Leaf samples were collected eight hours after herbicide application[10]. For each treatment, three leaves of the fourth trifoliate were collected from the three plants present in each pot, immediately frozen in liquid nitrogen and stored at -80 °C. Therefore, each treatment contained three biological pools of
three different plants, which were used for the transcriptomic analysis (Figure 1).

**Library construction, sequencing and mapping**

Total RNA was isolated using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The purity of RNA was measured using Nanodrop 2000 (Thermo Scientific), and the integrity of RNA was determined by agarose gel electrophoresis. Samples of 1-2 μg mRNA were used for library construction and sequencing. Libraries were constructed by Novogene Corporation (Sacramento, CA). Briefly, mRNA was enriched from total RNA using oligo (dT) beads. The mRNA was then randomly fragmented and cDNA was synthesized using random hexamers. After cDNA synthesis and library construction (terminal repair, A-tailing, ligation of sequencing adapters, size selection and PCR enrichment). Library concentration was quantified and then diluted to 1 ng/μl. The libraries were sequenced on the Illumina HiSeq platform (PE150). Raw reads are filtered to get rid of reads containing adapter or with low quality to get the clean reads for analysis. The Hisat2 v2.0.4 algorithm was used to map the filtered sequenced reads to the reference genome [17].

The mapped transcripts were annotated and counted generating normalized readcount values in terms of fragments per kilobase of transcript per million mapped reads (FPKM). Differentially expressed genes (DEGs) between the different treatments were determined using DESeq [18]. Only genes with FDR adjusted p-value (q-value) < 0.05 after Benjamini-Hochberg correction for multiple-testing [19] were considered as significant DEGs.

**Functional annotation of DEGs and bioinformatics**

The DEGs were annotated for gene ontology (GO) terms using Blast2GO v2.5 [20] and categorized into Molecular Function (MF), Cellular Component (CC), and Biological Process (BP) categories. The DEGs were annotated using KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis aiming to identify significantly enriched metabolic pathways or signal transduction pathways affected by the glyphosate treatment in both single and stacked varieties. Pathways with q-value < 0.05 were significantly enriched. A heatmap clustering analysis of the log10(FPKM+1) values was conducted using pheatmap library [21] in R [22] aiming to find gene expression patterns across the different treatments. Genes were also converted to proteins and used as input for producing flow
charts of DEGs and pathways significantly affected by using STRING v11 Protein-Protein Interaction Networks software (http://string-db.org) [23].

**EPSPS Expression by qRT-PCR**

The qRT-PCR analysis was performed aiming to quantify the expression of native and transgenic EPSPS in both single and stacked soybean varieties. Primers were designed based on the predicted coding sequence of each of the three target EPSPS genes (MON89788-1; MON4032-6; and EPSPS native) by using the online tool PrimerQuest (Integrated DNA Technologies Inc., Skokie, IL, USA) (Table S1). The cDNA was synthesized using 100 ng of total RNA from each biological replicate and the Superscript VILO cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. Real-time PCR reactions were performed using 10-fold diluted cDNA product and set up using the Power SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s directions and run on a StepOnePlus Real-Time PCR system (Applied Biosystems). Thermocycling conditions were: 50°C for 2 min; 95°C for 10 min; and 40 cycles at 95°C for 15 s, 60°C for 1 min, with melt curve set at 95°C for 15 s, 60°C for 1 min, 95°C for 30 s, and 60°C for 1 min. There were three biological replicates (pool of three plants) of each sample; in addition, three technical replicates were run for each biological replicate. To normalize the gene expression, 60S ribosomal protein was selected as housekeeping gene. Relative expression levels (Rq) were calculated, based on the average cycle threshold (Ct) of the technical replicates for each biological replicate, by the ΔΔCt method using the StepOnePlus Software v2.3 (Applied Biosystems). Statistical significance was assessed through t-test (p < 0.05) [24].

**Results**

**Transcriptome assembly and gene expression**

An average of 44,921,097 (98.18% > Q20; 43.63% GC content) and 45,172,415 (98.12% > Q20; 44.39% GC content) reads after filtering were generated from treated and control single variety samples, respectively. Similarly, treated stacked samples generated 43,768,427 (98.10% > Q20; 44.39% GC content), while stacked control generated 45,793,340 (98.17% > Q20; 43.38% GC content) clean reads (Table 1). Robust analysis were performed once approximately 96% of the reads
were mapped to the soybean reference genome (PRJNA19861, Glycine_max_v2.1) and ~1.8 to 2.7 million reads were mapped to multiple regions.

**Table 1.** Summary of RNA-Seq assembly performed for single and stacked soybean varieties under herbicide treatment.

| Sample          | Clean Reads   | > Q20 (%) | GC (%) | Total Mapped (%) |
|-----------------|---------------|-----------|--------|------------------|
| Single Control 1| 43,752,142    | 98.52     | 43.88  | 95.48            |
| Single Control 2| 44,706,616    | 98.12     | 43.82  | 96.03            |
| Single Control 3| 46,304,534    | 97.90     | 43.24  | 95.76            |
| Single Treated 1| 49,058,110    | 98.46     | 43.90  | 96.11            |
| Single Treated 2| 43,749,124    | 98.01     | 44.50  | 95.86            |
| Single Treated 3| 42,710,012    | 97.91     | 44.77  | 94.01            |
| Stacked Control 1| 52,509,694   | 98.50     | 43.58  | 96.33            |
| Stacked Control 2| 44,592,750    | 98.06     | 43.07  | 96.58            |
| Stacked Control 3| 40,277,576    | 97.96     | 43.50  | 96.00            |
| Stacked Treated 1| 45,299,654    | 98.48     | 44.51  | 96.42            |
| Stacked Treated 2| 42,526,766    | 97.89     | 44.49  | 95.92            |
| Stacked Treated 3| 43,478,860    | 97.93     | 44.17  | 96.17            |
| Average         | 44,913,820    | 98.14     | 43.95  | 95.89            |

In order to isolate the effect of herbicide and not account for those generated by differences in the genotype and phenotype of the varieties used, we have examined each variety separately. Therefore, differentially expressed genes (DEGs) were examined for single and stacked varieties under herbicide treatment with glyphosate commercial formula Roundup Transorb®. A total of 1425 (1024 up-regulated; 401 down-regulated) and 547 (522 up-regulated; 25 down-regulated) were identified as DEGs in response to herbicide treatment for the single and stacked variety respectively, when
compared to the control (Figure 2).

Additionally, we have further explored the data by running a hierarchical clustering analysis of DEGs aiming to find genes with similar expression patterns across the different varieties and treatments. The heatmap showed that gene expression data clustered according to treatment, which means that the factor ‘herbicide’ resulted in a major effect of variability as compared to the ‘variety’ factor (Figure 3). Although we have no precise information regarding the genetic background of the two commercial hybrids we have used in this experiment, such result was expected since its already known that soybean genetic diversity in Brazil is relatively low. For instance, most of the Brazilian soybean germplasm is derived from four main genotypes (CNS, S-100, Roanoke and Tokyo), which contributed to more than a half of the genetic base of all commercial cultivars released in Brazil [25] [26].

DEGs profiles for the single and stacked varieties under herbicide application were analyzed by Gene Ontology (GO) and enriched into three main domains: Biological Process (BP); Cellular Component (CG), and Molecular Function (MF) (Figure 4). In the single variety, herbicide application resulted in 16 significant Biological Process terms being up-regulated, in which the most predominant was protein metabolic processes including protein phosphorylation. A total of 6 BP terms were annotated as significantly down-regulated and the most enriched terms were photosynthesis and response to auxin (Fig. 4a). Similarly, the stacked variety showed 10 BP terms up-regulated, all related to protein metabolism. No significant BP terms were found down-regulated (Fig. 4b). Interestingly, when looking at the Molecular Function domain, DEGs of single and stacked varieties showed different enriched GO terms in response to herbicide application. The single variety presented protein kinase activity term as the most up-regulated enriched MF terms out of 13, and copper ion binding as the only significant down-regulated MF term. Whereas the stacked variety showed a total of 8 MF terms that were significantly up-regulated, in which the most enriched terms were related to catalytic activity (i.e. oxidoreductase and fatty acid) and nucleic acid binding. No terms for down-regulated DEGs were significantly annotated. Under the Cellular Component domain, the single variety showed endoplasmatic reticulum term annotated as up-regulated, while 8 significant down-regulated terms
were cellular components related to the Photosystem II. The stacked variety showed nuclear chromosome and extracellular matrix CC terms being up-regulated, and no terms were annotated as significantly down-regulated.

Aiming to understand the biological pathways activated in response to herbicide treatment, DEGs were mapped against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. We have considered only DEGs with FDR adjusted p-value (q-value) < 0.05 after Benjamin-Hochberg correction, as well as pathways were significantly affected when q-value < 0.05 in the KEGG enrichment analysis. Pathway enrichment analysis of DEGs helped us to investigate whether Roundup Transorb® induced metabolic changes in soybean examining the effect of maximum herbicide dosage (2.2 kg a.i. ha) in each single and staked variety. When looking at the single variety, the most affected pathways were: protein processing in endoplasmatic reticulum, photosynthesis – antenna proteins, phagosome, protein export, and plant-pathogen interaction; and for the stacked variety: phenylpropanoid biosynthesis, glutathione metabolism, plant-pathogen interaction, as well as protein process in endoplasmatic and plant-pathogen interaction (Figure 5).

For the single variety, the altered components are directly related to defense response mobilization. Notably, the herbicide treatment up-regulated energy and defense-related pathways while down-regulated growth-related pathways (Figure 5). For instance, carbon metabolism (22 DEGs out of 488 genes, 4.5%); N-Glycan biosynthesis (7/74, 9.45%); carbon fixation (9/129, 7%); propanoate metabolism (6/56, 10.7%), protein processing in endoplasmic reticulum (55/375, 14.6%), protein export (13/91, 14.3%), phagosome (13/265, 5.1%), monoterpenoid biosynthesis (4/14, 28.6%), and biosynthesis of amino acids (20/249, 8%) were pathways significantly up-regulated in response to the herbicide stress. Key genes involved in plant defense mobilization across such pathways could be identified: molecular chaperones from the endoplasmatic reticulum, calnexin [100810595 (log2FC = 1.4302); 547851 (log2FC= 1.6079); 100802236 (log2FC = 1.2665)] and calreticulin [100811997 (log2FC = 1.0754); 100037475 (log2FC = 1.1854)]; calmodulin like proteins [100775336 (log2FC = 2.8053); 100791253 (log2FC = 2.333); 100527439 (log2FC = 1.1821)]; WRKY transcription factors
[100776837 (log2FC = 1.7459); 100811997 (log2FC = 1.0754)]; as well as glycerol kinases [547750 (log2FC = 1.3814)]. Also, important energy-related genes involved in primary metabolism were identified as being up-regulated: fructose-bisphosphate aldolase (ALDO) [100802732 (log2FC = 1.7658); 100786504 (log2FC = 1.6327)]; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [100789951 (log2FC = 1.3482)]; malate dehydrogenase [100782096 (log2FC = 1.1071)]; alanine aminotransferase [100127413 (log2FC = 0.98378)]; phosphoenolpyruvate (PEP) carboxylase [100807407 (log2FC = 1.0789)] and carboxykinase [100786257 (log2FC = 0.91215)]. On the other hand, photosynthesis and growth-related pathways showed to be significantly down-regulated under the herbicide treatment: photosynthesis - antenna proteins (13/34, 38.2%), photosynthesis (10/120, 8.3%), porphyrin and chlorophyll metabolism (6/85, 7%), pentose and glucuronate interconversion (6/194, 3.1%) (Figure 4). Most genes repressed by the herbicide treatment in the single variety are involved in the light-harvesting chlorophyll complex (LHCa and LHCb proteins) [106797220 (log2FC = -2.1825); 100802138 (log2FC = -0.92404); 100808379 (log2FC = -0.8179); 100798548 (log2FC = -0.80487); 100799813 (log2FC = -2.8689); 547819 (log2FC = -1.7924); 100805310 (log2FC = -1.3478); 100800351 (log2FC = -1.52)] in both Photosystems I and II; as well as key genes encoding for pectinases - polygalacturonase [100776623 (log2FC = -1.3251); 100786608 (log2FC = -1.5456)], pectate lyase [100781845 (log2FC = -3.2966); 100101868 (log2FC = -2.7063); 100808253 (log2FC = -2.4293); 100779940 (log2FC = -2.4338)] and pectinesterase [100776623 (log2FC = -1.3251)] - acting in the cell wall degradation from the pentose and glucuronate interconversion pathway.

Differently, the stacked variety revealed up-regulation of pathways related to the biosynthesis of secondary metabolites under herbicide application, a well-known defense response of plants under a range of stress conditions. Phenylpropanoid biosynthesis (22/332 genes, 6.63%), Glutathione metabolism (15/158, 9.5%), and Plant-pathogen interaction (18/325, 5.54%) were the most enriched up-regulated pathways in response to the herbicide stress, followed by others, such as: Cyanoamino acid metabolism (8/84, 9.5%); Flavonoid (8/86, 9.30%) and Isoflavonoid (4/17 genes, 23.53%) biosynthesis; Protein processing in endoplasmatic reticulum (12/375, 3.2%); Circadian rhythm (6/98, 6.12%); Cysteine and methionine metabolism (7/195, 3.6%); as well as sugar (starch and sucrose)
metabolism (11/452, 2.43%). Not expected, Phenylalanine metabolism pathway (5/72, 8.3%), which is responsible for the synthesis of phenylalanine, one of the three aromatic amino acids inhibited by glyphosate in sensitive plants, was also significantly up-regulated for the stacked variety in the presence of glyphosate-based herbicide. More specifically, a group of genes encoding phenylalanine ammonia lyase (PAL) [100787902 (log2FC = 5.04); 100788438 (log2FC = 4.69); 100803857 (log2FC = 1.1305); 100811101 (log2FC = 2.9862)], an enzyme which eliminates ammonia from phenylalanine to form trans-cinnamic acid, a precursor of lignins and flavonoids, represented the main genes being up-regulated in the pathway. Again differently, the stacked variety under herbicide treatment showed repression of only the pathway related to protein processing in endoplasmic reticulum (4/375, 1.1%).

We analyzed the transcript levels of native and transgenic EPSPS aiming to quantify their relative expression in both single and stacked varieties under glyphosate treatment compared to control. RT-PCR relative expression data showed that native EPSPS was significantly up-regulated in Single Treated (1.49 ± 0.34 Rq; p = 0.001), as well as in Stacked Treated (1.43 ± 0.16 Rq; p = 1.66e-5) samples, compared to their respective controls (Fig. 6a and b). Similar results were found when we looked at expression levels of native EPSPS from the RNA-seq analysis. For the single variety, herbicide treated samples showed significant up-regulation of native EPSPS transcript (27.08 ± 5.84 FPKM) compared to control plants (14.35 ± 2.46 FPKM). The same trend was found for the stacked variety in which samples presented 28.57 ± 1.04 FPKM, against 18.22 ± 4.72 FPKM for control samples.

On the other hand, relative expression of transgenic EPSPS in the single variety (MON4032-6) showed significant down-regulation after herbicide application (0.79 ± 0.16 Rq; p = 9.67e-7) compared to control samples (Fig. 6c). The same pattern was found for the transgenic EPSPS present in the stacked variety (MON89788-1), in which expression levels in Stacked Treated samples was cut by half (0.47 ± 0.36 Rq; p = 5.33e-11) compared to untreated control samples (Fig. 6d).

Discussion

Impact of glyphosate in the shikimate pathway and cascade effects

Glyphosate affects sensitive plants by inhibiting the 5-enolpyruvylshikimate-3-phosphate synthase
(EPSPS; EC 2.5.1.19), an enzyme from the shikimate pathway, which leads to prevention of the biosynthesis of the amino acids phenylalanine, tyrosine, and tryptophan [27]. In transgenic resistant plants, a synthetic low-affinity EPSPS gene sequence (*Agrobacterium* sp. Strain CP4) is inserted into the genome of commercial crop plants, thus making them tolerant to glyphosate [28].

Despite the widespread use of glyphosate in agriculture, major questions remain concerning how this herbicide affects cell metabolism and physiology in glyphosate-resistant plants and, more importantly, if there are antagonistic or synergistic effects in stacked transgenic varieties. In order to address these questions, we have profiled the transcriptomic changes after glyphosate treatment in stacked versus single glyphosate-resistant soybean varieties and characterized the interactions between amino acid metabolism through the shikimate pathway and other unsupervised metabolic pathways, as determined by changes in statistically enriched defense, carbon, cellular redox and photosynthesis related metabolic pathways.

We were interested in glyphosate effects at native and transgenic EPSPS protein expression levels and whether changes might have a cascade impact in other metabolic pathways. Therefore, we have quantified EPSPS transcripts through RNA-Seq and real-time qPCR using primers located at distinct sequences in both EPSPS versions. Our results showed that while native EPSPS expression was up-regulated at the same level for both single and stacked events (approx. 1.5 log2FC); transgenic CP4-EPSPS showed a decrease in transcript accumulation also in both single and stacked varieties (approx. 60% expression level). Although stably integrated into the genome, variable and non-directional levels of CP4 EPSPS was observed with other factors like genetic background, trait stacking, growing region or season [29]. But the extent to which detection protocols can differentiate both versions of EPSPS is unclear in previous studies and might partially explain the deviations. It is uncertain why cp4-EPSPS is down-regulated after glyphosate spray.

Interestingly, while cp4-EPSPS has been modulated in the same manner in both single and stacked varieties; differences in the metabolism has been observed and thus suggests it is not related to either native or transgenic EPSPS modulation.

The impact of directly related metabolic pathways to shikimate pathway has been observed in both
single and stacked variety. However, in the stacked variety the impact on the isoflavonoid, flavonoid, glutathione, cysteine and methionine and phenylalanine metabolism were most prominent (Figure 7). In addition, alteration in the jasmonic acid metabolism was also observed. Increased levels of jasmonic acid have been also observed after glyphosate and drought stress application in NK603 herbicide resistant GM maize [13]. Glyphosate was also shown to interfere with other hormones such as ethylene [30] and abscisic acid (ABA) [10]. Although extremely important, the effects of glyphosate in plant hormone metabolism are still unclear.

**Strong Defense Response triggered by Glyphosate**

Defense imposes a substantial demand for resources that can negatively impact growth and diminish the overall set of energy reserves and/or promote resource diversion for growth, defense, and reduction of photosynthesis [31]. Previous transcriptome studies using microarray technique to investigate the metabolic impact of glyphosate treatment in susceptible and resistant soybean, Arabidopsis and brassica showed that most affected pathways are involved with defense metabolism [12][32][10]. In this study, genes related to defense metabolism were up-regulated, indicating that glyphosate may be related to alterations in gene cascades and unexpected pathways.

To defend themselves from adverse situations, plants need to mobilize a rapid response. Increases in calcium (Ca 2+) rates are essential to coordinate adaptive responses in various species[33][34]. The single and stacked varieties signaled a defense response with increased prominent calcium-related pathways. Calmodulin protein families were found for both single (two altered genes, average 2.10 log2FC) and stacked (five altered genes, average 3.3 log2FC) varieties. Previous studies with glyphosate application in sensitive soybean also observed changes in calcium-related genes regardless of herbicide concentrations and collection time after application (4 and 24 hours) [35][10]. The Ca2+/CaM complex play key roles in plant metabolism as it is a signal transduction pathway involved in turgor regulation[36]. In addition, cytosolic Ca2+ concentration controlled by ion channels in the plasma membrane of guard cells can modulate further cellular responses by promoting stomatal closure [37][38]. Considering calcium as one of the fundamental actors for the full functioning of the stoma, its accumulation may be involved in the imbalance between stomatal
opening and closing.

Rapid recognition of injuries by cellular signal transduction pathways occurs through various signaling molecules, including calcium, protein phosphorylation and ROS, which are well-known triggers of stress resistance in plants[39]. Herbicides are considered abiotic stressors that can disrupt the balance between the production and elimination of reactive oxygen species (ROS) [40]. There is a close relationship between calcium-dependent ROS production and a specific group of genes. For example, the respiratory burst oxidase homolog (Rboh) gene family. Activation of this group occurs after the recognition of pathogens and a variety of other processes [41][42]. We observed strong up-regulation of the Rboh group (3.58 log2FC) in the stacked variety. Such oxidases have been reported as key factors in activating innate and mobilized immunity during oxidative stress damage [43]. Another example of defense regulatory circuit was the identification of WRKY transcription factors that is phosphorylated by MAPK and a W-box in the promoter region of Nicotiana tabacum Rboh, interconnecting the phosphorylation events of MAPK in response to pathogen recognition with the accumulation of Rboh protein [42]. Strict regulation and fine-tuning of WRKY proteins are directly linked to plant stress signaling responses [44][45], such as saline stress[46], drought[47][48] and heat stress [49]. We observed up-regulation of WRKY genes in both varieties, with higher expression and number of genes in the staked variety (five genes with an average of 2.5 fold change). WRKY genes have not yet been found affected by glyphosate.

The pathogenesis-related proteins (PR), known as an indispensable component of innate immune responses in plants under biotic or abiotic stress conditions were also observed in this study. In the single-variety, we find one gene PR1, up-regulated with a 2.9 log2FC. These proteins are also involved in hypersensitive response or systemic acquired resistance against a variety of plant infections [50] and an important response mechanism to multiple stresses [51]. PR proteins are considered the signature genes of salicylic acid and jasmonic acid pathways in many crop plants [52][53][51][54]. Immune sensors in plants are well-known substrates for heat shock proteins, such as heat shock protein 90 (Hsp90). To recognize potential pathogens, higher eukaryotic organisms use extra- or intracellular sensors as the initial switch in the induction of disease defense responses[55][56].
Beyond defense, the environmentally responsive HSP90 chaperone complex is suggested to be involved in multiple signaling cascades, with the potential to be of great importance for sensing the environment and mediating appropriate phenotypic plasticity [57]. In our study, Hsp90 gene families were found up-regulated in the both single and stacked varieties. In soybean, Hsp90 gene was induced by heat, salt, and osmotic stresses but the response times and expression abundances were diverse [58].

Many plant defense compounds are used in a non-active glycosylated form suitable for storage in the vacuole and further protection from toxic side-effects as a consequence of its defense system [59]. These are recognized as class of secondary metabolites called phytoanticipins. When plant tissue in which they are present is disrupted, the phytoanticipins are bio-activated by the enzymatic removal of a protecting glucose group by a β-glucosidase. These are binary systems in which two sets of components that, when separated, are relatively inert provide plants with an immediate chemical defense against protruding herbivores and pathogens [60]. Strikingly, the stacked variety up-regulated seven β-glucosidase-related genes with an average of 5 log2FC. We also found a regulated isoflavone 7-O-methyltransferase gene found 8.5 log2FC. This specific change in the metabolism of secondary metabolites has not been reported before in plants treated with glyphosate. Thus, suggesting that the presence of rCry1Ac transgenic cassette has an impact on the defense metabolism when glyphosate is sprayed.

**Changes in Carbon Allocation**

The insertion of transgenes controlled by strong promoters has been always a concern as to the potential physiological effects on carbon allocation metabolism. In this paper, we applied glyphosate, an inhibitor of the enzyme EPSPS, present in the shikimate pathway as an abiotic stressor at concentrations present in real HR crop fields.

While under normal growth conditions, more than 20% of plant-fixed carbon flows through the shikimate pathway [61] [62]. Under stress, plants mobilize their carbon stocks to transform energy and resist harmful effects on cells. In our study, in the single variety, we observed the up-regulation of enzymes related to energy transformation processes and structural functions, such as lactate
dehydrogenase, which participates in the process of transforming glucose into energy formed from pyruvate [63] and N-acetylglucosamine, a cellulose analogous structural polysaccharide, involved in structural roles on the cell surface [64].

The total biomass production of soybean depends on energy supplied by photosynthesis for synthesizing carbon compounds [66]. Alteration in carbon metabolism has been already observed after glyphosate application. This occurs because the inhibition of EPSPS deregulates the pathway, which results in an uncontrolled flow of carbon and subsequent massive accumulation of shikimate and other acids in metabolic sinks such as leaves and nodules of legumes [67]. Two of the major metabolic checkpoints co-ordinating primary nitrogen and carbon assimilation in leaves are nitrate reductase (NR) and PEPC [68].

The enzyme PEPC has been found up-regulate and is biologically related to maintaining load balance during the upward flow of xylem sap in vegetables [69] [70] and in the supply of substrates for symbiotic organism, developing a central role for biological nitrogen fixation [71]. Our data demonstrate a relationship of up-regulated genes that involve balancing the energy supply to the nodules and retaining sufficient carbon for growth, at a level of change beyond the PECP enzyme. Several PEPC isoforms are controlled by an interaction between allosteric regulation and reversible phosphorylation. In legume leaves and root nodules, these regulatory functions of PEPC are governed primarily by phosphoenolpyruvate carboxykinase (PEPc Kinase) (Also found as altered in this study) [72] that promotes reversible protein phosphorylation of major importance in controlling legume nodule carbon metabolism and related metabolite transport [73]. PEPc Kinase is a member of the Ca2+/calmodulin-regulated group of protein kinases. However, it lacks the auto-inhibitory region and EF-hands of plant Ca2+-dependent protein kinases [74]. Regulatory mechanisms related to the formation and maintenance of root nodules for biological nitrogen fixation are fundamental for the proper adjustment of metabolic flow between host plants and symbolic organisms [75]. In the cytoplasmic compartment of plants, glucose and fructose-free hexoses (two genes for fructose bisphosphate aldolase were found with an average of 1.7 log2FC) are phosphorylated by glucose or pentose pathways [76][77]. PEPC and malate dehydrogenase (a gene also found in this study with a
1.7 log2FC) convert the carbon flux of glycolysis to malate [78] which is used as carbon skeletons for N2 amino acid synthesis [79] [80]. Although malate is the main source of energy for symbiotic organisms, high volume malate may inhibit N2 fixation and nitrogen uptake [81]. This leads to the belief that in the single variety there was a change in carbon metabolism relative to storage strategies. Both in the structural form and in the carbon flow demand required by nodular organisms. This may be involved in the change in carbon flow required for growth and development. In the stacked variety, on the other hand, carbon appears to be stored as starch, the carbohydrate used as the energy source for the defense response. We find altered starch and sucrose metabolism, especially gene related to trehalose-phosphate with 3.6 log2FC. Sucrose and starch management and balance promote optimization of growth rates [82][83]. Trehalose (α-d-glucopyranosyl-1,1-α-d-glucopyranoside) is a nonreducing disaccharide that is found in many organisms and has various functions: osmolyte, storage reserve, transport sugar, and stress protectant [84][85]. It is also involved in growth and development metabolism [86] with clear links to abscisic acid and auxin signaling [87] as well as to the activation of starch synthesis [88]. The levels of trehalose increase in response to osmotic stress [89] as well as to dehydration stress tolerance [90][89]. Trehalose is one of the most effective osmoregulatory sugars in terms of the minimal concentration required to establish a normal balance [91]. Many plants accumulate substantial starch reserves in their leaves to provide carbon and energy for maintenance and growth [92][93]. Therefore, the accumulation of soluble sugars, such as trehalose, is suggested to be a protective mechanism under oxidative stress conditions [94][95].

Wingler et al.[96] showed a strong accumulation of starch in response to trehalose [96]. In this study, in the stacked variety we suggest that the trehalose transcripts may also be involved in the accumulation of starch, carbohydrate required to cope in the energy balance due to the need for response observed by up-regulation in various secondary metabolites (flavonoids, isoflavonoids, monoterpenoids).

*Altered Cellular Redox Homeostasis*

Exposure to glyphosate-based herbicides is directly linked to accumulation of antioxidant enzymes,
indicating that glyphosate treatment might result in oxidative stress [85]. Glutathione (GSH) is a key of the complex antioxidant network in plants, acting to control ROS accumulation and facilitating cellular redox homeostasis especially under stress conditions [86]. For instance, GSH plays an important role in herbicide detoxification via the glutathione S-transferase (GST) system [87]. We found evidence for cellular detoxification response through significant up-regulation of GST in both soybean varieties under herbicide stress (Single: average log2FC = 3.1; Stacked: average log2FC = 3.5).

On the other hand, other genes encoding important enzymes related to glutathione metabolism showed to be differently affected in the single and stacked varieties, revealing that both genotypes may respond in a different manner in response to oxidative stress. For instance, we found glucose 6-phosphate dehydrogenase (G6PDH) - an enzyme participating in the first two reactions of oxidative pentose phosphate pathway - being significantly down-regulated in the single variety. Reduced levels of G6PDH is related to glutathione depletion and consequent high oxidative stress in the cell [88]. It is known that reduced glutathione (GSH) is required to combat oxidative stress and maintain the normal reduced state in the cell, a phenomenon known as the redox homeostasis [85][86][11]. Oxidized glutathione (GSSG) is reduced to GSH by NADPH generated by G6PDH in the pentose phosphate pathway [89]. Complete depletion of glutathione in its reduced form (GSH), or the production of GSSG from GSH, with concomitant accumulation of formaldehyde have already been reported as signs of undergoing oxidative stress in single-event GM soybean varieties as compared to its non-GM isogenic line [14][90]. On the other hand, for the stacked variety, although G6PDH gene expression has not been significantly affected, herbicide treatment up-regulated the expression of 6-phosphogluconate dehydrogenase (6PGDH) gene (log2FC = 1.25). 6PGDH, a second enzyme participating in the OPPP, catalyses the NADP-dependent oxidative decarboxylation of 6-phosphogluconate generating NADPH and ribulose-5-phosphate, a precursor for the synthesis of nucleotides and nucleic acids [91]. We hypothesize that the production of such reducing equivalents is being used in further reductive reactions in stacked plants, such as keeping GSH in its reduced form, aiming at maintaining the cell redox homeostasis.
Our results also showed protein processing in endoplasmatic reticulum (ER) as one of the most up-regulated pathways in both, single and stacked varieties when glyphosate is applied. Glutathione homeostasis in response to oxidative stress has been also described as active in the ER [92]. A diverse range of genes encoding important molecular chaperones guiding secretory folding proteins, as well as ubiquitin-proteasomes responsible for exporting and degradation of misfolded proteins, were shown to be significantly up-regulated in the presence of glyphosate. Interestingly, the ER protein processing-related genes was much higher in terms of number of genes and expression levels in the single variety when compared to the stacked one. For instance, chaperones/folding enzymes [i.e. calreticulin, protein disulfide-isomerase (PDI), and glucose regulated protein 94 (GRP94)], enzymes involved in the cytosol-to-ER and ER-to-cytosol transport of glutathione [i.e. endoplasmic reticulum oxidoreductin-1 (Ero1), binding immunoglobulin protein (BiP), B-cell receptor associated protein 31 (Bap31), and protein transport Sec 61] were exclusively up-regulated in the single variety.

In agreement, ER was annotated as the most up-regulated cellular component term under the GO enrichment analysis for the single variety. The only ER genes substantially affected in the stacked variety were those encoding for ER-associated degradation (ERAD) enzymes, such as derlin, Hsp40, Hsp70, Hsp90, and small heat shock factors (sHSF), which were also affected in the single variety.

Abiotic stress causes significant increase in protein unfolding metabolism, leading to the accumulation of misfolded proteins in the ER [93]. Such accumulation triggers the increase in degradation capacity of ERAD system aiming to maintain ER homeostasis. Over time, this process can lead to a variety of cellular signaling pathways which determine the state and fate of cell, which can include autophagy, apoptosis, inflammation, and even activation of cell death under severe conditions [94][95]. In our study, the metabolic responses to the oxidative stress caused by glyphosate seems to be highly correlated to ER-related genes; most probably due to GSH depletion or elevated production of GSSG as already suggested by previous studies [14][90]. Since glutathione is oxidized, transport proteins must export GSSG from the ER to the cytosol aiming to reach an ideal glutathione homeostasis [92]. Conversely, the stacked variety showed evidence of oxidative stress responses due to the up-regulation of cytosolic glutathione genes (GST log2FC = 3.5; 6PGDH log2FC = 1.25), while
only genes encoding ERAD enzymes were significantly up-regulated in ER. Vivancos et al. [11] have also found effects of herbicide on cellular redox homeostasis of single event glyphosate-resistant soybean variety. More specifically, the authors reported that accumulation of high levels of glyphosate in GM tolerant plants have enhanced cellular oxidation, possibly through mechanisms involving increasing of photorespiratory pathway [11]. Moreover, a recent integrative in silico model of C1 metabolism in single event glyphosate-resistant GM soybean, predicted complete depletion of glutathione and accumulation of formaldehyde as a result of oxidative stress compared to its non-GM counterpart. The authors alert on how a single event modification can potentially create a large perturbation to molecular system equilibria [14]. According to our findings, single and stacked GM soybean showed oxidative stress at different levels and cellular components.

*Photosynthesis imbalance*

Glyphosate has been shown to have detrimental effects on many plant physiological and biochemical processes, which reduce photosynthesis efficiency and inhibits chlorophyll function [96][97][98]. Chlorophyll is essential in photosynthesis and provides matter and energy for plant growth [99]. The concentration of total chlorophyll is the sum of chlorophyll A and chlorophyll B [100][101]. In our study, the single variety showed a decrease in the light-harvesting chlorophyll A and B contend (complex I of class LhCA 2,3 and 4 with four genes involved, and the complex II of class LhCB 1,2,3 and 6 with nine genes involved). These findings are supported by Li et al.[102], who also observed a decline in the content of chlorophyll A and B in GM and conventional soybean varieties under glyphosate treatment [102].

After light excitation of chlorophyll molecules in the light-harvesting complexes, the energy is transferred to the reaction centers of photosystems I (PSI) and II (PSII) [103]. The electron transfer chain, which mediates the transmembrane charge separation, is the functionally most important part of photosystem [104]. Ferredoxins (FDXs) in chloroplasts are electron transfer proteins that deliver reducing equivalents from PSI in photosynthetic organisms [105]. Electrons from reduced FDXs are accepted by FDX-NADPH-oxidoreductase to generate NADPH, which is required for carbon assimilation in the Calvin cycle [106]. Limited capacity of electron transport after glyphosate exposure was shown
by [96][11]. In this study, we find two genes down-regulated related to putative FDX. The amount of FDX is also decreased in tobacco under various stresses, including those from herbicide treatment [107].

In general, we have observed, down-regulation of genes involved in photosynthesis and this is in agreement with previous studies when glyphosate application is performed. Iquebal et al. [108] observed that genes involved in the photosynthetic pathway were deregulated after exposure to herbicides in resistant chickpea variety [108]. In Lolium perene sensitive plants, chlorophyll fluorescence was also affected by glyphosate [109].

Relevance to risk assessment of stacked GM crops

Worldwide, a growing number of GM crops with stacked transgenic traits are being developed to confer resistance to herbicide active ingredients and some insect species. For most varieties, the single events might never reach market and pre-market risk assessment. Therefore, an assessment of the risks of a stacked GM plant to cause combinatorial and cumulative effects should be considered in the context of the closely related non-modified recipient organism in the receiving environment. Omics profiling analysis can contribute to the identification of combinatorial effects that may occur due to interactions among the proteins and metabolites produced by the transgenes or endogenous genes of a stacked GM plant. In addition, interactions between the stacked transgenes or their products, or interactions among the physiological pathways in which the transgenes are involved, taking into account the possibility that these interactions could result in potentially harmful substances, such as anti-nutritional factors, some of which may persist or accumulate in the environment should be also considered.

Stacked GM plants can be produced through different approaches. In addition to the cross-breeding of two GM plants, multiple traits can be also achieved by the natural cross of transgenic lines that have been found in crop field boundaries [110][111], such as feral transgenic canola outside of cultivation [112][113]. Accordingly, it is reasonable to anticipate future occurrence of stacked traits within ruderal and wild populations. Despite the potential for the formation of feral populations with multiple transgenes, we
have little understanding of how these traits could migrate, evolve or influence native and naturalized plant communities. Thus, such profiling studies could generate useful information to assist risk assessment of stacked GM crops and potential feral populations.

Conclusions
In conclusions, the RT-PCR results showed that native EPSPS expression was regulated for single and stacked events. Although modulated in the same way, differences in metabolism were observed and therefore suggest a cascade of effects of various metabolic pathways resulting from glyphosate application. Our RNA-seq data supports this information. We show that although the varieties tested have similar responses in some ways when we analyze route by route, it is possible to suggest that the single-variety exhibited similar overall behavior to susceptible plants when glyphosate is applied, which is expected as a physiological response when plants are subjected to stress (increased sugar content, significant decrease in light harvest of chlorophyll A and B and impairment of redox cell balance). On the other hand, the stacked variety showed strong up-regulation of secondary metabolite accumulation as well as defense components and redox equilibrium mechanisms. We found Ca2+ signaling responses and several up-regulated molecular chaperones in both varieties. However, distinct stress responses were found among the varieties. The stacked variety showed a more pronounced stress response (activation of specific stress defense proteins (Rboh, WRKY) and secondary compounds (β-glucosidase, isoflavone 7-O-methyltransferase). Both varieties were energetically impacted, the single showed changes in carbon metabolism about storage and accumulation strategies, while the stacked variety showed starch accumulation we have found a regulated response to trehalose and suggest that this carbohydrate is being stored to help regulate energy balance due to the regulation of some secondary metabolites (flavonoids, isoflavonoids, monoterpenoids). There were responses to cellular detoxification through significant up-regulation of GST in both varieties, but in the stacked, we hypothesized that the production of some reducing equivalents is being used in additional reductive reactions as a way to keep GST low to maintain cellular redox homeostasis.

Similar results to ours for the single variety were also found using single GM with glyphosate
application and omic approaches. However, this is one of the first studies to use the RNA-seq approach on bet varieties to track transcriptomic profiles. Profile approaches that allow unbiased comparisons contribute to the knowledge of biological processes that regulate plant composition and the interactions between genes, RNA, proteins, and metabolites of a stacked GM plant. In addition, they have been shown to have biological relevance to the possible safety implications of such changes in genetically modified plants.

Abbreviations
GM: genetically modified; EU: Europe Union; GMOs: Organism genetically modified; EPSPS: 5-enolpyruvylshikimate-3-phosphate-synthase; PEPC: phosphoenolpyruvate carboxylase; CP4-EPSPS: Agrobacterium tumefaciens strain CP4 5-enolpyruvylshikimate-3-phosphate-synthase; CRY: Crystal Baccilus turigiensis; RNA: ribonucleic acid; mRNA: messenger ribonucleic acid; cDNA: complementary deoxyribonucleic acid; PCR: reverse transcription polymerase cGM: genetically modified; EU: Europe Union; GMOs: Organism genetically modified; EPSPS: 5-enolpyruvylshikimate-3-phosphate-synthase; PEPC: phosphoenolpyruvate carboxylase; PEPc Kinase: phosphoenolpyruvate carboxykinase; CP4-EPSPS: Agrobacterium tumefaciens strain CP4 5-enolpyruvylshikimate-3-phosphate-synthase; CRY: Crystal Baccilus turigiensis; RNA: ribonucleic acid; mRNA: messenger ribonucleic acid; cDNA: complementary deoxyribonucleic acid; PCR: reverse transcription polymerase chain reactions; FPKM: fragments per kilobase of transcript per million mapped reads; DEGs: Differentially expressed genes; GO: gene ontology; MF: Molecular Function; CC: Cellular Component; BP: Biological Process; KEGG: Kyoto Encyclopedia of Genes and Genomes; Rq: Relative expression levels; Ct: cycle threshold; PAL: Phenylalanine ammonia lyase; CaMCML: calcium-related pathways/ Calmodulin protein families; Ca2+: calcium-related genes; ROS: reactive oxygen species; Rboh: respiratory burst oxidase homolog; NADPH oxidase: Reduced nicotinamide adenine dinucleotide phosphate oxidase; WRKY: transcription factors; HR: hypersensitive response; SAR: systemic acquired resistance; SA: salicylic acid; JA: jasmonic acid; PR: pathogenesis-related proteins; LDH: Lactate dehydrogenase; MDH: Malate dehydrogenase; Nitrate reductase (NR); GSH: Glutathione; GST: Glutathione S-transferase; OPPP: Reactions of oxidative pentose phosphate pathway; G6PDH: Glucose 6-phosphate dehydrogenase;
6PGDH: 6-phosphogluconate dehydrogenase; FDR: False discovery rate; FLNC: Full-length non-chimeric reads; RNA-Seq: RNA sequencing, RT-qPCR: Quantitative reverse transcription PCR, SRA: Sequence Read Archive.

Declarations
Ethics approval and consent to participate
Not applicable.
Consent for publication
Not applicable.
Availability of data and material
The full sequencing dataset (Illumina) is available through the Sequence Read Archive (SRA) at NCBI under BioProject number XXXXXXXX.
Competing interests
The authors declare that they have no competing interests.
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Authors' contributions
SZA, RON, CBZ, and RFB conceived the research. SZA, RON, CBZ, and RFB designed the experiments.
CBZ and RFB conducted the bioinformatics analyses. RFB performed the RT-qPCRs. SZA, CBZ, and RFB wrote the manuscript. SZA, RON, CBZ, and RFB revised the draft of the manuscript. All authors read and approved the final manuscript.
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Figures

A schematic drawing of how the application and collection were performed. At the phenological stage V2, Roundup Transorb R was applied in its commercial formulation with a hand sprayer (4.5 L/ha; 2.2 kg a.i./ha). Eight hours after application, the middle leaf of the second trifolium of each plant in the pot was collected. The leaves were rolled and stored in cryotubes and immediately placed in liquid nitrogen at -80°C.
Volcano plots of significantly differentially expressed genes, DEGs (p-adjusted value ≤ 0.05 and |FC| ≥ 1.5; red, up-regulated; green, down-regulated) when applied herbicide. The X-axis represents fold change; Y-axis represents significance.

Hierarchical clustering of differentially expressed genes across samples in this study: staked control, single control, staked herbicide treated, single herbicide treated (p adjusted value ≤ 0.05). Each row of the heatmap represents the z-score transformed log2(1 + FPKM) values of one differentially expressed gene across all samples (blue, low expression; red, high expression).
Ontological classification of DEGs for glyphosate application in single (a) and stacked (b) GM soybean varieties. The X-axis indicates GO terms, while the Y-axis indicates the number of genes. The results are divided into three main categories or domains: biological process, cellular and molecular function. Note: * is significantly enriched term.

KEGG enrichment scatter plots from up and down-regulated DEGs in single and stacked varieties. Only statistically significant enriched pathways (p-adjusted < 0.05). Circles in red are up-regulated and circles in green are down-regulated pathways. Rich factor is the ratio of DEGs counts to this pathway in the annotated genes counts. The more the Rich factor is, the higher is the degree of enrichment.
Figure 6

Levels of EPSPS transgene expression in single and stacked varieties under glyphosate
treatment. Note: Bars show standard deviation.

Figure 7

Changes caused due to the spray of glyphosate in the stacked variety. The picture shows that the herbicide triggered a series of events. There were mainly changes in the pathways and products related to the biosynthesis of secondary compounds.