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Root vitality and protein homeostasis determine divergent strategies against autotoxicity in cassava cultivars

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Abstract: Autotoxic stress can disturb cassava (*Manihot esculenta* Crantz) endogenous hormones, thus hindering plant growth and development. However, the mechanisms by which cassava responds to autotoxic stress are still unknown. Hydroxyacetone, 2,4-di-tert-butylphenol, and acetic acid, previously confirmed as the main autotoxins, were applied in this study to simulate autotoxic stress on cassava using a tissue culture approach. Three genotypes, SC205, NZ199, and XX048, were used as plant materials. The allelopathic effects varied with genotype. Under autotoxicity stress, XX048 showed stronger negative allelopathy than SC205 and NZ199, with root length and biomass significantly reduced. Under identical stress conditions, SC205 and XX048 yielded 696 and 545 differentially expressed proteins (DEPs), respectively, which were functionally enriched in the catalytic/binding, metabolic, cellular, single-organism, and stress-defense categories. Cassava's defence responses to autotoxic stress appeared to be initiated by apoplastic glutathione, while salicylic acid served as the primary signalling hormone, modulating glutathione metabolism systemically. Protein-protein interaction network analysis revealed that under identical stress conditions, the two varieties shared conserved basal defences but diverged in their adaptive strategies, revealing fundamental molecular distinctions between the genotypes.

Keywords: continue cropping obstacle; chronic sub-lethal stress; oxidative balance; resource allocation trade-off
xidative balance

Cassava (*Manihot esculenta* Crantz) is cultivated for its starchy roots and is considered a staple crop for as many as one billion people in tropical and sub-tropical regions (Ceballos et al. 2004, Pan et al. 2021). In China, cassava is mainly used as a starch and bio-fuel feedstock in the south, where it is continuously cultivated on marginal land for years (Liang et al. 2016). The theoretical yield of cassava is 120 t/ha. The reported farmland yield in China was as high as 75.0 t/ha (Liang et al. 2017), whereas the average cassava yield is only 16.4 t/ha (<https://www.fao.org/>

[faostat/en/](https://www.fao.org/), accessed on 31 October 2024). To reveal the causes of yield gaps across diverse fields and management practices, previous studies have reported that continuous cropping obstacles (CCO) – which have become prominent under long-term monoculture as replant diseases, soil sickness, and yield penalties – serve as a critical constraint on cassava yield improvement in China (El-Sharkawy and Cadavid 2002, Liang et al. 2017). To reduce the gap between the theoretical yield and the actual output of cassava, measures to alleviate CCO are therefore needed.

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The effects of CCO on crops are exacerbated by an increase in the number of consecutive cropping years (Bai et al. 2019, Ma et al. 2023). The formation mechanisms of CCO vary across crops and are primarily associated with soil conditions (Hiddink et al. 2010, Ma et al. 2023). Previous studies indicated that soil nutrient deficiency, decline in pH and microbial community diversity, increase in soil-borne disease, and accumulation of autotoxic substances in rhizosphere soil are the main reasons that cause CCO in most crops (Zhou and Wu 2012, Yin et al. 2016, Bai et al. 2019, Liao et al. 2026). Among them, the autotoxic substances in root exudates that accumulate in rhizosphere soil have been proven to be the key factor causing soil degradation in continuous cropping systems (Yang et al. 2015). Under autotoxic substance stress, plant hormones vary, disrupting the division and elongation of plant cells and destroying cell membranes and cell wall structures, leading to delayed plant growth and development, including weak roots and dwarfing. Ginsenoside is the autotoxic substance of Sanqi ginseng (*Panax notoginseng*), which is a highly valuable medicinal herb in China. It can inhibit the root growth of Sanqi ginseng by disrupting the root cell membrane and cell wall, while genes regulating cell wall and membrane degradation and reactive oxygen species (ROS) metabolism are all up-regulated in the root cells (Yang et al. 2018). Phthalic acid is the main autotoxin from root exudates of Lanzhou lily (*Lilium davidii* var. *unicolour*). It can induce oxidative stress and oxidative damage to the root system, thereby increasing membrane lipid peroxidation, resulting in lower fresh weight, plant height, and root oxygen free radical absorption capacity in Lanzhou lily under phthalic acid stress (Hua et al. 2019). When germinated rice seeds were treated with different concentrations of ferulic acid (an autotoxin from rice straw), the root length of rice was inhibited after 3 days of treatment, and transcriptome analysis revealed that ferulic acid inhibited root growth by mediating ethylene- and abscisic acid-related genes (Chi et al. 2013). Baicalin was found not only to induce autotoxicity stress in *Scutellaria baicalensis* Georgi but also to promote the growth of soil-borne pathogens (Zhang et al. 2010). Thus, to reveal the formation mechanisms of CCO of cassava, the autotoxic substances of cassava should be identified.

Cassava roots release sugars and amino acids, which are utilised by rhizosphere decomposers, making it impossible to obtain the initial root

exudates of cassava in the field or other open cultivation methods. Thus, the tissue culture method was used to characterise the autotoxic substances from cassava root exudates in previous studies (Han et al. 2022, Han et al. 2023). A total of 78 chemical substances have been identified in cassava root exudates, and their relative contents varied with genotype and cultivation rounds (Han et al. 2022). Hydroxyacetone, 2,4-di-tert-butylphenol, and acetic acid were proven to be the main autotoxins of cassava, while 2,4-di-tert-butylphenol showed the strongest influence on cassava plantlets among these three substances (Han et al. 2023). However, the mechanisms by which cassava responds to these autotoxins are still unknown. Thus, in the present study, an autotoxicity stress experiment was simulated based on the relative proportions of these three autotoxins in the root exudates of different cassava varieties. Subsequent analyses of physiological parameters and proteomic profiles in stressed plants allowed us to test the hypothesis that different cassava varieties exhibit significantly distinct response strategies to autotoxic stress. The findings elucidated the mechanisms of how different cassava genotypes respond to autotoxic stress at the proteomic level.

MATERIAL AND METHODS

Plant materials. Three varieties, Xinxuan 048 (XX048), South China 205 (SC205), and Nanzhi 199 (NZ199), were used as plant materials. When cultivated in the same field, XX048 usually shows stronger plant architecture, longer root length, and significantly higher yields of biomass (27~47 t/ha DM, dry mass) and tuberous roots (19~29 t/ha DM) than NZ199 (15~28 t/ha DM for biomass, 10~21 t/ha DM for roots) and SC205 (14~27 t/ha DM for biomass, 8~19 t/ha DM for roots) (Luo et al. 2026). Cassava plantlets of these three cultivars were provided by Guangxi Academy of Agricultural Sciences.

Experimental design. Hydroxyacetone, 2,4-di-tert-butylphenol (2,4-DTBP), and acetic acid have been identified as the dominant autotoxins in cassava, with 2,4-DTBP exhibiting the strongest inhibition and proving lethal to cassava plantlets at concentrations $\geq 10^{-6}$ mol/L (Han et al. 2023). However, under *in vitro* culture conditions, the relative contents and ratios of these three autotoxins in the medium differ among cultivars (Han et al. 2022). Because

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autotoxic substances exert long-lasting stress on plants, the stress level must be set within the tolerance range of tissue-cultured plantlets to ensure their growth in agar medium. Accordingly, 2,4-DTBP at 10^{-8} mol/L was used as the baseline for stress establishment, and autotoxicity stress was simulated at different levels based on the relative proportions of the three autotoxins in the root exudates of three cassava cultivars (Table 1), as reported in our previous study (Han et al. 2022). Experiments 1, 2, and 3 were set as independent controls for each cultivar. In experiments 4, 5, and 6, the three cultivars were tested under the same stress level, with the concentrations of these three chemicals set based on their relative proportions in the root exudates of NZ199 (hydroxyacetone:2,4-di-tert-butylphenol:acetic acid $\approx$ 10:1:20), while experiments 7 and 8 were set based on the relative contents of these three chemicals in the root exudates of SC205 (6:1:15) and XX048 (4:1:10), respectively. Each treatment included 30 bottles, each containing five secondary axillary buds. All buds were cultured in 1/2 Murashige-Skoog (MS) medium (20.00 g/L sucrose and 0.02 mg/L NAA) in glass bottles (Zeng et al. 2015) at 27 °C under an illumination intensity of 8 000 lx and a 12/12 h light/dark cycle for 30 days.

Sampling. After the stress, 30 plants per experiment were randomly sampled (one plant was randomly selected from each bottle) and rinsed with sterile water. The plant height, aboveground biomass, root length, and root biomass were then measured. The 60 plants for each experiment (two plants were randomly selected from each bottle) were sampled, cleaned, well mixed, and then divided into two portions. Twenty-five cassava plantlets were randomly selected from the 60 plants and immediately rinsed with phosphate-buffered saline, frozen in liquid nitrogen, and stored at -80 °C for proteomics analysis.

The left plantlets were rinsed with sterile water, frozen in liquid nitrogen, and stored at -80 °C to determine root activity and the contents of proline (PRO), malondialdehyde (MDA), soluble sugars, soluble proteins, and total chlorophyll.

Characterisation of morphological and physiological parameters. The physiological parameters of cassava plantlet roots were measured using physiological assay kits (Suzhou Grace Biotechnology Co., Ltd., Jiangsu, China). Concentrations of MDA, PRO, soluble sugar, and soluble protein were measured with three technical replicates using the thiobarbituric acid-reactive substance method, the ninhydrin method, the anthrone-sulfuric acid method, and the Bradford method, respectively (Bandurska et al. 2017, Yang et al. 2025). The total chlorophyll content in fresh leaves was extracted using an acetone-ethanol mixture (1:1) and then measured by ultraviolet spectrophotometry (SHIMADZU, Kyoto, Japan) (Ardıç et al. 2023). The allelopathic effect index (AEI) of cassava plantlets under autotoxicity stress was calculated according to the protocol described by Williamson and Richardson (1988). The AEI was defined as $1-(C/T)$ (if $T \geq C$) or as $(T/C)-1$ (if $T < C$), where C is the control and T is the treatment. The comprehensive index of allelopathic effect (CIAE) was the average value of AEIs of plant height, root length, aboveground biomass, and root biomass. Given the small biomass of tissue-cultured plantlets, water content was determined mainly by the weighing method, and such small biomass may introduce considerable measurement error. Therefore, physiological parameters were expressed on a fresh weight basis.

Proteomics and bioinformatics analyses. Based on the AEI of cassava plantlets across all treat-

Table 1. The experimental design of autotoxicity stress on different cassava cultivars

Experiment	ID	Variety	Hydroxyacetone	2, 4-Di-tert-butylphenol	Acetic acid
1	CK199	NZ199	0	0	0
2	CK205	SC205	0	0	0
3	CK048	XX048	0	0	0
4	D1-199	NZ199	1×10^{-7} mol/L	1×10^{-8} mol/L	2×10^{-7} mol/L
5	D1-205	SC205	1×10^{-7} mol/L	1×10^{-8} mol/L	2×10^{-7} mol/L
6	D1-048	XX048	1×10^{-7} mol/L	1×10^{-8} mol/L	2×10^{-7} mol/L
7	D2-205	SC205	6×10^{-8} mol/L	1×10^{-8} mol/L	1.5×10^{-7} mol/L
8	D3-048	XX048	4×10^{-8} mol/L	1×10^{-8} mol/L	1×10^{-7} mol/L

NZ199, SC205 and XX048 represent the cassava cultivars Nanzhi 199, South China 205 and Xinxuan 048, respectively

ments, varieties SC205 and XX048 under the same stress level (Exp. 5 and 6) and their independent controls (Exp. 2 and 3) were selected for proteomic analysis using whole-plant tissue-culture seedlings. Due to limited materials, two technical replicates from well-mixed fractions of the twenty-five cassava plantlets for each treatment were prepared for the proteomics analysis, which was performed using DIA (Data Independent Acquisition) technology (Bilbao et al. 2015) by Gene Denovo Biotechnology Co. (Guangzhou, China).

Protein extraction and digestion. Protein extraction, digestion, and identification from cassava tissue were conducted according to protocols released by Liu et al. (2020). Total protein in each sample was extracted using the cold acetone method. Cassava samples were ground into powder in liquid nitrogen and dissolved in 2 mL lysis buffer (100 mmol EDTA, 50 mmol borax, 50 mmol vitamin C, 30% sugar, 100 mmol Tris, 1% Triton X-100, 1–5% PVPP, 1x Protease Inhibitor Cocktail (Roche Ltd. Basel, Switzerland), 5 mmol DTT, pH 8.0), followed by sonication on ice for 30 min and centrifugation at 13 000 rpm for 30 min at 4 °C. The supernatant was transferred to a new tube, and proteins were precipitated with ice-cold methanol containing ammonium acetate at –20 °C overnight. The precipitate was cleaned with 90% acetone three times, redissolved in lysis buffer (8 mol/L urea, 1% SDS, 1x Protease Inhibitor Cocktail) by sonication on ice, and centrifuged at 13 000 rpm for 20 min at 4 °C. The protein quality was then examined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After that, proteins were digested using trypsin digestion buffer from an iST kit (PreOmics, Martinsried, Germany) and the mixture was incubated at 37 °C and 500 rpm for 2 h. The termination buffer was then added to terminate the enzymatic reaction. The iST cartridge was applied for peptide desalting. The peptide segments were then eluted with 2 × 100 µL elution buffer and vacuum-dried before being stored at –80 °C.

Establishment of the spectrogram database. The freeze-dried peptide segments were dissolved in buffer A (20 mmol ammonium formate solution, with the pH adjusted to 10.0 using ammonium hydroxide). The peptides were separated using a reversed-phase column (XBridge C18 column, 4.6 mm × 250 mm, 5 µm, Waters Corporation, Milford, USA) interfaced with an Ultimate 3000

system (Thermo Fisher Scientific, Waltham, USA). The samples were separated using a linear gradient from 5–45% solution B (20 mmol ammonium formate in 80% acetonitrile, with the pH adjusted to 10.0 using ammonium hydroxide) over 40 min. The chromatographic column was equilibrated under initial conditions for 15 min. The column flow rate was set to 1 mL/min, and the temperature was set to 30 °C. Six fractions were collected and dried in a vacuum concentrator.

The fractions were dissolved in 0.1% formic acid solution and analysed by on-line nanospray LC-MS/MS on an Orbitrap Lumos mass spectrometer (Thermo Fisher Scientific, Waltham, USA) coupled with an EASY-nLC 1200 system. Peptide samples (3 µL) were loaded onto the analytical column (Acclaim PepMap C18, 75 µm × 25 cm). The linear gradient separation lasted 120 min, using a 5–35% buffer C (0.1% formic acid in aqueous acetonitrile) gradient. The column flow rate was controlled at 200 nL/min, and the electrospray voltage was 2 kV. The Orbitrap Lumos mass spectrometer was operated in data-dependent mode, switching automatically between MS and MS/MS acquisition. Survey full-scan MS spectra (*m/z* 350–1 500) with a mass resolution of 120K were acquired. The resolution of the higher-energy collisional dissociation (HCD) MS/MS scans was set at 15 K. The dynamic exclusion time was 30 s.

The DDA raw data were processed by Spectronaut Pulsar 11.0 (Biognosys AG) (Kim et al. 2018). Assuming trypsin as the digestive enzyme, Pulsar was set up to search the *Manihot esculenta* database (https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_001659605.2/). Carbamidomethylation (C) was specified as the fixed modification. Oxidation (M) was specified as the variable modification. The false discovery rate (FDR) at both the precursor-ion and peptide levels was set to 1%. The DDA reference database was built using the Pulsar software based on data obtained from the DDA collection mode.

DIA data collection: nano-HPLC-MS/MS analysis. The fractions from 2.5.2.1 were added to 30 µL of 0.1% formic acid solution to create suspensions. For each sample, 9 µL of suspension was well mixed with 1 µL of 10× iRT peptide and then analysed by online nanospray LC-MS/MS using the same system as in 2.5.2.2. The Orbitrap Lumos mass spectrometer was operated in data-independent mode, switching automatically between MS and MS/MS acquisition. Survey full-scan MS spectra (*m/z* 350–1 500) with a mass resolution of 120K were acquired. The

<https://doi.org/10.17221/336/2026-PSE>

resolution of HCD MS/MS was set to 30K. For the variable window acquisition, 60 windows were set, with each window overlapping by 1 m/z. The raw data were processed by Spectronaut Pulsar with default settings. To identify proteins, the DIA data were analysed against the DDA reference database. A precursor threshold of 1.0% FDR and a protein threshold of 1.0% FDR were set for the qualitative analysis of peptides and proteins, respectively. For protein quantification, the overall peak intensity of the sample spectrum was normalised using the local normalisation method in Pulsar. The average peak area of the first three peptide segments, sorted by the lowest FDR value, was used to quantify protein abundance.

Bioinformatics analysis. For each treatment, the Pearson correlation coefficient of detected proteins between the two technical replicates exceeded 0.988, demonstrating good reproducibility and justifying further analysis. Differentially expressed proteins (DEPs) between control and treatment were filtered using the following selection criteria: $|\text{fold change (FC)}| \geq 1.5$ and $Q\text{-value} < 0.05$ (Student's t -test). Gene Ontology (GO) annotation of the proteome was determined using the Gene Ontology resource knowledgebase (<https://www.geneontology.org/>). Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of DEPs was performed as described by Okuda et al. (2008). Pfam (<https://pfam.xfam.org/>) was used for protein domain annotation based on protein sequence alignment and the domain database. The PPI network of the symbols of significant DEPs (Confidence Scores > 0.9) was constructed using the STRING database (<https://string-db.org/>, accessed on 28 January 2025), and *Manihot esculenta* was used as the reference. All raw data

obtained were deposited in the ProteomeXchange Consortium (<https://proteomecentral.proteomexchange.org>) and are accessible *via* PXD050939.

Quantitative real-time PCR (qRT-PCR) analysis. To validate the proteomic results, the expression levels of genes encoding eight randomly selected DEPs were analysed by qRT-PCR in XX048 plantlets. The qRT-PCR protocol was well described in a previous study (Yang et al. 2023). Total RNA from cassava was extracted using the FastPure® Plant Total RNA Isolation Kit (Vazyme, China) according to the manufacturer's instructions. First-strand cDNA was synthesised from 1 µg of total RNA using TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix (Vazyme) according to the manufacturer's instructions. RT-qPCR was performed using ChamQ Universal SYBR qPCR Master Mix (Vazyme) on a qTOWER2.2 real-time PCR system (Analytik Jena, Germany) according to the manufacturer's instructions. The total volume of the 20 µL qRT-PCR reaction was used, including 10 µL ChamQ Universal SYBR qPCR Master Mix, 0.4 µL of forward primer (10 µmol), 0.4 µL of reverse primer (10 µmol), 1 µL of cDNA, and 8.2 µL of ddH₂O. The cycling conditions were 95 °C for 30 s, followed by 42 cycles of 95 °C for 10 s and 60 °C for 30 s. Relative gene expression levels were analysed using the $2^{-\Delta\Delta C_t}$ method. Each experiment included three independent biological replicates. The cassava actin gene was used as an internal control to normalise the data. All primers used in this study are listed in Table 2.

Statistical analysis. The data were processed using Microsoft Excel 2021 (Microsoft Corporation, Redmond, USA) and are presented as mean $\pm$ standard error (SE). Multiple comparisons were conducted using Fisher's least significant difference (LSD) test, and a significant difference between

Table 2. Primers used for qRT-PCR of 8 randomly selected DEGs

Gene names	Forward primer (5'-3')	Reverse primer (5'-3')
<i>MeRLK7</i>	AATGGGAGCTTATGGGACCG	GACTGGTCTGTCGCATCCAT
<i>MeDEAH</i>	GCGTCTTGATGCTGAGTTGC	CCAATCAAGACTATTACCTGCTGC
<i>MePEX16</i>	TGGAATTGAGGATCGGGCTC	CCAGCGAGTGGACATAGTCT
<i>MeAL4</i>	TTGAGTTACGGACATGGAGGG	TTGATAGAACTCTTCCACATCAGTG
<i>MeLOX2.1</i>	AAAGTTACAGTTGGTGGGCTC	TAGCCCCGTTTTGGGATCA
<i>MeASIL1</i>	TTGGAAATGGAGAGAATGCGGA	TGCATCTTGTGAAGTGCGGA
<i>MeDF1.1</i>	TCTTTGGGATGAGGTTTCCAGATTA	TGTCTTGCCATTTGACTTGCT
<i>MeDF1.2</i>	ACCGATGGCCTAGACAGGAA	ACCCTTTACACTGGCATCGC
<i>MeACTIN</i>	CGTTTGTGTATGCGTGCGAT	ATCCTGCGTCTATCACTGCG

two groups was established at $P < 0.05$. OriginPro 2023 (Northampton, USA) and Cytoscape 3.7.2 (San Diego, USA) were used for statistical analysis and data visualisation.

RESULTS

Morphological traits and the allelopathic effect of cassava seedlings under autotoxicity stress. Compared to the independent control, the plant heights of cassava seedlings in the D1-205, D1-048, and D3-048 treatments were lower than their controls, respectively, while the plant height of the D1-199 treatment was slightly taller than its control (Figures 1A, B and 2). Similar trends were also found for root lengths. The root biomass of XX048 under all stress treatments was significantly lower than that of its control (Figure 1C).

To evaluate the allelopathic effects of cassava under autotoxicity stress, the AEI for each treatment was also calculated using morphological traits (Table 3). The AEI of aboveground biomass and root biomass for the D1-199 treatment were -0.06 and -0.13 , respectively, while the AEI for plant height and root length were both larger than zero, resulting in a positive CIAE. For variety SC205, the AEI of root length and root biomass of the D2-205 treatment were -0.10 and -0.30 , respectively, resulting in a negative comprehensive effect of AEI (-0.08). As stress levels increased, negative effects were observed in the D1-205 treatment across all examined traits, and its CIAE decreased to -0.22 . These results indicate that autotoxins inhibit the growth of SC205 seedlings and that this influence increases with the stress level. The AEI of all morphological traits for D3-048 and

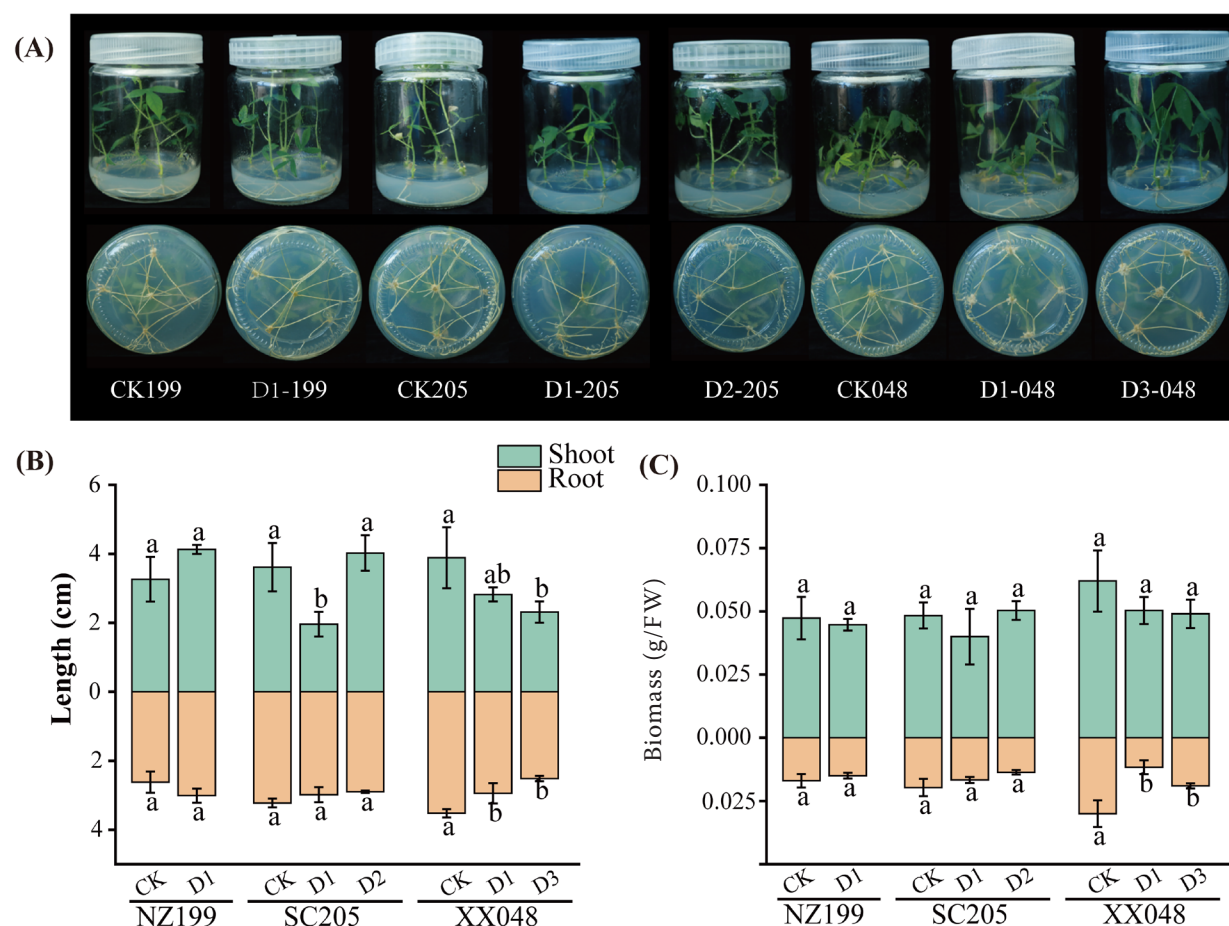


Figure 1. Morphological traits of cassava seedlings under autotoxicity stress varied with genotype. (A) Phenotypic characteristics; (B) root length and (C) biomass of cassava seedlings under autotoxicity stress. Values are presented as mean $\pm$ SE. Fisher's *LSD* test was used for multiple comparisons. The lowercase letters indicate that the mean values of different treatment groups differ significantly ($P < 0.05$); the same applies below. FW – fresh weight

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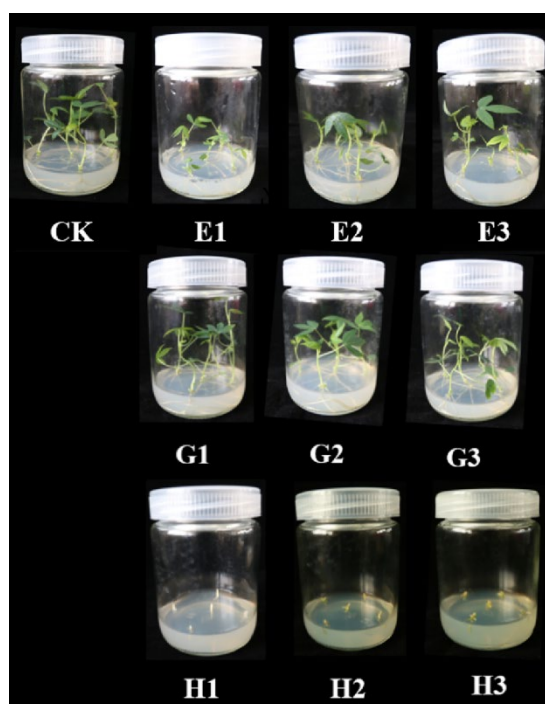


Figure 2. Cassava tissue culture seedlings under different stress levels of hydroxyacetone, acetic acid, and 2,4-di-tert-butylphenol, respectively. CK – control; E, G, and H – treatments of hydroxyacetone, acetic acid, and 2,4-di-tert-butylphenol, respectively. Number 1, 2, and 3 are concentrations at 1×10^{-3} mol/L $\approx 1 \times 10^{-4}$ mol/L $\approx 1 \times 10^{-5}$ mol/L, respectively

D1-048 were negative, resulting in the lowest CIAE (–0.32) among these three varieties, which indicates that the allelopathic effects on cassava tissue culture seedlings of variety XX048 were stronger than those on SC205 and NZ199 under the same stress level.

Physiological response of cassava under autotoxicity stress. For variety NZ199, the MDA content, root vitality, and total chlorophyll content

of the D1-199 treatment were lower than those of CK199, while PRO content was higher than that of the control (Table 4). The total chlorophyll content in the NZ199 leaves declined significantly under stress, indicating that photosynthesis of NZ199 was affected by autotoxicity stress to some extent. For variety XX048, the PRO content and root vitality of cassava under autotoxicity stress increased significantly, while the total chlorophyll content of cassava leaves under severe stress (D1-048) was lower than that of the light stress treatment (D3-048) and the control (CK048). The MDA and soluble protein contents of XX048 under light stress treatment were higher than those under severe stress or the control. Furthermore, the PRO content of SC205 under the light stress level (D2-205) was significantly lower than that of the severe stress level (D1-205) and the control (CK205), whereas the total chlorophyll content and root vitality showed the opposite trends. The soluble protein content of SC205 under severe stress was higher than that of D2-205 and CK205. In addition, a reduced ability of autotoxic substances to be reabsorbed from root exudates may cause less stress on plants. Under stress, the root vitality of NZ199 decreased, whereas that of SC205 and XX048 increased significantly; this may be the primary reason for the differing responses of different cassava varieties to autotoxicity stress.

Identification of DEPs of cassava in response to autotoxicity stress. Among the three varieties, XX048 and SC205 were significantly influenced by autotoxicity stress. Thus, to further elucidate the mechanisms by which cassava responds to autotoxicity stress, the proteomic profiles of independent controls (CK048 and CK205) and the autotoxicity stress treatments of these two varieties at the same level (D1-205 and D1-XX048) were

Table 3. The allelopathic effect index (AEI) and its comprehensive index of allelopathic effect (CIAE) of cassava under autotoxicity stress

Treatment	Plant height	Root length	Above-ground biomass	Root biomass	CIAE
D1-199	0.17 ± 0.08^a	0.10 ± 0.07^a	-0.06 ± 0.07^a	-0.13 ± 0.08^a	0.02 ± 0.06^a
D2-205	0.06 ± 0.11^a	-0.10 ± 0.05^b	0.02 ± 0.08^a	-0.30 ± 0.05^a	-0.08 ± 0.07^{ab}
D3-048	-0.41 ± 0.09^b	-0.28 ± 0.02^c	-0.21 ± 0.06^a	-0.36 ± 0.03^a	-0.32 ± 0.04^c
D1-205	-0.46 ± 0.12^b	-0.08 ± 0.07^b	-0.18 ± 0.18^a	-0.16 ± 0.12^a	-0.22 ± 0.08^{bc}
D1-048	-0.27 ± 0.10^b	-0.16 ± 0.07^{bc}	-0.19 ± 0.06^a	-0.61 ± 0.10^b	-0.31 ± 0.06^c

Values are presented as mean $\pm$ SE. Fisher's *LSD* test was used for multiple comparisons; the lowercase letters indicate that the mean values of different treatment groups have significant differences ($P < 0.05$); the same applies below

Table 4. Physiological parameters of different cassava genotypes under autotoxicity stress

Index	NZ199		SC205			XX048		
	CK	D1	CK	D1	D2	CK	D1	D3
Malondialdehyde (nmol/g FW)	1.04 ± 0.02 ^a	0.70 ± 0.06 ^b	0.70 ± 0.03 ^a	0.72 ± 0.07 ^a	0.57 ± 0.01 ^a	0.26 ± 0.03 ^b	0.28 ± 0.04 ^b	0.47 ± 0.03 ^a
Proline content (µg/g)	2.14 ± 0.07 ^b	2.94 ± 0.07 ^a	3.42 ± 0.15 ^a	3.43 ± 0.12 ^a	2.34 ± 0.08 ^b	2.76 ± 0.05 ^b	3.45 ± 0.05 ^a	3.59 ± 0.12 ^a
Soluble sugar (%)	5.13 ± 0.31 ^a	3.62 ± 1.21 ^a	7.89 ± 0.76 ^a	8.51 ± 1.70 ^a	6.74 ± 0.34 ^a	8.98 ± 1.13 ^a	7.05 ± 0.73 ^a	6.01 ± 2.00 ^a
Total chlorophyll (mg/g FW)	1.76 ± 0.14 ^a	0.49 ± 0.06 ^b	1.15 ± 0.06 ^b	1.36 ± 0.05 ^b	1.72 ± 0.12 ^a	1.20 ± 0.04 ^a	0.98 ± 0.07 ^b	1.35 ± 0.05 ^a
Soluble protein (mg/g)	15.6 ± 0.35 ^a	17.2 ± 0.84 ^a	12.8 ± 1.18 ^b	17.2 ± 0.56 ^a	11.7 ± 1.68 ^b	13.7 ± 0.85 ^b	17.1 ± 0.74 ^b	21.0 ± 1.56 ^a
Root vitality (µg/h/g FW)	1.88 ± 0.12 ^a	1.38 ± 0.10 ^b	1.69 ± 0.05 ^c	2.63 ± 0.26 ^b	3.34 ± 0.23 ^a	1.95 ± 0.06 ^b	3.75 ± 0.20 ^a	4.27 ± 0.55 ^a

FW – fresh weight

analysed. In total, 85 378 peptides were obtained, while 10 312 protein groups and 16 654 proteins

were identified (Figure 3). To avoid bias from using the same quantitative values across mass spectra

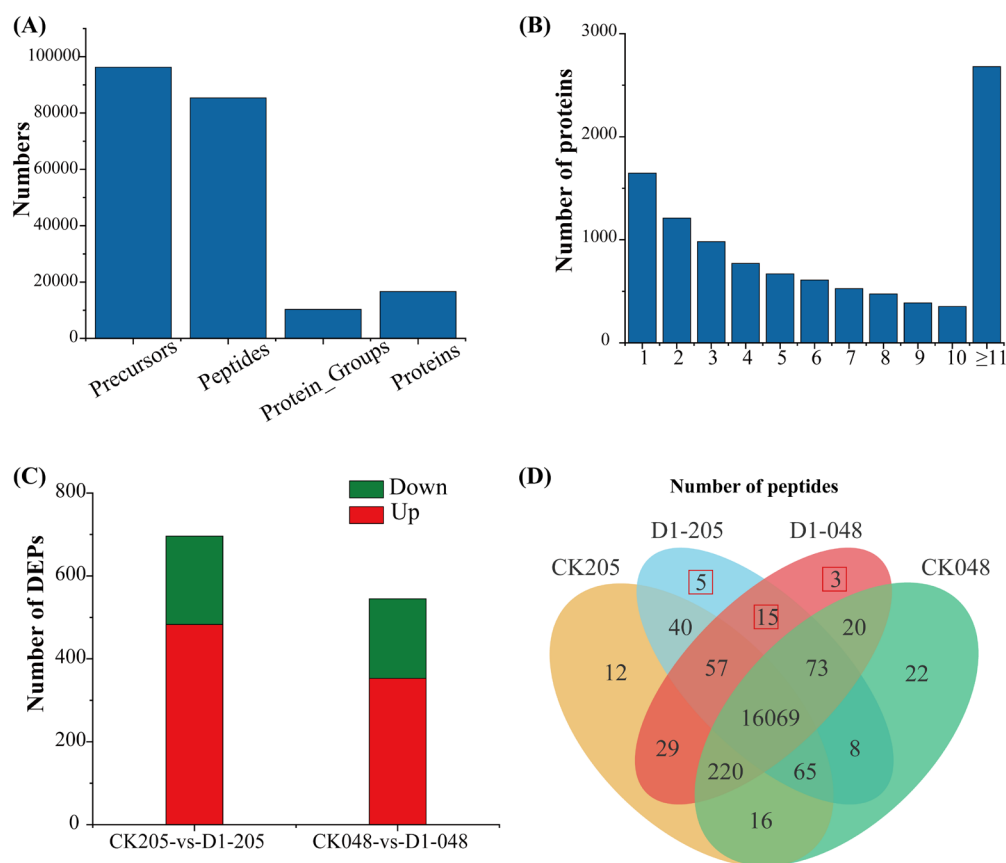


Figure 3. Results of the tandem mass tag (TMT)-based liquid chromatography-tandem mass spectrometry (LCMS/MS) identification of the proteins of cassava seedlings under autotoxicity stress. (A) Classification of the items used for identifying DEPs; (B) number and length of the identified peptides; (C) number of differentially expressed proteins in the CK205 vs D1-205 and CK048 vs D1-048 comparison groups. Red dots: up-regulated proteins; green dots: down-regulated proteins, and (D) a Venn diagram was applied to show the correlations of the identified proteins among the examined samples

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and multiple accession numbers identified as the same protein annotation for GO or KEGG enrichment pathways, protein group data were used as the identified proteins for each treatment in subsequent analysis. Among all samples, 1647, 1211, 11, 115, and 2681 proteins contained 1, 2, 3–10, and ≥ 11 peptides, respectively (Figure 3B). Compared with the control, 696 DEPs were found in SC205, with 69.4% up-regulated, while 545 DEPs were identified in XX048, with 64.8% up-regulated (Figure 3C).

Venn diagram analysis was used to compare detected proteins across the four treatments (Figure 3D), selecting the protein with the highest coverage as the representative of each group to avoid bias. Overall, 5 proteins (3 groups) and 3 proteins (3 groups) were detected exclusively in the D1-205 and D1-048 treatments, respectively, while 15 proteins (9 groups) were shared between the two va-

rieties (Table 5). Shared DEPs indicate conserved core defense and metabolic pathways. Both varieties reprogrammed transcription *via* ASIL1-like and Mediator 22b-like proteins. RNA and protein modifications (pseudouridine synthase and GCN5-related N-acetyltransferase) and membrane/structure remodeling (tetraspanin-19 and HAIKU2) were also shared strategies to maintain proteostasis and cellular homeostasis under stress. Unique proteins revealed divergent strategies. SC205 specifically activated a disease resistance protein (At4g27190), a splicing factor (RSZ21), and a glutathione transport system permease (gsiD). The reliably identified gsiD (with unique peptides) suggests enhanced glutathione detoxification, while RSZ21 and At4g27190 indicate transcript remodeling *via* alternative splicing and defense signaling initiation, respectively. In contrast, XX048 specifically activated HAIKU2 (membrane

Table 5. The DEPs were identified as lipoxygenase or glutathione sulfur transferase in SC205 and XX048.

Var.	Protein id	log2 (fc)	Coverage (%)	Symbol	Description
SC205	XP_021625458.1	0.6	35.90	LOX2.1	linoleate 13S-lipoxygenase 2-1, chloroplastic [<i>Manihot esculenta</i>]
	XP_021626234.1	1.9	52.40	LOX2.1	linoleate 13S-lipoxygenase 2-1, chloroplastic [<i>M. esculenta</i>]
	XP_021630634.1	-0.71	30.20	LOX2.1	lipoxygenase 6, chloroplastic [<i>M. esculenta</i>]
	XP_021596240.1	-0.71	35.70	GSTU19	glutathione S-transferase U19 [<i>M. esculenta</i>]
	XP_021601210.1	1.1	53.20	GSTF11	glutathione S-transferase [<i>M. esculenta</i>]
	XP_021603763.1	1.36	33.60	GSTF11	glutathione S-transferase F11 [<i>M. esculenta</i>]
	XP_021606585.1	-0.81	29.30	HSP26-A	probable glutathione S-transferase [<i>M. esculenta</i>]
	XP_021608465.1	-1.19	33.60	PRP1	probable glutathione S-transferase [<i>M. esculenta</i>]
	XP_021617725.1	-0.82	31.70	HSP26-A	probable glutathione S-transferase [<i>M. esculenta</i>]
	XP_021618319.1	0.67	32.80	GSTL3	glutathione S-transferase L3 isoform X1 [<i>M. esculenta</i>]; protein IN2-1 homolog B isoform X2 [<i>M. esculenta</i>]
	XP_021626265.1	-0.87	25.80	HSP26-A	probable glutathione S-transferase [<i>M. esculenta</i>]
	XP_021626283.1	-3.17	20.90	HSP26-A	probable glutathione S-transferase [<i>M. esculenta</i>]
	XP_043817838.1	-0.72	21.90	GST3	glutathione S-transferase 3-like [<i>M. esculenta</i>]
	XP_021626234.1	1.66	52.40	LOX2.1	linoleate 13S-lipoxygenase 2-1, chloroplastic [<i>M. esculenta</i>]
XX048	XP_021603734.1	-0.82	52.20	GSTU7	glutathione S-transferase U7-like [<i>M. esculenta</i>]
	XP_021603736.1	-1.25	25.70	GSTU7	glutathione S-transferase U7-like [<i>M. esculenta</i>]
	XP_021603763.1	-0.93	33.60	GSTF11	glutathione S-transferase F11 [<i>M. esculenta</i>]
	XP_021603922.1	0.6	30.90	GSTU17	glutathione S-transferase U17-like [<i>M. esculenta</i>]
	XP_021606585.1	-1.01	29.30	HSP26-A	probable glutathione S-transferase [<i>M. esculenta</i>]
	XP_021608465.1	-0.84	33.60	PRP1	probable glutathione S-transferase [<i>M. esculenta</i>]
	XP_021617725.1	-1.13	31.70	HSP26-A	probable glutathione S-transferase [<i>M. esculenta</i>]
	XP_021618319.1	0.78	32.80	GSTL3	glutathione S-transferase L3 isoform X1 [<i>M. esculenta</i>]; protein IN2-1 homolog B isoform X2 [<i>M. esculenta</i>]
	XP_021619647.1	-0.65	49.60	GSTL3	glutathione S-transferase L3-like isoform X2 [<i>M. esculenta</i>]
	XP_043816311.1	-1.83	5.40	PARC	probable glutathione S-transferase parC [<i>M. esculenta</i>]

Table 6. The DEPs enriched to the immune system process in SC205 and XX048 under autotoxicity stress

Var.	Protein id	log2(fc)	Coverage (%)	Symbol	Description
SC205	XP_021625935.1	1.80	81.50	RCA1	ribulose biphosphate carboxylase/oxygenase activase, chloroplastic isoform X1 [<i>Manihot esculenta</i>]
	XP_021633936.1	1.15	74.60	PGK1	phosphoglycerate kinase, chloroplastic [<i>M. esculenta</i>]
	XP_021624073.1	1.37	42.30	RCA1	ribulose biphosphate carboxylase/oxygenase activase, chloroplastic-like [<i>M. esculenta</i>]
	XP_021625703.1	1.06	38.30	At3g55800	sedoheptulose-1,7-bisphosphatase, chloroplastic-like [<i>M. esculenta</i>]
	XP_021595497.1	1.54	57.90	BCA1	carbonic anhydrase, chloroplastic-like isoform X1 [<i>M. esculenta</i>]
	XP_021612038.1	0.65	43.80	STR4	rhodanese-like domain-containing protein 4, chloroplastic [<i>M. esculenta</i>]
	XP_021621162.1	0.70	42.50	FFC	signal recognition particle 54 kDa protein, chloroplastic isoform X1 [<i>M. esculenta</i>]
	XP_021621163.1	0.70	42.50	FFC	signal recognition particle 54 kDa protein, chloroplastic isoform X1 [<i>M. esculenta</i>]
	XP_021596302.1	1.10	30.00	ATPG	ATP synthase subunit b', chloroplastic [<i>M. esculenta</i>]
	XP_021618564.1	1.27	30.80	At3g55800	sedoheptulose-1,7-bisphosphatase, chloroplastic [<i>M. esculenta</i>]
	XP_021595917.1	1.07	30.00	At1g32060	phosphoribulokinase, chloroplastic-like [<i>M. esculenta</i>]
	XP_021601132.1	-0.70	47.00	DHAPS-1	phospho-2-dehydro-3-deoxyheptonate aldolase 1, chloroplastic-like [<i>M. esculenta</i>]
	XP_021620177.1	1.02	48.30	RPI3	probable ribose-5-phosphate isomerase 3, chloroplastic [<i>M. esculenta</i>]
	XP_021604454.1	-0.73	21.20	STT3B	dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3B [<i>M. esculenta</i>]
	XP_021624198.1	0.74	54.90	SARED1	Sanguinarine reductase [<i>Capsicum annuum</i>]
	XP_021602374.1	1.00	64.90	At1g32060	phosphoribulokinase, chloroplastic [<i>M. esculenta</i>]
	XP_021628916.1	0.70	38.30	HEMC	porphobilinogen deaminase, chloroplastic [<i>M. esculenta</i>]
	XP_021621945.1	1.53	70.30	CSP41B	chloroplast stem-loop binding protein of 41 kDa b, chloroplastic [<i>M. esculenta</i>]
	XP_021633157.1	1.06	77.50	SHM1	serine hydroxymethyltransferase, mitochondrial-like [<i>M. esculenta</i>]
	XP_021633158.1	1.06	77.50	SHM1	serine hydroxymethyltransferase, mitochondrial-like [<i>M. esculenta</i>]
	XP_021592948.2	0.77	48.20	petC	cytochrome b6-f complex iron-sulfur subunit 2, chloroplastic [<i>M. esculenta</i>]
	XP_021605404.1	0.60	32.60	PSBP	oxygen-evolving enhancer protein 2, chloroplastic-like [<i>M. esculenta</i>]
	XP_021631480.1	-0.71	71.00	HNL	(S)-hydroxynitrile lyase-like [<i>M. esculenta</i>]
	XP_021604215.1	1.01	48.90	PETH	ferredoxin--NADP reductase, leaf isozyme, chloroplastic [<i>M. esculenta</i>]
	XP_021597940.1	-0.67	34.80	SDT	spermidine sinapoyl-CoA acyltransferase [<i>M. esculenta</i>]
	XP_021611821.2	0.87	29.90	STR4	rhodanese-like domain-containing protein 4, chloroplastic [<i>Jatropha curcas</i>]
	XP_021616362.1	0.96	17.10	PETH	ferredoxin--NADP reductase, leaf isozyme, chloroplastic-like [<i>M. esculenta</i>]
	XP_021603763.1	1.36	33.60	GSTF11	glutathione S-transferase F11-like [<i>M. esculenta</i>]
	XP_021634507.1	1.82	20.60	At4g02530	thylakoid luminal 16.5 kDa protein, chloroplastic-like isoform X1 [<i>M. esculenta</i>]
	XP_021634508.1	1.82	22.80	At4g02530	thylakoid luminal 16.5 kDa protein, chloroplastic-like isoform X2 [<i>M. esculenta</i>]
	XP_021619511.1	0.71	41.00	SARED1	Sanguinarine reductase [<i>Hibiscus syriacus</i>]
	XP_021598352.1	1.56	19.80	BCA1	carbonic anhydrase 2 [<i>M. esculenta</i>]
	XP_021619389.1	-1.13	28.60	--	DSBA domain-containing protein [<i>Gossypium australe</i>]
	XP_021619404.1	-1.13	28.60	--	DSBA domain-containing protein [<i>G. australe</i>]
	XP_043813015.1	-1.13	28.60	--	DSBA domain-containing protein [<i>G. australe</i>]
	XP_021605924.1	1.07	9.00	ATPG	ATP synthase subunit b', chloroplastic-like [<i>M. esculenta</i>]
	XP_021629743.1	-1.15	11.00	SABP2	salicylic acid-binding protein 2-like [<i>M. esculenta</i>]
	XP_021625750.1	0.66	10.60	CLPR1	ATP-dependent Clp protease proteolytic subunit-related protein 1, chloroplastic-like [<i>M. esculenta</i>]
	XP_021619621.1	-1.61	37.30	GNS1	glucan endo-1,3-beta-glucosidase, basic isoform-like [<i>M. esculenta</i>]
	XP_021611370.1	-0.85	26.60	NQR	NADPH:quinone oxidoreductase-like [<i>M. esculenta</i>]
	XP_021625936.1	2.16	5.00	RCA	ribulose biphosphate carboxylase/oxygenase activase, chloroplastic isoform X2 [<i>M. esculenta</i>]
	XP_021594687.1	-0.65	26.80	--	HR-like lesion-inducer [<i>Parasponia andersonii</i>]
	XP_021607547.1	-0.66	13.20	SDE3	probable RNA helicase SDE3 [<i>M. esculenta</i>]
	XP_021607548.1	-0.66	13.20	SDE3	probable RNA helicase SDE3 [<i>M. esculenta</i>]
	XP_043811077.1	-0.66	13.20	SDE3	probable RNA helicase SDE3 [<i>M. esculenta</i>]
	XP_021623789.1	1.10	27.10	RPI3	probable ribose-5-phosphate isomerase 3, chloroplastic [<i>M. esculenta</i>]
	XP_021632879.1	-0.63	15.90	SHKB	phospho-2-dehydro-3-deoxyheptonate aldolase 2, chloroplastic [<i>M. esculenta</i>]
	XP_021603146.1	-0.90	12.90	S-ACP-DES6	stearoyl-[acyl-carrier-protein] 9-desaturase 6, chloroplastic-like [<i>M. esculenta</i>]
	XP_021605523.1	1.13	53.30	ATPD	ATP synthase subunit delta, chloroplastic [<i>M. esculenta</i>]
	XP_021627696.1	0.61	35.30	EP3	endochitinase EP3-like [<i>M. esculenta</i>]
	XP_021598686.1	-1.89	1.40	DOX1	alpha-dioxygenase 1 [<i>M. esculenta</i>]

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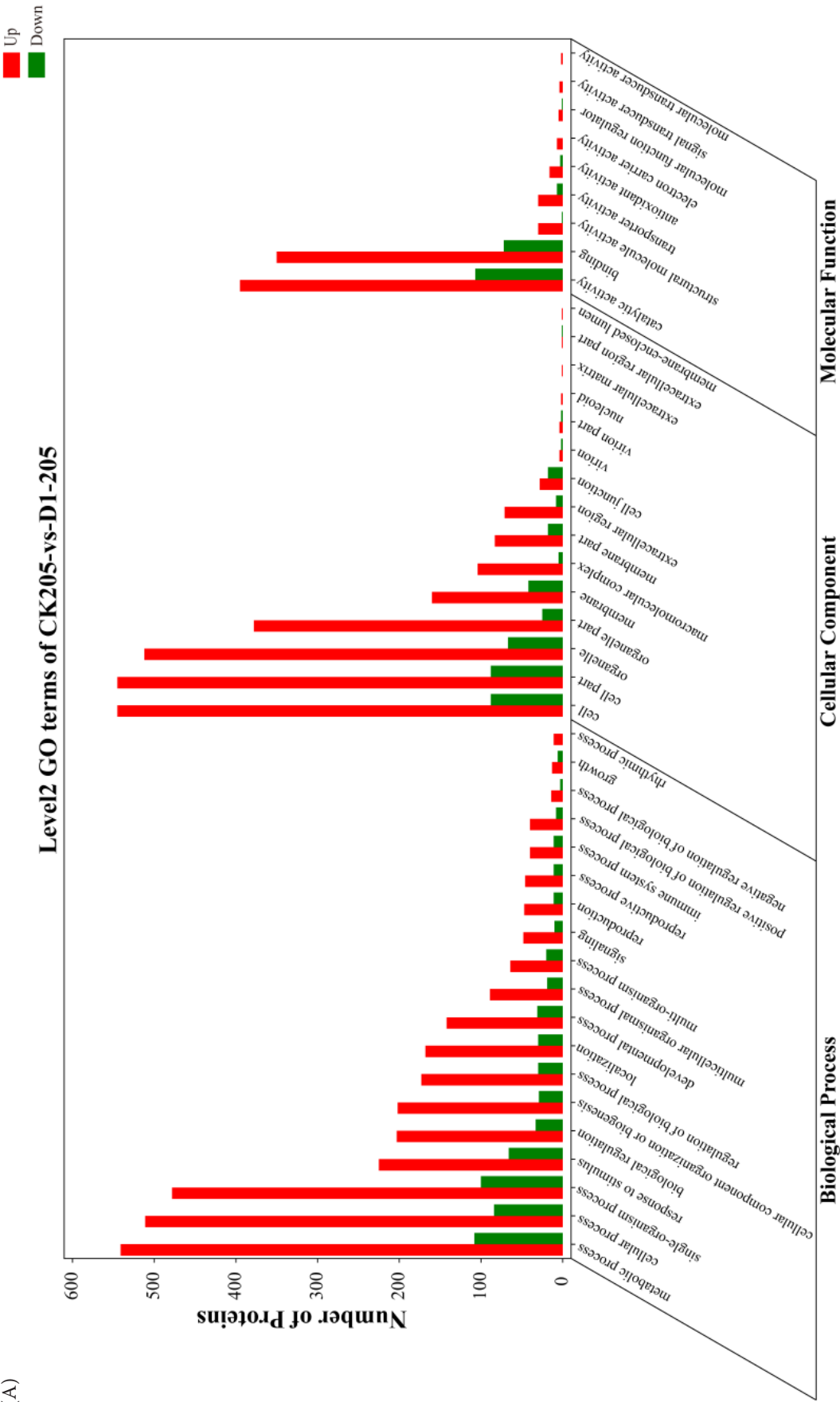
Continued Table 6. The DEPs enriched to the immune system process in SC205 and XX048 under autotoxicity stress

Var.	Protein id	log2(fc)	Coverage (%)	Symbol	Description
XX048	XP_021625703.1	0.82	38.30	At3g55800	sedoheptulose-1,7-bisphosphatase, chloroplastic-like [<i>Manihot esculenta</i>]
	XP_021595497.1	1.15	57.90	BCA1	carbonic anhydrase, chloroplastic-like isoform X1 [<i>M. esculenta</i>]
	XP_021625935.1	0.95	81.50	RCA1	ribulose bisphosphate carboxylase/oxygenase activase, chloroplastic isoform X1 [<i>M. esculenta</i>]
	XP_021633936.1	0.82	74.60	PGK1	phosphoglycerate kinase, chloroplastic [<i>M. esculenta</i>]
	XP_021620905.1	0.97	43.50	DJ1E	DJ-1 protein homolog E-like [<i>M. esculenta</i>]
	XP_021627696.1	2.85	35.30	EP3	endochitinase EP3-like [<i>M. esculenta</i>]
	XP_021618564.1	1.15	30.80	At3g55800	sedoheptulose-1,7-bisphosphatase, chloroplastic [<i>M. esculenta</i>]
	XP_021596388.1	−0.86	42.70	ALDH2C4	aldehyde dehydrogenase family 2 member C4-like [<i>M. esculenta</i>]
	XP_021605523.1	0.78	53.30	ATPD	ATP synthase subunit delta, chloroplastic [<i>M. esculenta</i>]
	XP_021595917.1	0.87	30.00	At1g32060	phosphoribulokinase, chloroplastic-like [<i>M. esculenta</i>]
	XP_021598352.1	1.18	19.80	BCA1	carbonic anhydrase 2 [<i>M. esculenta</i>]
	XP_021592948.2	0.61	48.20	petC	cytochrome b6-f complex iron-sulfur subunit 2, chloroplastic [<i>M. esculenta</i>]
	XP_021633157.1	1.04	77.50	SHM1	serine hydroxymethyltransferase, mitochondrial-like [<i>M. esculenta</i>]
	XP_021633158.1	1.04	77.50	SHM1	serine hydroxymethyltransferase, mitochondrial-like [<i>M. esculenta</i>]
	XP_021620177.1	0.67	48.30	RPI3	probable ribose-5-phosphate isomerase 3, chloroplastic [<i>M. esculenta</i>]
	XP_021596302.1	0.73	30.00	ATPG	ATP synthase subunit b', chloroplastic [<i>M. esculenta</i>]
	XP_021611821.2	0.78	29.90	STR4	rhodanese-like domain-containing protein 4, chloroplastic [<i>Jatropha curcas</i>]
	XP_021593456.1	0.78	42.50	PSBP	oxygen-evolving enhancer protein 2, chloroplastic-like [<i>M. esculenta</i>]
	XP_021619511.1	0.63	41.00	SARED1	Sanguinarine reductase [<i>Hibiscus syriacus</i>]
	XP_021621945.1	0.90	70.30	CSP41B	chloroplast stem-loop binding protein of 41 kDa b, chloroplastic [<i>M. esculenta</i>]
	XP_021630872.1	0.95	25.00	SHM1	serine hydroxymethyltransferase, mitochondrial [<i>M. esculenta</i>]
	XP_021598483.1	0.71	43.80	At1g29670	GDSL esterase/lipase At5g45670-like [<i>M. esculenta</i>]
	XP_021607352.1	0.69	8.80	NRPD2	DNA-directed RNA polymerases IV and V subunit 2-like [<i>M. esculenta</i>]
	XP_021595041.1	0.75	7.30	IWS1	protein IWS1 homolog 1 [<i>M. esculenta</i>]
	XP_021634507.1	0.95	20.60	At4g02530	thylakoid lumenal 16.5 kDa protein, chloroplastic-like isoform X1 [<i>M. esculenta</i>]
	XP_021634508.1	0.95	22.80	At4g02530	thylakoid lumenal 16.5 kDa protein, chloroplastic-like isoform X2 [<i>M. esculenta</i>]
	XP_021603763.1	−0.93	33.60	GSTF11	glutathione S-transferase F11-like [<i>M. esculenta</i>]
	XP_021604215.1	0.63	48.90	PETH	ferredoxin--NADP reductase, leaf isozyme, chloroplastic [<i>M. esculenta</i>]
	XP_021617728.1	−1.32	7.80	BXL5	probable beta-D-xylosidase 5 [<i>M. esculenta</i>]
	XP_021593999.1	0.85	50.60	GDCHS	glycine cleavage system H protein, mitochondrial-like [<i>M. esculenta</i>]
	XP_021619621.1	−1.11	37.30	GNS1	glucan endo-1,3-beta-glucosidase, basic isoform-like [<i>M. esculenta</i>]
	XP_021605404.1	0.65	32.60	PSBP	oxygen-evolving enhancer protein 2, chloroplastic-like [<i>M. esculenta</i>]
	XP_021609297.1	−0.68	54.50	SUS2	sucrose synthase 2 isoform X1 [<i>M. esculenta</i>]
	XP_021621280.1	1.00	37.90	CP29B	29 kDa ribonucleoprotein, chloroplastic-like [<i>M. esculenta</i>]
	XP_043805361.1	0.85	14.80	petC	cytochrome b6-f complex iron-sulfur subunit, chloroplastic-like, partial [<i>M. esculenta</i>]

signal perception), a peroxisome biogenesis protein (involved in ROS metabolism), and a chloroplastic RNA helicase (involved in RNA metabolism). This indicates a distinct strategy focused on membrane signal perception, peroxisomal ROS balance, and chloroplast RNA regulation.

Since lipoxygenase (LOX) and glutathione sulfur transferase (GST) are recognised as stress-related

proteins, all detected DEPs between SC205 and XX048 annotated as either enzyme were compiled in Table 6. LOX is the initiating enzyme of the lipoxygenase pathway, participating in the synthesis of stress signaling molecules such as jasmonic acid (JA), and is also associated with membrane lipid peroxidation. This may suggest that SC205 tends to exhibit stronger activation of membrane



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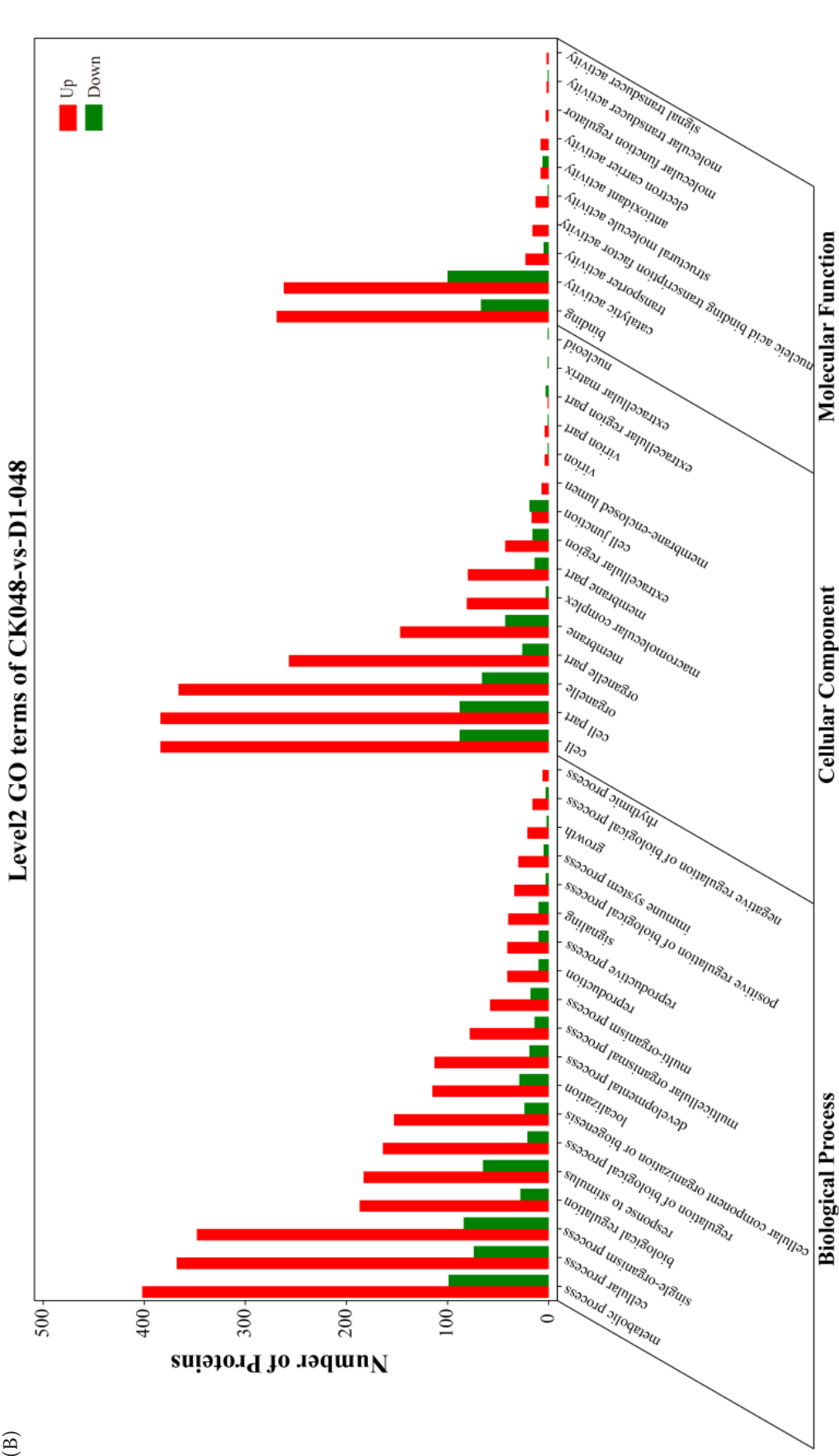


Figure 4. Gene Ontology functional classification of differentially expressed proteins (DEPs) of (A) SC205 and (B) XX048 under the same autotoxicity stress level. In the figure legends, "up" and "down" represent upregulated and downregulated DEPs, respectively, compared with the control

lipid signal transduction and the jasmonic acid defense pathway under autotoxicity stress, potentially facilitating the more rapid initiation of downstream defense gene expression. In contrast, the LOX response of XX048 was relatively limited and less pronounced, suggesting that its signal amplification capacity may be weaker than that of SC205. In addition, GST is a core enzyme of the glutathione detoxification system, catalysing the conjugation of toxic substances with glutathione to promote their excretion or further metabolism. The two varieties had similar numbers of GST DEPs (10 in SC205 and 11 in XX048), but their regulatory patterns differed markedly. SC205 had more up-regulated GST members with greater magnitudes, indicating that its glutathione detoxification capacity was more effectively activated under autotoxicity stress. Although XX048 had slightly more differentially expressed GST proteins overall, most were down-regulated, possibly reflecting suppressed or insufficient detoxification responses under stress, leading to the accumulation of toxic intermediates and aggravated autotoxic damage (Table 3).

GO functional classification of DEPs of cassava under autotoxicity stress. GO functional classification (biological process, cellular component, and molecular function) was performed on SC205 and XX048 (Figure 4). In the CK205-vs-D1-205 comparison, up-regulated proteins substantially outnumbered down-regulated ones across almost all GO Level 2 categories, indicating an overall activated proteomic response in SC205. The largest up-regulated categories (metabolic/cellular processes, catalytic activ-

ity, and binding) suggest metabolic and cellular remodeling. Furthermore, the up-regulation of stress-related categories (response to stimulus, biological regulation, antioxidant, transporter, and signal transducer activities) corroborates the activation of unique proteins (gsiD, RSZ21, At4g27190) and LOX/GST, reflecting an active initiation of detoxification and defense. In contrast, XX048 exhibited smaller overall scales of up-regulation in metabolic and cellular processes. Its up-regulated stress-related proteins (response to stimulus, signaling, and immune system) were markedly fewer than those of SC205 (Table 7), and only one pathogenesis-related protein was up-regulated compared to four in SC205 (Table 7). Similarly, major cellular components (cell, cell part, and organelle) showed lower up-regulation in XX048. At the molecular function level, although catalytic activity and binding dominated, the numbers of up-regulated antioxidant (~5 vs. ~25) and transporter (~20 vs. ~35) proteins were far lower in XX048 than in SC205, consistent with its GST down-regulation and absence of gsiD. Overall, the GO results demonstrate that SC205 possesses stronger capacities for metabolic activation, transport-detoxification, and signaling regulation under autotoxicity stress.

To better understand the functional characteristics of DEPs in response to autotoxicity stress, GO enrichment based on clustering analysis was performed using differential protein abundance, divided into four aliquots: Q1 (0–25%), Q2 (25–50%), Q3 (50–75%), and Q4 (75–100%) (Figure 5 and 6). In the biological process category, in XX048, DEPs classified into salicylic acid (SA) metabolic pro-

Table 4. The DEPs identified as pathogenesis-related in SC205 and XX048 under autotoxicity stress

Var.	Protein id	log2(fc)	Coverage (%)	Symbol	Description
SC205	XP_021605202.1	0.17	3.20	At1g75040	pathogenesis-related protein 5-like isoform X1 [<i>Manihot esculenta</i>]
	XP_021605203.1	0.17	3.70	At1g75040	pathogenesis-related protein 5-like isoform X2 [<i>M. esculenta</i>]
	XP_021624799.1	−0.19	4.30	At1g75040	pathogenesis-related protein 5-like [<i>M. esculenta</i>]
	XP_021615388.1	1.23	12.40	PR-1	pathogenesis-related protein PR-1 [<i>M. esculenta</i>]
	XP_021621262.1	0.36	7.60	WIN1	pathogenesis-related protein PR-4-like [<i>M. esculenta</i>]
XX048	XP_021605202.1	−0.25	3.20	At1g75040	pathogenesis-related protein 5-like isoform X1 [<i>M. esculenta</i>]
	XP_021605203.1	−0.25	3.70	At1g75040	pathogenesis-related protein 5-like isoform X2 [<i>M. esculenta</i>]
	XP_021624799.1	−0.52	4.30	At1g75040	pathogenesis-related protein 5-like [<i>M. esculenta</i>]
	XP_021615388.1	−0.51	12.40	PR-1	pathogenesis-related protein PR-1 [<i>M. esculenta</i>]
	XP_021621262.1	1.21	7.60%	WIN1	pathogenesis-related protein PR-4-like [<i>M. esculenta</i>]

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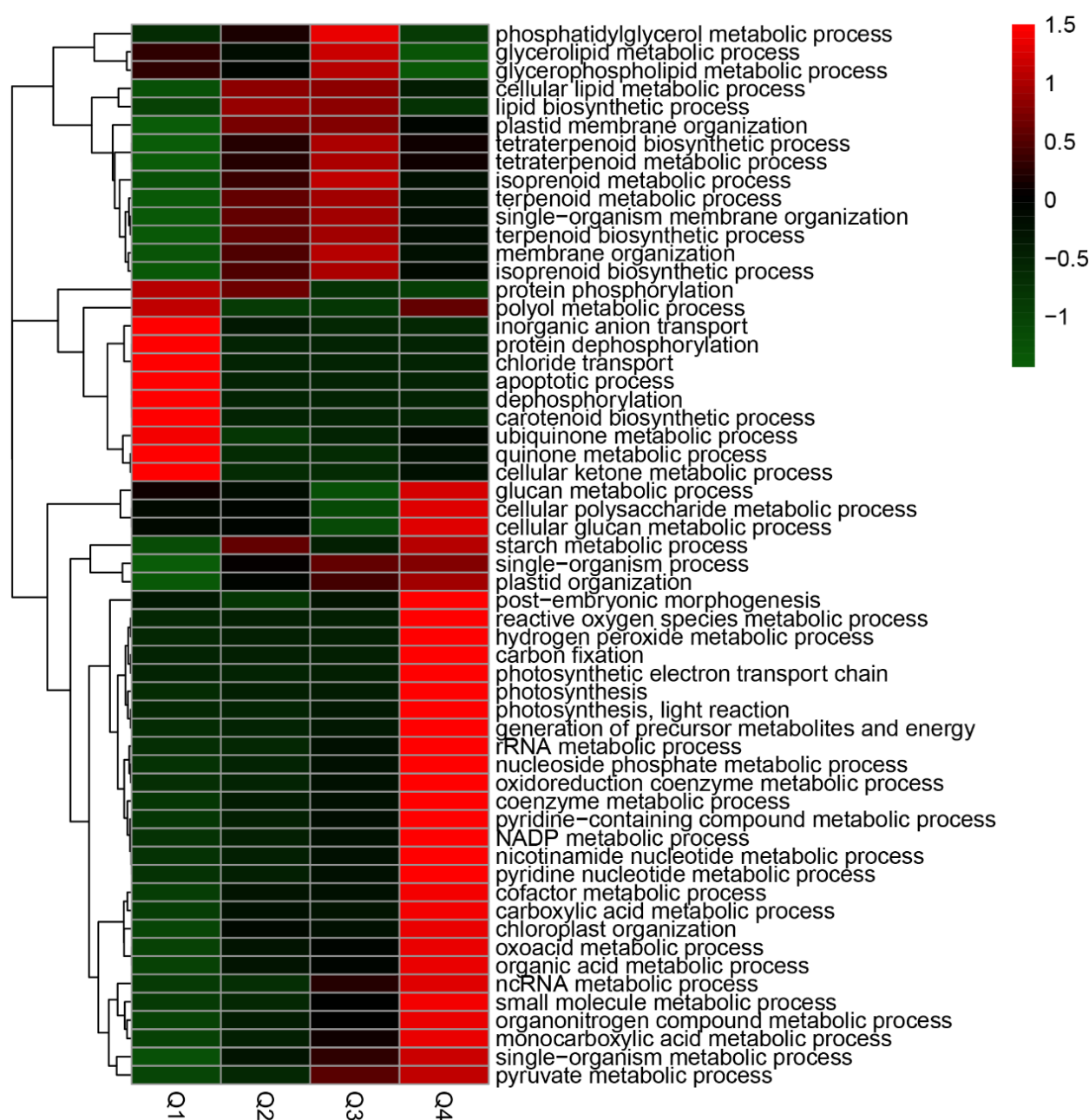


Figure 6. Heat map of biological process of differentially expressed proteins of SC205 under the autotoxicity stress

cess (22 DEPs), response to SA (21 DEPs), cellular response to SA stimulus process (21 DEPs), and SA mediated signaling pathway process (21 DEPs) were among the top 20 highly enriched in Q4 (Figure 5, while in SC205 these processes were among the top 30 enriched processes. These results indicate both varieties use SA as the major defense hormone and signal molecule, and that SA-mediated defense responses constitute a conserved mechanism for cassava against autotoxicity stress. For SC205, it simultaneously enriched ROS

metabolism, oxidative stress response, starch/carbon metabolism, organic acid metabolism, protein phosphorylation, and programmed cell death in Q4, forming a complete network spanning signal perception, metabolic reprogramming, and detoxification defense (Figure 5). In contrast to SC205 (Figure 6), the red highly enriched regions of XX048 were markedly concentrated in Q1–Q2 (low-abundance intervals), enriching processes such as transcriptional regulation, RNA processing, histone acetylation, and chromatin remodeling,

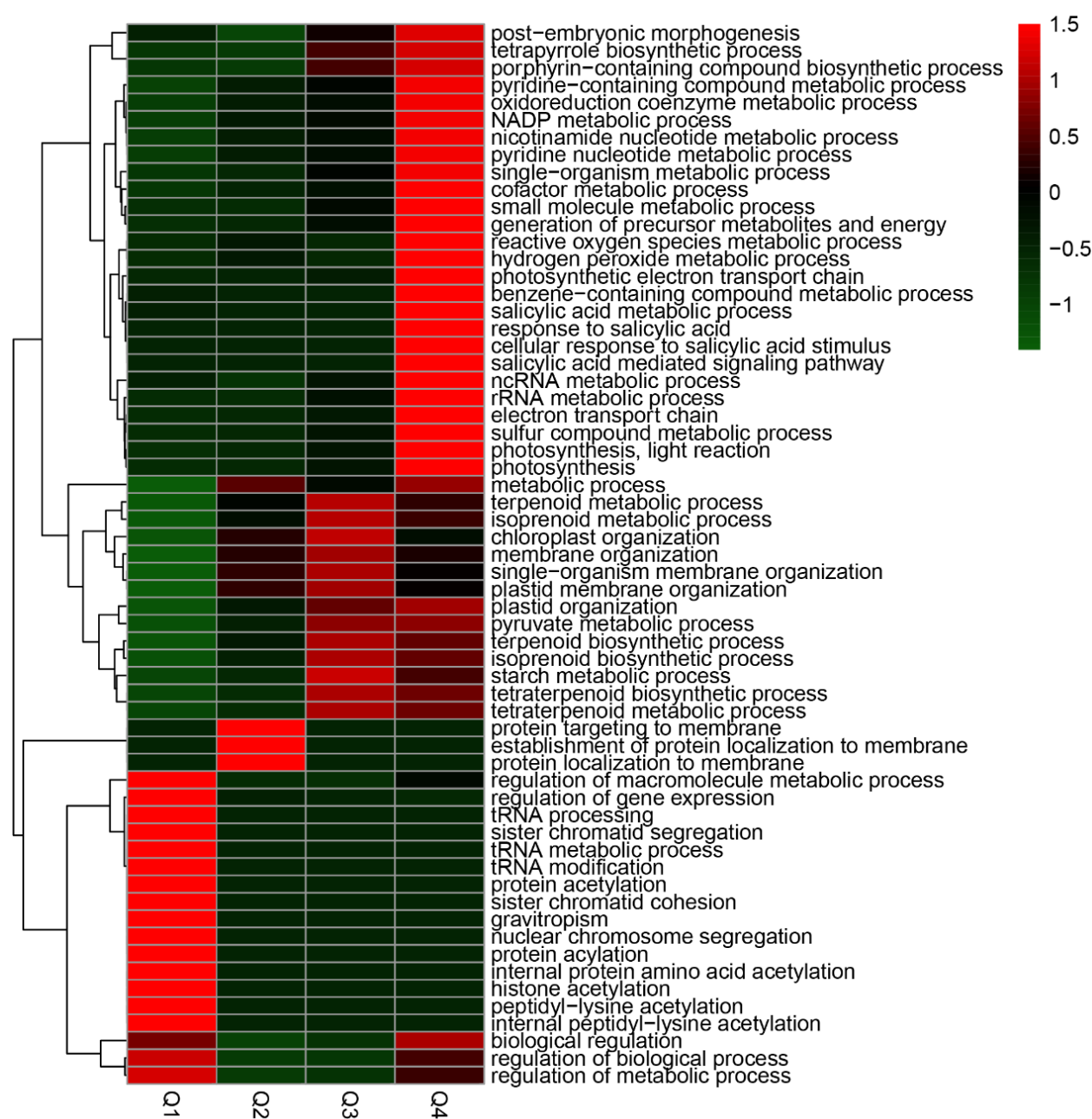


Figure 6. Heat map of the biological processes of differentially expressed proteins of XX048 under the autotoxicity stress

indicating that XX048 initiates a gene expression regulatory network at the low-abundance level; however, Q4 (high-abundance intervals) was predominantly green, lacking enrichment of defense-related processes such as ROS metabolism, oxidative stress response, starch/carbon metabolism, and isoprenoid biosynthesis. Moreover, most GST proteins were down-regulated and *gsiD* was absent, resulting in insufficient detoxification and antioxidant capacity. This indicates that XX048

mobilises regulatory processes such as transcription, RNA processing, and chromatin remodeling at the low-abundance level, but has insufficient enrichment of high-abundance defense proteins and limited detoxification and antioxidant capacity.

Protein domain analysis of the DEPs. To further understand the domain features of DEPs, domain annotation analysis was also performed (Figure 7). Both varieties enriched domains such as GST, copper/zinc superoxide dismutase (SODC),

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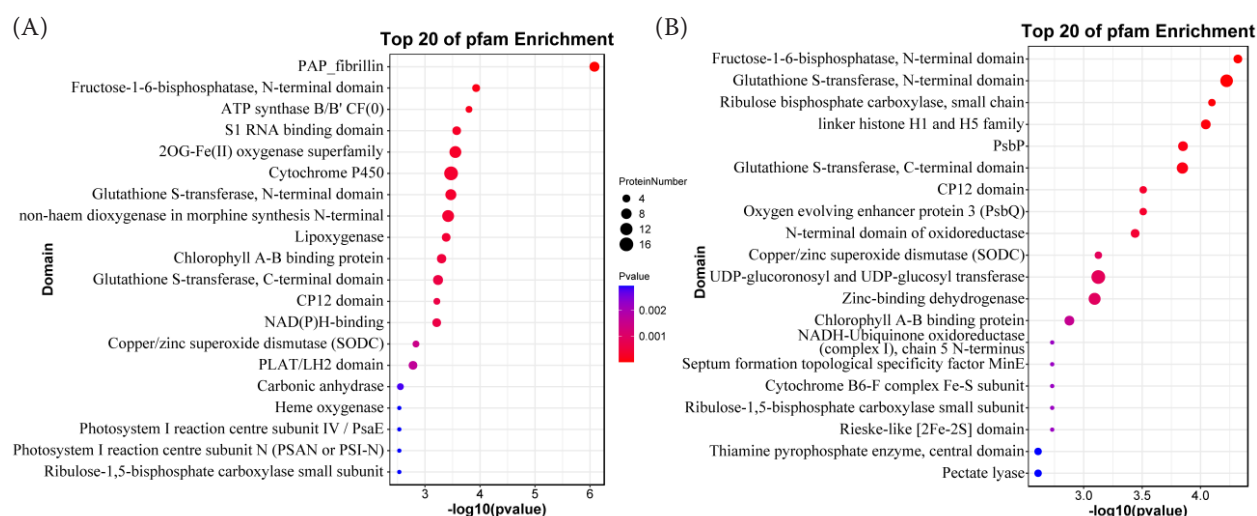


Figure 7. The top 20 enriched Pfam domains of differentially expressed proteins (DEPs) in (A) SC205 and (B) XX048 under the same autotoxicity stress level. Pfam enrichment analysis was performed to identify significantly enriched protein domains. The x -axis represents the statistical significance of enrichment (P -value), and the y -axis lists the top 20 enriched Pfam domains. The dot size indicates the number of DEPs annotated to each domain, and the colour represents the enrichment significance (P -value), with red indicating higher significance and blue indicating lower significance. Only domains with $P < 0.05$ were considered significantly enriched

and carbon fixation, suggesting that glutathione detoxification, antioxidant defence, and carbon metabolic reprogramming may represent conserved mechanisms by which cassava responds to autotoxicity stress. SC205 showed greater enrichment and a larger number of DEPs in defence-related domains such as GST, SODC, LOX, and cytochrome P450 (CYP450), indicating that its

detoxification and antioxidant defence are more fully activated at the protein domain level, consistent with its stronger resistance. Although XX048 also enriched some defence-related domains, the significance was lower, and it preferentially enriched domains related to chromatin regulation and cell wall remodelling, such as linker histone H1, glucosyltransferase, and pectate lyase, sug-

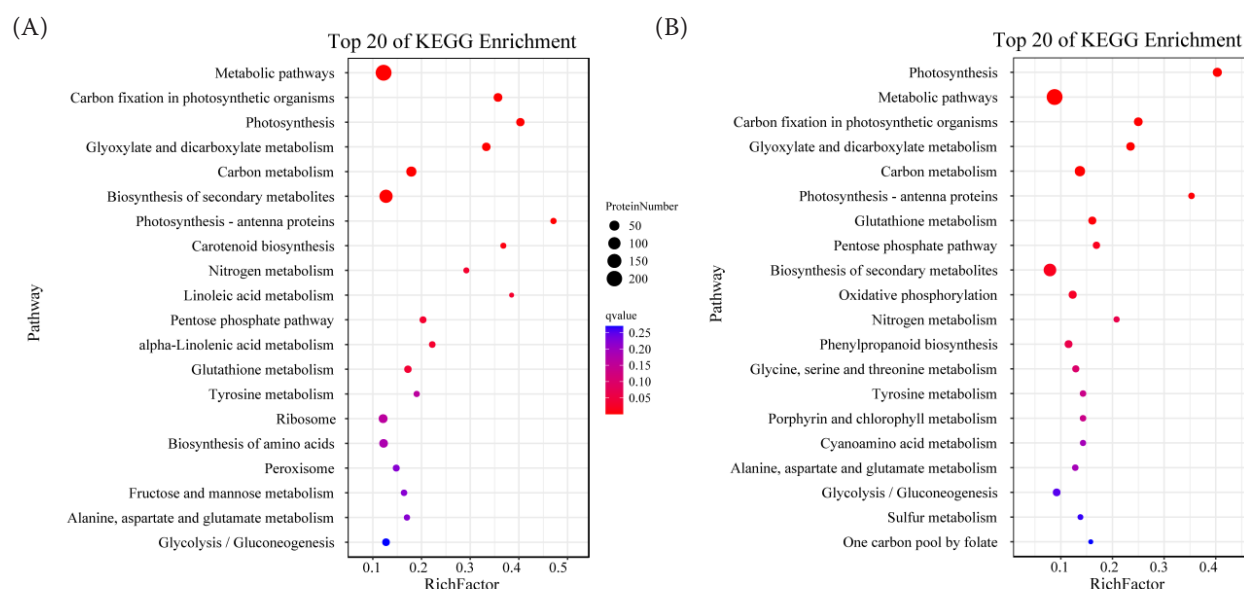


Figure 8. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of differentially expressed proteins of (A) SC205 and (B) XX048 under the same autotoxicity stress level

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(A)

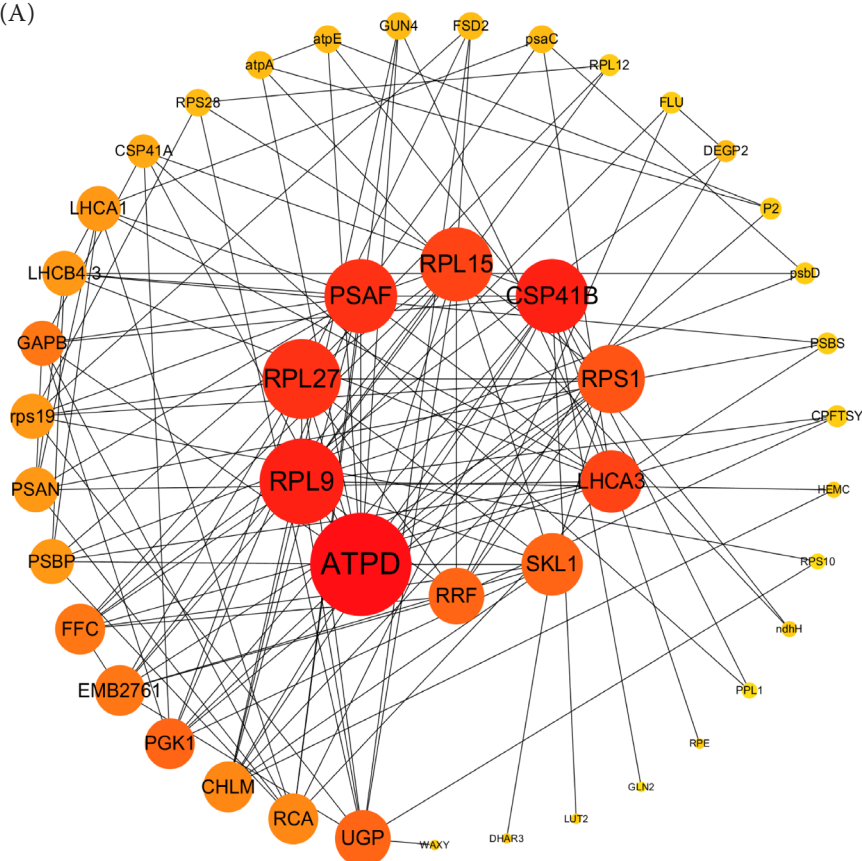
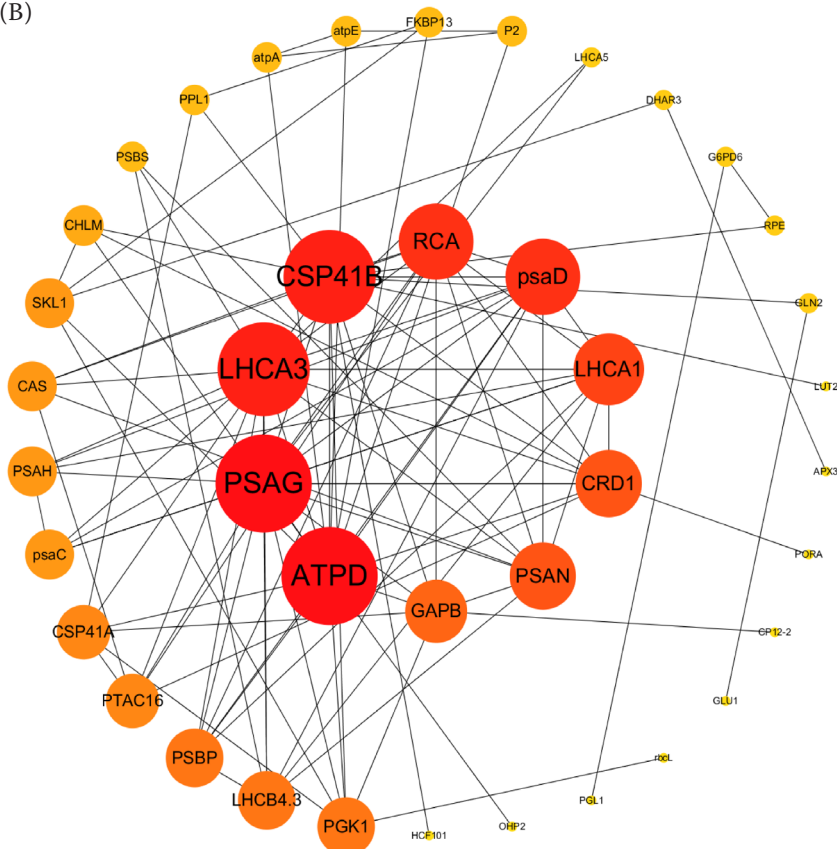


Figure 9. Protein-protein interaction network analysis of differentially expressed proteins of (A) SC205 and (B) XX048 under the same autotoxicity stress level. Circles are proteins. The larger and darker the circle, the more important the protein in the interaction network

(B)



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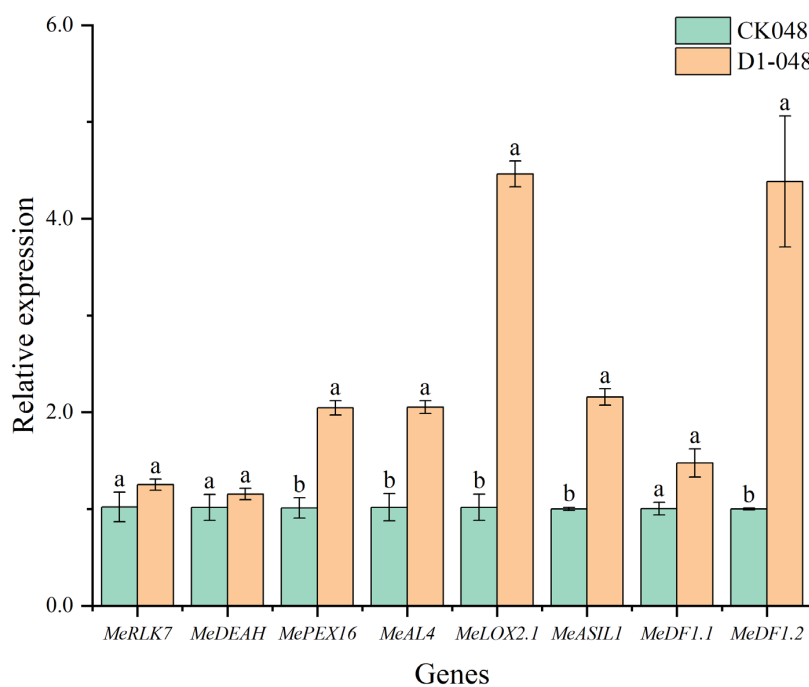


Figure 10. Relative gene expression levels of DEPs by qRT-PCR. CK048 represents XX048 without autotoxic stress, and D1 represents XX048 under 10–8 mol/L with 2, 4-Di-tert-butylphenol stress

gesting that its response is more biased toward regulation and structural remodelling than toward high-intensity detoxification defence.

KEGG pathway analysis of DEPs of cassava under autotoxicity stress. KEGG pathway enrichment analysis further validated the results of GO enrichment, Pfam domain, and DEP expression patterns at the pathway level (Figure 8). Both varieties showed enrichment in pathways such as photosynthesis, carbon fixation, carbon metabolism, secondary metabolism, and amino acid metabolism, indicating that carbon metabolic reprogramming and basal metabolic adjustment are conserved mechanisms for cassava in response to autotoxic stress. The molecular basis for SC205's superior resistance compared to XX048 is supported by the full activation of its detoxification, antioxidant, and lipoxygenase pathways (Figure 8A). Although XX048 also enriched pathways related to photosynthesis, carbon metabolism, and secondary metabolism, it does not significantly enrich defence-related pathways. Instead, it enriched more pathways associated with energy metabolism and cell wall remodelling, such as oxidative phosphorylation and phenylpropanoid biosynthesis (Figure 8B). This suggests that its response is more oriented towards maintaining basal metabolism and structural remodelling rather than intensive detoxification and defence, with limited detoxification and antioxidant capacity.

Protein-protein interaction (PPI) network analysis of DEPs in cassava under autotoxicity stress.

To identify the hub proteins of the DEPs, their PPI network was constructed using cytoHubba (Figure 9). Under identical levels of stress, the two varieties not only shared fundamental defence mechanisms but also exhibited divergent specific response strategies. ATPD, RPL5, RPL27, and CSP41B were unequivocally identified as topologically central hubs within both interactomes. This conserved network architecture strongly suggests a shared basal defensive response that encompasses energy metabolism (ATP/ATP synthase) and ribosomal- and translation-oriented pathways. The unique core proteins of SC205 were RPL9, PSAP, and RPL3 (Figure 9A). Given that both RPL9 and RPL3 were ribosomal proteins, it was suggested that SC205 might have primarily focused on regulating ribosome assembly or optimising translation efficiency. The unique core proteins of XX048 were identified as CRA1, HSCA, PSAG, and RPL3A (Figure 9B). Among them, the presence of HSCA (a heat shock homolog/protein chaperone) was considered particularly critical, suggesting that under similar stress, XX048 might have initiated a more robust protein-folding protection or heat shock response; conversely, PSAG was a subunit of photosystem I (PSI), pointing to its close involvement in photosynthetic regulation. These differences among the core nodes revealed fundamental distinctions in the molecular-level adaptive regulatory strategies between the two genotypes when they were exposed to identical adverse conditions.

Validation of proteomic results by qRT-PCR. To validate the proteomic results at the transcriptional level, eight DEPs were randomly selected from the proteomic analysis, and the expression levels of their corresponding coding genes in XX048 were measured. The selected DEPs were encoded by the following genes: receptor-like protein kinase HAIKU2 (*MeRLK7*), ATP-dependent RNA helicase DEAH12 (*MeDEAH*), peroxisome biogenesis protein 16 (*MePEX16*), PHD finger protein ALFIN-LIKE 4 (*MeAL4*), linoleate 13S-lipoxygenase 2-1 (*MeLOX2.1*), trihelix transcription factor ASIL1 (*MeASIL1*), GTL1 (*MeDF1.1*), and GT-2 (*MeDF1.2*). The qRT-PCR results indicated that the expression trends of these eight genes were largely consistent with the abundance changes of their corresponding proteins (Figure 10). Among them, six genes showed significantly increased expression in cassava under autotoxic stress. These findings further confirm that the proteomic data reliably reflect cassava's molecular response to autotoxic stress.

DISCUSSION

The allelopathic effect of cassava seedlings under autotoxicity stress. Plant autotoxins are mainly identified in root exudates (Yang et al. 2015, Hua et al. 2019). The root cause of self-toxic stress lies in plants' reabsorption of autotoxic substances from root exudates. If the vitality of plant root systems (including respiratory metabolism and active absorption capacity) declines significantly, their ability to actively absorb water and nutrients in the culture medium is impaired, and the uptake of autotoxins decreases. After adding autotoxins to the culture medium, cassava growth and biomass yield were inhibited, leading to negative allelopathic effects (Figure 1, Table 3), especially for root biomass, which decreased across all treatments, resulting in negative AEs (Table 3). Similar results can be found in alfalfa and melon, where root exudates were used as the source of autotoxic stress (Zhang et al. 2020, Wang et al. 2022). In alfalfa, after 21 days of incubation in its root exudates, the fresh weight penalty increased with the concentration of root exudates (Wang et al. 2022). The root growth of melon seedlings was significantly inhibited after adding the root exudates to the cultivation substrate for 24 h (Zhang et al. 2020). In addition, the allelopathic effects of cassava under autotoxicity stress varied among genotypes (Table 3), which

may be partly attributed to differences in genotypes' capacity to reabsorb autotoxic substances from root exudates. The autotoxicity stress level was simulated based on the relative proportions of the three main autotoxins of NZ199, which were also treated as the severe stress level for SC205 and XX048 (Table 1). Under severe stress, the root vitality of XX048 and SC205 remained at a consistently high level, whereas that of NZ199 declined significantly (Table 3). Although high root vitality is generally associated with plant robustness, under autotoxic stress it may instead reflect enhanced passive uptake of rhizosphere-derived autotoxins. In NZ199, root vitality decreased significantly, which may reflect an active avoidance detoxification strategy to reduce passive uptake of toxic substances. However, this avoidance also leads to a substantial reduction in the uptake of water and mineral nutrients. Due to the lack of the nutrient basis required for the synthesis of the antioxidant enzyme system, we inferred that the capacity of its enzymatic antioxidant system is correspondingly weakened. To compensate for this deficiency, the plant expends energy to synthesise more osmoregulatory substances, such as proline, for non-enzymatic protection (proline content is significantly increased). In addition, nutrient and water deficiencies lead to a significant decrease in chlorophyll content. Because the avoidance strategy greatly reduced toxin entry into the plant and overall cellular metabolism was in a low-activity state, the burst of ROS was effectively suppressed, preventing an increase in MDA content due to decreased antioxidant enzyme activity. Instead, it showed a significant downward trend (Table 3, Figure 1). In contrast, XX048 and SC205 maintained high root vitality, which may have led to continuous toxin uptake and excessive toxin accumulation in the above-ground tissues. However, their root vitality was preserved, overall biomass loss was more severe. This apparent trade-off between toxin avoidance and tolerance suggests that cassava genotypes may adopt two distinct adaptive strategies in response to autotoxic stress, although direct evidence (e.g., labeled toxin uptake analysis) is needed to confirm this hypothesis.

The mechanisms by which cassava responds to autotoxicity stress. Plants encounter numerous abiotic and biotic stresses throughout their lifetime. As sessile organisms, they must develop

<https://doi.org/10.17221/336/2026-PSE>

strategies to overcome these stresses or compensate for their effects. To mount defenses efficiently, plants prioritise first-line defense strategies in the apoplastic space before cellular processes are affected (Du et al. 2024). Apoplastic defense relies on antioxidants (ascorbate, glutathione), antimicrobial peptides (defensins, small peptides), secondary metabolites, and enzymes like class III peroxidases and NADPH oxidase. Glutathione, the long-distance transport form of reduced sulfur, is present in the apoplastic space at relatively low concentrations yet plays a crucial role in signaling and activating plant defenses against infection (Köstner et al. 1998, Zechmann 2020, Ito and Ohkama-Ohtsu 2023). Oxidised glutathione can be reduced back to glutathione by glutathione reductase in an NADPH-dependent reaction within the apoplastic space, after which it is transported across the plasmalemma from the cytoplasm (Foyer and Noctor 2011). Among detoxification enzymes, glutathione S-transferases [EC:2.5.1.18] play a major role in cellular protection against oxidative stress (Edwards et al. 2000, Dong et al. 2016). Ten glutathione S-transferase gene family members were found to be significantly differentially expressed in roots and leaves of melon seedlings under cinnamic acid stress (Wang et al. 2020). In addition, class I plant defensins, containing both an N-terminal signal sequence and a mature defensin domain, are cytoplasmically synthesised, constitutively expressed, and partially allocated into the apoplastic space, demonstrating broad-spectrum protective effects against microbial pathogens (Parisi et al. 2019, Mammari et al. 2021). Along with other peptides and proteins, apoplastic defensins function as mediators of cell-to-cell communication and long-distance signaling (Farvardin et al. 2020). In our study, ten and eleven DEPs were identified as glutathione S-transferases in SC205 and XX048, respectively. Among these, three were upregulated in SC205 and two in XX048 (Table 6). Fructose-1-6-bisphosphatase (N-terminal domain) and glutathione S-transferase were found as common domains of DEPs (Figure 7). Thus, we inferred that defense reactions of cassava to autotoxic stress were likely initiated by glutathione in the apoplastic space.

In most cases, apoplastic and symplastic processes work together when stress occurs (Du et al. 2024). If the apoplastic strategies fail to overcome the stress, the ROS generated in intracellular com-

partments need to be detoxified by antioxidants or enzymes to prevent potential damage caused by ROS accumulation. As the most abundant intracellular antioxidants, ascorbate and glutathione can effectively scavenge ROS. Enzymatic reactions catalysed by SOD, catalase, glutathione peroxidase, peroxiredoxins and thioredoxins can directly scavenge the subcellular ROS at the sites of generation (Dumanović et al. 2021). When defense takes place at the organ and whole-plant levels, defense mechanisms such as stomatal regulation, hypersensitive response, and systemic responses enable plants to mitigate and adapt to various stresses. The hypersensitive response acts as a localised resistance reaction, triggering localised cell death at infection sites to prevent the further spread of biotic stress (Balint-Kurti 2019). Beyond local responses, long-distance signaling and systemic responses induced by abiotic and biotic stresses play fundamental roles in plant resistance, acclimation, and survival (Zandalinas et al. 2020). By triggering defense mechanisms in uninfected tissues, systemic responses provide rapid and long-lasting protection that contributes to both short-term acclimation and long-term survival in challenging environments, which is termed systemic acquired resistance (SAR) (Durrant and Dong 2004). These signaling molecules encompass: (1) reactive species (ROS, NO, S-nitrosoglutathione); (2) biomolecules (proteins, peptides, RNAs, metabolites); (3) second messengers; (4) phytohormones; (5) sulfate compounds; and (6) physical signals (hydraulic and electrical waves) (Du et al. 2024). The phytohormone SA enhances plant tolerance to abiotic stress by regulating glutathione metabolism (Guo et al. 2016), and serves as a crucial signaling molecule for SAR. Associated with the accumulation of pathogenesis-related (PR) proteins, SAR is characterised by the increased expression of pathogenesis-related genes in both local and whole-plant tissues (Durrant and Dong 2004). In the current study, DEPs related to SA metabolic processes, response to SA, cellular response to SA stimulus, and SA mediated signaling pathways process were among the top 20 and top 30 enriched categories in Q4 for XX048 and SC205, respectively (Figures 5 and 6). Both the N-terminal and C-terminal domains of glutathione S-transferase were found in the DEPs of cassava (Figure 7), indicating that SA is the primary signaling hormone in cassava in response to autotoxicity

stress by regulating glutathione metabolism. Based on the results of the GO functional classification (Table 6 and 7, Figures 4–6) and KEGG pathway analysis (Figure 8), we inferred that cassava employs defense strategies against autotoxic stress at multiple levels: extracellular (apoplast), intracellular (symplast), organ, and whole plant. The autotoxic substances first stimulate the roots, which promotes a hypersensitive response. An oxidative burst occurs (Figure 5 and 6) and glutathione scavenges ROS in apoplastic and symplastic processes. When ROS is overloaded, the involved detoxification processes require additional transport systems. Long-distance signaling and systemic responses are induced and the biosynthesis of secondary metabolites is activated. Endogenous SA and H₂O₂ trigger the transcriptional upregulation of a number of genes, including those encoding PR proteins such as glucanase, glutathione S-transferases, PR protein 5-like isoforms X1 and X2, PR-1 and PR-4-like proteins, and serine/arginine-rich splicing factor RSZ21-like isoform X3 in the cassava plantlets (Table 6 and 7; Figure 8, 5 and 6), thus activating the SAR of cassava that confers long-lasting protection against autotoxicity stress (Chen et al. 1993, Vernooij et al. 1994, Bolwell 1999, Zhu et al. 2014). However, the activation of SAR suppressed the expression of ABA-related genes, which has been demonstrated to antagonise the onset of SA-dependent defenses and SAR (Mohr and Cahill 2007, Yasuda et al. 2008). The receptor-like protein kinase HAIKU2 (XP_021600950.1) was only found in XX048 (Table 4), which has been proven to be a positive ABA-signaling component in guard cell movement (Liu et al. 2023). The biosynthesis of metabolites and enzymes for SAR is a costly and energy-consuming process. As a high-yield cultivar, XX048 appears to minimise defense costs by attenuating the activation of SA-dependent defenses and SAR under prolonged autotoxicity stress.

Furthermore, PPI analysis revealed that SC205 and XX048 shared conserved core defense mechanisms involving energy metabolism and basal translation, yet their specific molecular defense strategies were clearly distinct (Figure 9). SC205 established a global homeostasis-maintenance network centered on ribosomal proteins (RPL9, RPL15, RPL27, RPS1), energy metabolism (ATPD), and photosystem I (PSAF). The clustering of ribosomal nodes indicates highly active protein translation machinery, consistent with the significant increase in soluble proteins, as SC205 rapidly synthesised stress-resistant proteins

(e.g., detoxifying GST and LOX) to mitigate toxicity (Table 5). Meanwhile, the PSAF and CSP41B hubs ensured photosystem I stability and precise synthesis of photosynthetic proteins, forming a robust network that maintained chlorophyll content under severe stress (Table 3). The central hub ATPD, together with carbon fixation and glycolysis enzymes (RCA, PGK1), suggests that the active defense and protein synthesis in SC205 were powered by a highly robust energy metabolism, corroborated by its significantly higher root vitality (Table 3). Although XX048 is recognised as a high-yield cultivar, its superior productivity under optimal conditions does not confer enhanced photosynthetic resilience under autotoxic stress. Its network was dominated by chloroplast/photosynthesis-related proteins (PSAG, LHCA3, psaD) and the chloroplast RNA regulatory factor CSP41B. This indicates a highly energy-consuming compensatory mechanism: XX048 prioritised repairing damaged photosynthetic structures (as evidenced by decreased chlorophyll in Table 3) by rapidly replenishing depleted proteins *via* CSP41B-mediated RNA processing. Additionally, the ATPD nodes in XX048 were more concentrated in these repair and carbon metabolic pathways, forcing the plant to divert substantial energy toward rescuing damaged photosynthetic machinery. In summary, while SC205 maintained unperturbed protein synthesis and balanced photosynthesis and growth under the stress, XX048 sacrificed growth to preserve photosynthesis. This high-energy-consuming compensatory network, resulting in severe energy depletion and growth arrest (reduced root biomass), profoundly explains the molecular basis for XX048's weaker stress resistance compared with SC205.

Limitations and future perspectives. Despite the insights provided by this study, several limitations should be acknowledged. While the tissue culture simulation system does not fully replicate field conditions, particularly regarding root architecture differences between tissue-cultured plantlets and field-grown cassava, it offers clear advantages for isolating specific variables and minimising confounding factors, and its validity is supported by prior field observations (Luo et al. 2026). First, this study employed a simulated autotoxicity experiment using exogenous application of autotoxins rather than measuring endogenous autotoxin concentrations in planta; although this approach allows controlled manipulation of stress levels, it may not fully replicate

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the complex dynamics of autotoxin accumulation, transport, and metabolism in natural soil systems, and the dose-response relationships observed here should therefore be interpreted with caution. Second, because individual tissue-cultured plantlets are small and variable, we pooled 25 plantlets per sample and processed two technical replicates independently. These replicates showed high consistency in protein identification and quantification; however, technical replicates cannot capture biological variability. Therefore, the proteomic results should be regarded as exploratory and indicative rather than definitive, and the conclusions have been worded accordingly. The transcript-level validation was performed only in XX048, and future work should include SC205. Third, certain data, such as the actual uptake rate of autotoxins and the long-term field performance of the tested genotypes, were not available, which limits our ability to fully elucidate the proposed "avoidance" mechanism that remains a hypothesis rather than a definitive conclusion. Addressing these gaps through direct quantification of endogenous autotoxins (e.g., *via* HPLC-MS/MS or isotope labeling), adequate biological replicates, and integrated multi-omics and field experiments will be a key direction for future research.

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