Effects of medium-induced osmotic and nutrient stress on cyanogenic glycosides in Flax (Linum usitatissimum)

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Research Article

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Abstract

Background

Cyanogenic glycosides (CGs) are multifunctional plant metabolites involved in defense, nitrogen turnover, and stress responses. Study examined effects of medium-induced osmotic and nutrient stress on cyanogenic glycosides in flax (Linum usitatissimum L.)cv. Linola. Plants were cultivated under mannitol-induced osmotic stress or on media with modified sulfate or nitrate supply, and sampled throughout early development. Transcript levels of genes involved in CGs biosynthesis, degradation, and cyanide detoxification were analyzed by real-time RT-PCR, together with cyanogenic potential, free cyanide content, β-cyanoalanine synthase (β-CAS) activity.

Results

Drought caused the strongest response, leading to late-stage induction of CG-related genes, increased β-CAS activity, higher cyanogenic potential, and elevated free cyanide under prolonged stress, indicating intensified CGs turnover and a link between cyanide metabolism and drought responses. Sulfur availability exerted a stronger influence on CGs homeostasis than nitrogen, primarily affecting detoxification capacity and overall pathway balance, whereas nitrogen effects were more subtle and reflected modulation of temporal dynamics rather than sustained changes in CGs accumulation. In both cases, transcriptional responses were not consistently mirrored at the enzymatic or metabolite levels, highlighting a partial decoupling across regulatory layers. In particular, β-CAS activity did not closely follow transcript abundance, supporting the concept of substrate-driven regulation of cyanide detoxification, whereby enzyme activity is primarily governed by substrate availability rather than transcriptional control.

Conclusions

These findings indicate that CGs metabolism in flax is developmentally regulated and highly responsive to environmental and nutritional cues, with its functional output shaped by integrated transcriptional, post-transcriptional, and metabolic buffering mechanisms.

Introduction

Flax (Linum usitatissimum L.) is a highly versatile and historically significant crop with diverse uses that utilize its unique features, including being used as a fiber crop, source of industrial oil, and nutraceutical. Despite well-documented health benefits, flaxseed also contains cyanogenic glycosides (CGs), compounds with the potential to release toxic hydrogen cyanide (HCN) under certain conditions, which necessitates consideration of safety aspects in its consumption. Though such compounds are undesirable from the utilitarian point of view, they can be vital for the plant itself. CGs are supposed to

play significant physiological roles in the plant, including being involved in primary metabolism, nitrogen homeostasis, and mediating biotic as well as abiotic stress responses [1]. CGs are typically made up of three fundamental structural components: an aglycone moiety, a sugar molecule, and a nitrile functional group [2]. In flax the CGs linamarin and lotaustralin occur predominantly, as monoglucosides derived from valine and isoleucine based cyanohydrin precursors, respectively, whereas the corresponding diglucosides linustatin and neolinustatin represent their glycosylated storage forms, particularly abundant in seeds. CGs metabolism comprises biosynthesis, cyanogenesis, and cyanide detoxification, forming an integrated system that links secondary metabolism with nitrogen and sulfur homeostasis [3].Biosynthesis of CGs is initiated by the cytochrome P450 enzyme CYP79D, which catalyzes N-hydroxylation, decarboxylation, and deamination of amino acid precursors to produce the corresponding oximes (2-methylpropanal oxime and 2-methylbutanal oxime). These oximes are subsequently converted into unstable cyanohydrins through isomerization, dehydration, and C-hydroxylation reactions catalyzed by CYP71E, with both P450 enzymes operating in association with NADPH-dependent oxidoreductases [3, 4]. The cyanohydrin intermediates are stabilized through glycosylation mediated primarily by UDP-glucosyltransferase UGT85, followed by an additional glycosylation step performed by a more broadly acting UDP-glucosyltransferase [4], yielding the flax-specific diglucosides linustatin and neolinustatin [5]. Cyanogenesis is the process by which certain plants like flax synthesize CGs that can then be enzymatically degraded to HCN through an unstable cyanohydrin intermediate [5]. The enzymatic process is usually triggered by tissue damage, allowing the glucosides to come in contact with specific degradative enzymes [6]. However, controlled CGs turnover has also been reported in intact flax tissues, particularly during seed germination, indicating a developmental role [7]. In cyanogenic plants, including flax, α-hydroxynitrile lyase accelerates cyanohydrin decomposition under acidic conditions, whereas spontaneous dissociation into HCN and corresponding ketones occurs at pH values above 6 [8] and is promoted by elevated temperatures and low moisture availability [5]. Given the high toxicity of cyanide, flax relies on efficient detoxification mechanisms to maintain cellular homeostasis The β-CAS pathway functions as a vital connection between sulfur and nitrogen metabolic pathways while controlling cyanide levels in plant cells [9]. Cyanide is detoxified by β-CAS, a pyridoxal-5'-phosphate-dependent enzyme predominantly localized in mitochondria, which catalyzes the reaction of cyanide with cysteine to form β-cyanoalanine [10, 11]. Subsequent conversion of β-cyanoalanine into asparagine or aspartate by nitrilase activities facilitates nitrogen recovery and establishes a functional link between CGs metabolism and sulfur and nitrogen metabolic pathways in flax [9, 11].

The genetic adaptation of plant populations to specific ecological conditions together with individual plant physiological responses to abiotic stressors could influence the variability in CGs content [12]. CGs levels increase when plants face different abiotic stress factors including drought [13, 14], extreme temperatures (both high and low)[13], salinity [15], nutrient deficiency, and inadequate light availability [16]. Several mechanisms have been proposed to explain the increased CGs levels observed under abiotic stress: reduced biomass production with unchanged or sustained CGs biosynthesis efficiency, leading to a higher concentration per unit of tissue; delayed plant development, resulting in prolonged phases associated with inherently higher CGs levels; and active transcriptional upregulation of CGs

biosynthesis pathways in response to environmental stimuli [6]. Available evidence indicates that CGs contribute to a broad spectrum of abiotic stress response processes, although the underlying regulatory mechanisms, effective physiological thresholds and species specific response strategies remain largely unresolved. The need to clarify these mechanisms is particularly pronounced in flax, for which current insights into CGs mediated metabolic regulation under stress are fragmentary. In this context, the present study examines CGs dynamics in flax exposed to drought and controlled alterations in nutrient availability.

Materials and Methods

Plant growth conditions and stress treatments

Flax (*Linum usitatissimum L.*) cv. Linola was used in all experiments. Flaxseed (cv. Linola) was obtained from the Flax and Hemp Collection of the Institute of Natural Fibres, Poland. Seeds were pre-soaked in sterile water for 1 h, sterilized in 70% ethanol for 5 min followed by treatment with 50% Plant Preservation Mixture (PPM™) for 30 min, rinsed three times in sterile distilled water, and transferred aseptically to the respective media. For abiotic stresses analyses cultivation was performed on Murashige and Skoog (MS) based media supplemented with 2% (w/v) sucrose and 0,7% (w/v) agar, adjusted to pH 5,8 prior to sterilization. The composition of media variants used in this study is provided in Supplementary tables 1–3. Half-strength MS (½MS) medium served as the baseline control for nutrient-modified conditions. Stress treatments included (a) sulfur- or nitrogen-modulated media achieved by controlled adjustments of sulfate and nitrate salts, and (b) osmotic stress induced with 2% (w/v) mannitol. A detailed overview of the experimental workflows for each treatment is presented in Fig. 2. For each measured condition, tissues were collected from at least three independent culture vessels, with ≥ 6 plants per vessel. Samples were harvested at a consistent time of day, frozen in liquid nitrogen, and stored at − 80°C until analysis.

RNA extraction and quantitative PCR

Total RNA was isolated using the RNA Extracol (EURx) according to the manufacturer's instructions. Analysis of the obtained RNA, cDNA synthesis, and evaluation of the number of transcripts using the RT-QPCR technique were performed as described earlier [3]. All reactions were conducted in technical triplicates to ensure reproducibility and accuracy. Gene expression was analyzed using the relative quantification method, with actin serving as the reference gene for normalization. Primer sequences used in the study are listed in Supplementary table 4.

Free cyanide and cyanogenic potential assay

Plant tissue samples stored for no longer than one month at -80°C or fresh were ground to a fine powder in liquid nitrogen. 25 mg or 50 mg of frozen ground tissue was suspended in 2 ml of extraction buffer

containing 50 mM Tris-HCl (pH 8,0), 0,1% v/v Triton X-100, and 1 mM PMSF. In parallel, an equivalent mass of tissue was suspended in 5 M urea solution to inhibit endogenous β-glucosidase activity (control). Samples in extraction buffer were incubated at 26°C for 2 h with gentle agitation. Urea samples were incubated for 10 min at room temperature. After incubation, all samples were centrifuged at 14 000×g for 5 min at room temperature and supernatants were collected. For cyanide quantification, 96-well microplates were prepared by dispensing 50 µl of 1% chloramine-T into each well, followed by 50 µl of plant extract (diluted 2× for free cyanide and 10× for cyanogenic potential). Subsequently, 100 µl of freshly prepared γ-picoline barbituric acid reagent was added. The chromogenic reagent was prepared by dissolving 3 g barbituric acid, 3 ml of concentrated HCl, 15 ml 4-methylpyridine, and adjusting to the final volume 50 ml with distilled water. Wells were sealed with Parafilm immediately after reagent addition to prevent evaporation and contamination of neighboring sample. Absorbance was measured at 605 nm. Cyanide concentrations were calculated from KCN standard curves (1,5–460 pmol CN^-/µl). Free cyanide and cyanogenic potential were expressed as µmol HCN·g^{-1} FW or DW.

β-CAS enzymatic assay

Plant tissue was ground in liquid nitrogen. Powdered material (25 or 50 mg) was extracted in 0,8 ml of protein buffer (100 mM Tris, pH 9,5; 2 mM EDTA; 10 mM L-cysteine), vortexed, and incubated on ice for 15 min. Samples were centrifuged at 14,000×g for 5 min at 4°C, and supernatants were collected. Protein concentrations were determined by the Bradford method using BSA as the standard. For β-CAS activity, 50 µl of protein extract was mixed with 83,25 µl of freshly prepared substrate solution (25 mM KCN and 25 mM L-cysteine) and incubated at 28°C for 2 h. Reactions were stopped with 54 µl of 3 mM $FeCl_3$ in 4,2 M HCl, followed immediately by 54 µl of 15 mM DMPDA in 4,2 M HCl. After gentle mixing, samples were incubated for 20 min at room temperature in the dark. A 100-µl aliquot of each reaction was transferred to a 96-well plate, and absorbance was measured at 745 nm. H_2S quantification was based on Na_2S calibration standards (0–100 µM), and the amount of released H_2S was used to calculate β-CAS enzymatic activity.

Amino acid profiling (GC-FID)

Lyophilized plant tissue (25 or 50 mg) was incubated with 2 ml of 6 M HCl containing 0,01% (v/v) phenol. Norvaline (200 µg) was added as an internal standard. Vials were sealed and hydrolyzed at 110°C for 24 h. After cooling, samples were evaporated to dryness. Residues were derivatized with 200 µl BSTFA containing 1% TMCS at 100°C for 30 min. Reaction mixtures were cooled and transferred to autosampler vials for GC-FID analysis. GC-FID analyses were performed on an HP-5 (5% phenyl-methylpolysiloxane) column (30 m × 0,32 mm × 0,25 µm). Amino acids were identified by retention times of derivatized commercial standards and quantified using external calibration curves normalized to norvaline.

Statistical analysis

Statistical analyses and data visualization were performed using GraphPad Prism 9. Relative transcript changes ≥ 2-fold or reductions ≥ 50% were interpreted as biologically meaningful. Data are presented as mean ± SD with ≥ 3 biological replicates per condition. Statistical significance was assessed using two-way analysis of variance (two-way ANOVA), followed by Tukey's post hoc multiple comparison test. Differences were considered statistically significant at $P < 0.05$.

Results

Cyanogenic glycosides metabolism in drought stress

Figure 3A illustrates temporal changes in the expression of genes involved in CGs metabolism in flax subjected to severe drought stress. Transcript levels, expressed as $\log_2$ fold changes relative to developmentally matched control plants, indicate a coordinated but time-dependent transcriptional response. In flax, CGs metabolism naturally increases from germination until approximately day 7 after sowing, when maximal transcript abundance is typically observed. During early drought treatment, particularly at day 5, most CGs-related genes exhibited reduced transcript levels relative to the control, suggesting attenuation or temporal displacement of the developmental expression peak. At later time points, a contrasting pattern emerged. Genes involved in CGs biosynthesis showed pronounced induction, with CYP79 significantly upregulated at days 30 and 40 (2,93- and 4,89-fold of the control, respectively), while UGT85 exhibited increased transcript abundance from day 10 onward, reaching 3,40-fold at day 30 and 2,25-fold at day 40. Among genes associated with CGs degradation, HNL displayed a clear drought-responsive induction, with transcript levels significantly elevated at days 30 (3,94-fold) and 40 (3,00-fold). Genes involved in cyanide detoxification also contributed to this response. CAS showed progressive upregulation following initial repression at day 5, reaching significant induction at day 40 (2,61-fold), while OASTL, after marked downregulation at days 5 (0,47-fold) and 20 (0,31-fold), exhibited renewed induction at later stages, with a significant increase at day 30 (2,26-fold). This coordinated late-stage activation suggests enhanced CGs turnover and intensified cyanide detoxification during prolonged drought stress. In contrast, β-GLU linamarase and CAN remained transcriptionally stable throughout the experiment, indicating selective regulation within CGs degradation and detoxification modules. Nitrate reductase (NR) showed persistent repression under drought stress, with significant downregulation at days 5 (0,40-fold), 30 (0,49-fold), and 40 (0,33-fold), reflecting drought-induced constraints on nitrogen assimilation. Figure 3D-E presents temporal dynamics of cyanogenic potential and free cyanide concentration in Linola flax under severe drought stress. Cyanogenic potential increased from day 2 and reached a maximum at day 10 in both control and drought-treated plants, but values were higher under drought (49,1 ± 3,7 vs. 26,4 ± 4,3 μmol $CN^{-}\cdot g^{-1}$ FW). At day 2, cyanogenic potential reached 5,51 ± 0,49 μmol $CN^{-}\cdot g^{-1}$ FW under drought compared with 3,01 ± 0,66 μmol $CN^{-}\cdot g^{-1}$ FW in controls. After a decline at day 20, pronounced accumulation was observed at later stages, reaching 26,2 ± 3,3 vs. 10,8 ± 0,8 μmol $CN^{-}\cdot g^{-1}$ FW at day 30 (2,43-fold) and 33,7 ± 6,5 vs. 6,58 ± 0,31 μmol $CN^{-}\cdot g^{-1}$ FW at day 40 (5,12-fold). Free cyanide concentrations remained comparable to controls during early drought stages but increased markedly at day 40, reaching 8,26 ± 0,35 μmol $CN^{-}\cdot g^{-1}$ FW compared

with 1,99 ± 0,29 µmol $CN^{-}\cdot g^{-1}$ FW in control plants, suggesting either enhanced stress-related signaling or insufficient detoxification capacity under prolonged drought. Figure 3B-C shows β-CAS activity in shoots and roots under severe drought stress. In shoots, β-CAS activity was initially lower under drought at day 2, followed by a significant increase at day 5 in both drought-treated and control plants with a stronger response under stress. From day 20 onward, β-CAS activity remained consistently higher in drought-stressed plants, reaching 1,66 to 1,83-fold of the control at days 20 to 40, respectively. In roots, β-CAS activity was significantly elevated under drought at all analyzed time points, indicating sustained activation of cyanide detoxification under prolonged drought stress.

Cyanogenic glycoside metabolism under reduced nutrient availability

Under reduced nutrient strength (½ MS), genes associated with CGs metabolism exhibited time-dependent transcriptional patterns (Fig. 4A). A general tendency toward elevated transcript levels at day 2 was evident across the majority of analyzed genes, with the most pronounced changes observed for cyanide detoxification (CAS, CAN, OASTL). Between days 5 and 20, differences were characterized by a trend toward reduced transcript levels. At day 5, significant decreases were observed for *CYP79* (2,3-fold), *UGT85* (4,9-fold), *β-GLU vicianinase* (2,3-fold), *CAS* (2,4-fold), and *OASTL* (2,1-fold); at day 10, a significant reduction was detected for *CYP79* (2,2-fold); at day 20, transcript levels of *HNL* and *OASTL* were reduced by approximately 2,6-fold and 3,1-fold, respectively. At later stages of cultivation, transcriptional differences between ½ MS and MS were generally limited, indicating that reduced nutrient strength primarily influenced the timing of transcriptional dynamics within the CGs metabolic pathway. After day 20, when the pathway is relatively stable, no consistent differences in transcript levels were observed between MS and ½ MS, suggesting that reduced medium strength does not substantially affect CGs metabolism at this stage; this is further supported by the lack of significant changes in β-CAS activity and cyanogenic potential(Fig. 4B). Despite trace levels of CGs in flax roots, β-CAS activity in this tissue was comparable to that in green tissues, suggesting an active cyanide detoxification capacity independent of glycoside content. Cyanogenic potential and free cyanide levels expressed as µmol $HCN\cdot g^{-1}$ FW (Fig. 4D-E). Cyanogenic potential increased markedly from days 2 to 10 post-germination, rising from 3,0 and 2,4 µmol $HCN\cdot g^{-1}$ FW (MS and ½ MS, respectively) to peak values of 26,36 and 35,15 µmol $HCN\cdot g^{-1}$ FW on day 10, followed by a gradual decline toward day 40. No statistically significant differences in cyanogenic potential were detected between media at any time point. Similarly, free cyanide content did not differ significantly between media and exhibited a distinct temporal profile, remaining low at early stages and increasing at later time points

Because amino acids serve as substrates for CGs biosynthesis and participate in cyanide detoxification, amino acid profiles were analyzed. Results are expressed as fold changes in amino acid content in ½ MS relative to MS for each sampling day (Fig. 5). No statistically significant differences were detected for most amino acids at days 2 and 5 after sowing, except for isoleucine and proline, which were reduced at day 5 in plants grown on ½ MS. At later time points, both amino acids showed significantly higher levels

under ½ MS compared with MS. Lysine, tyrosine, and methionine did not differ significantly between treatments at any time point. In contrast, alanine, valine, leucine, serine, threonine, ornithine, aspartate, and asparagine accumulated to significantly higher levels in plants grown on ½ MS at days 10–30. A significant increase in glycine (1,29-fold) and hydroxyproline (1,21-fold) was detected exclusively at day 10 under ½ MS. Glutamate and glutamine also showed higher levels at day 10 (1,39- fold), followed by a decrease below MS levels by day 20 (0,83-fold).

Effect of sulfur availability in medium on cyanogenic glycosides metabolism

Sulfur availability markedly affected the temporal expression of genes involved in CGs metabolism (Fig. 6A). Under sulfur-deficient conditions (S−), significant transcriptional induction was observed mainly at intermediate (day 10, 20) time points. At day 20, strong upregulation was detected for several detoxification-related genes, including HNL, CAS, CAN, OASTL, and NR (approximately 2,6–2,9-fold), accompanied by increased expression of CGs degradation–associated genes, including two β-glucosidases and HNL. At earlier stages, significant induction was limited to selected genes, such as CAN, HNL, and β-GLU. At later stages, transcript levels generally returned toward control values, with the exception of CYP79. Sulfur supplementation (S+) (Fig. 6B) resulted in a distinct transcriptional pattern characterized by pronounced repression at early time points, with multiple genes showing strong downregulation, including CYP79, β-GLU vicianinase, and HNL. The strongest responses under S+ conditions were observed at later stages, when marked upregulation of HNL (day 40, 5,62-fold), CAN (day 40, 2,38-fold), β-GLU vicianinase (day 20, 3,25-fold), and CYP79 (day 40, 4,22-fold) occurred. Overall, sulfur deficiency primarily induced mid-stage activation of detoxification pathways, whereas sulfur excess led to delayed but pronounced transcriptional activation of both detoxification and biosynthetic components of the CGs pathway. Figure 6C-D summarizes β-CAS activity in Linola flax cultivated under sulfur-depleted, sulfur-supplemented, and control (½ MS) conditions. Across all treatments, β-CAS activity followed a developmental pattern characteristic for flax, with a pronounced peak at day 5, coinciding with the highest cyanogenic potential and maximal transcriptional activity of CGs related genes. Activity under sulfur supplementation was significantly higher compared with both the control and sulfur-depleted treatments. At subsequent stages (days 10–30), no statistically significant differences in β-CAS activity were observed between treatments. At day 40, β-CAS activity in aerial tissues amounted to 8,35 in control plants, 5,64 under sulfur depletion, and 8,54 under sulfur supplementation, with a significant difference detected only between sulfur-depleted and sulfur-supplemented plants. In roots, β-CAS activity did not differ significantly between treatments at days 20 and 30. However, at day 40, significant differences were observed, with activities of 7,13 in control plants, 5,82 under sulfur depletion, and 8,45 under sulfur supplementation. In sulfur-supplemented plants, cyanogenic potential (Fig. 6E) was significantly reduced relative to the control on day 2 and day 10, while no differences were observed on day 5. By day 20, values in sulfur-supplemented plants exceeded those of the control (23,29 vs 22,86), reaching statistical significance on day 30 (15,57 vs 10,81) and remaining elevated on day 40. Plants cultivated under sulfur-deficient conditions exhibited

lower cyanogenic potential than the control on day 10 and day 20. In both sulfur-deficient and sulfur-supplemented treatments, the temporal profile of cyanogenic potential was characterized by a lower, broadened developmental peak compared to the sharp maximum observed in control plants. Free cyanide content (Fig. 6F) differed significantly between treatments only on day 30, when plants grown in sulfur-supplemented medium showed higher concentrations than the control .

Nitrogen depletion and supplementation

Under nitrogen-deficient conditions (N–), significant transcriptional repression dominated the early phase of cultivation, particularly on day 5, when strong downregulation was observed for CYP79 (0,18-fold), UGT85 (0,40-fold), β-GLU (0,33-fold), β-GLU vicianinase (0,18-fold), HNL (0,16-fold), CAS (0,37-fold), CAN (0,30-fold), and OASTL (0,33-fold) (Fig. 7A). At later stages, only slight transcriptional induction became evident, with significant upregulation of HNL (2,01-fold), CYP79 (2,12-fold) observed between days 20 and 40, indicating delayed activation of CGs related pathways under nitrogen limitation. In contrast, nitrogen supplementation (N+) resulted in a different transcriptional profile (Fig. 7B). Early time points showed repression, restricted mainly to UGT85 (0,54-fold at day 2) and NR (0,29-fold at day 2). Transcriptional induction occurred at later stages, with UGT85 strongly upregulated at day 10 (2,31-fold), CYP79 at days 30 and 40 (2,26- and 2,08-fold, respectively). Overall, nitrogen deficiency was associated with strong early repression followed by delayed activation of genes involved in CGs metabolism, whereas nitrogen supplementation promoted later-stage induction, particularly affecting biosynthetic components of the pathway. The enzymatic activity of β-CAS ,presented in Fig. 7C-D, on day 5, plants grown under nitrogen supplementation exhibited significantly higher β-CAS activity compared to the control. In contrast, on day 10, plants under nitrogen deprivation showed significantly lower β-CAS activity than the control. In green tissues, statistically significant differences ($p < 0,05$) in β-CAS activity between plants exposed to nitrogen deprivation and nitrogen supplementation were detected on days 2, 10, and 40. For roots, a significant difference was observed only on day 40. Specifically, on day 40, β-CAS activity in roots of N- plants was significantly higher ($p < 0,01$) than in control plants (8,89 vs. 7,13). For free cyanide content (Fig. 7F), no statistically significant differences were observed between treatments at any of the analyzed time points. The cyanogenic potential (Fig. 7E) increased markedly from day 2 to day 5 in plants across all three treatments. On day 10, the cyanogenic potential was significantly lower in nitrate-deficient and nitrate-supplemented plants compared with control. By day 20, the cyanogenic potential in control plants decreased to 22,86 μmol $CN^{-}g^{-1}FW$, whereas in N+ plants it increased to 29,76 μmol $CN^{-}g^{-1}$ FW, representing a statistically significant rise relative to day 10. This pattern may indicate a shift in the early developmental peak of CGs metabolism intensity in N+ plants. At later stages (days 30 and 40), no significant differences in cyanogenic potential were observed between treatment.

Discussion

CGs are increasingly regarded as multifunctional metabolites, linked not only to defense but also to nitrogen turnover, sulfur metabolism, and broad stress responses. A thorough evaluation of whether CGs in flax represent a useful metabolic feature or an undesirable trait requires a deeper understanding of

their pathways and functions. Drought is one of the most severe abiotic stresses limiting agricultural productivity worldwide. Flax plants were exposed to stress treatments, among which drought stress was induced by mannitol (used as an osmotic agent to simulate drought stress in vitro). Across cyanogenic species, water limitation modulates CGs metabolism at both transcriptional and metabolite levels, with responses differing among species, genotypes, and developmental stages. In sorghum, drought stress promotes accumulation of the phenylalanine-derived CG dhurrin [17]. In cassava, drought is associated with elevated leaf linamarin levels, which may contribute to nitrogen reallocation when uptake and assimilation are limited, supporting survival under stress [18]. In white clover, moderate and recurrent drought stress confers higher fitness to cyanogenic plants compared with acyanogenic forms, as evidenced by increased number and size of inflorescences [19]. Transcriptional changes observed under severe drought conditions (Fig. 3A) similarly to moderate stress (Supplementary Fig. 1), remain transient across the time course. The characteristic developmental peak in transcript abundance of CGs pathway genes in early days after germination (for more details please read [3]) is attenuated for most analytes under severe drought. At later time points, transcript levels shift toward renewed increases, particularly for genes involved in CGs biosynthesis (CYP79, UGT85), as well as for HNL associated with CGs degradation and CAS and OASTL contributing to key steps of cyanide detoxification. Together, this coordinated upregulation may indicate an overall intensification of metabolic flux through the cyanogenic pathway. In Arabidopsis, water deficit induces cyanide release from CGs turnover and activates the β-cyanoalanine detoxification pathway, with the graded induction of this route under increasing drought intensity supporting a role for cyanide metabolism in drought signaling [10]. In agreement with this framework, under drought we observed higher β-CAS activity in flax (Fig. 3B), indicative of sustained detoxification demand that can arise both from accelerated CGs turnover and from endogenous cyanide co-produced during ethylene biosynthesis. The ethylene pathway generates HCN as an obligate co-product of the ACC oxidase reaction, and plant tissues possess substantial capacity to detoxify this cyanide through β-cyanoalanine formation, linking hormonal stress responses with cyanide metabolism under water limitation [20]. Under the severe drought regime, the appearance of elevated free cyanide at day 40 coincide with the progression of stress (Fig. 3E). Drought promotes senescence then ethylene biosynthesis rises and the ACC oxidase reaction generates cyanide. In parallel, senescing tissues may upregulate cyanide detoxification capacity, including β-CAS, indicating active handling of senescence associated cyanide [21]. These observations are consistent with a scenario in which late increases in free cyanide under severe drought arise primarily as a byproduct of intensified ethylene metabolism and sustained CGs turnover during stress induced senescence. Although our data do not demonstrate a signaling role for cyanide, the temporal concurrence with ethylene driven senescence raises the possibility that low, transient cyanide pulses may operate in parallel with ethylene as an auxiliary cue. HCN, long regarded solely as a toxic by-product, also functions as a signaling molecule. It has been shown to induce reactive oxygen species, activate AOX and ACC synthase, thereby integrating into ethylene signaling and adaptive mechanisms [22, 23]. During advanced stages of drought stress, signs of stress responses were apparent at the amino acid level (Supplementary Fig. 2), as on day 30 a pronounced increase in hydroxyproline content relative to control plants was detected. Hydroxyproline is a diagnostic component of hydroxyproline-rich glycoproteins

such as extensins, which undergo peroxidase-mediated crosslinking to reinforce and remodel the primary cell wall. This mechanism has been documented under drought and salt stress, where enhanced extensin deposition contributes to wall stabilization and adaptive plasticity [24]. In parallel, a decline in lysine content was observed, consistent with its catabolism to glutamate and further conversion to proline, an established osmoprotectant in drought adaptation. Some increases in proline and glutamate were also noted, and the reduced lysine levels may therefore be linked to enhanced flux through this pathway [25]. Taken together, findings point to several functions for CGs during drought. First, they can underpin rapid defense readiness while the plant experiences resource limitation. Second, they can act as dynamic reservoirs of reduced nitrogen and carbon that are drawn upon as stress intensifies, helping to buffer primary metabolism. Third, their controlled turnover yields cyanide pulses that participate in signaling networks that coordinate respiratory and hormonal responses to stress. The balance among these roles appears to shift with stress intensity and nutrient status.

In experiments with changed nitrogen and sulfur supply, to minimize confounding ionic and osmotic effects during nitrate or sulfate supplementation plants were cultured on ½ MS medium, which served as a control condition. A comparative analysis of CGs metabolism was conducted between plants grown on full-strength MS and half-strength MS medium (Fig. 4). The results showed no significant differences in cyanogenic potential, free cyanide content, or β-CAS activity, and the characteristic developmental patterns of CGs metabolism were preserved. In in vitro flax grown on both MS and ½ MS, root β-CAS activity remained substantial (Fig. 4C) despite the likely absence of de novo CGs biosynthesis in flax roots. Two factors may account for this observation. First, CGs can be delivered to roots from aerial tissues, so β-CAS may process cyanide released from turnover of imported cyanogens. Such basipetal transport is well documented in cassava and other cyanogenic species [26, 27]. Second, independent of cyanogen turnover, roots require continuous detoxification of cyanide that is produced stoichiometrically with ethylene during the ACC oxidase step; β-CAS constitutes the primary route for removing this endogenous cyanide by converting it with cysteine to β-cyanoalanine [28]. In contrast, amino acid profiling revealed higher levels of several amino acids in plants grown on ½ MS compared with full-strength MS. This trend was particularly evident at later time points of the experiment and concerned amino acids such as serine, threonine, proline, isoleucine, aspartate and asparagine, leucine, valine, and alanine. Since no differences were observed in CGs metabolism, it can be inferred that there was no substantial release of nitrogen from this pool, and the observed increase in amino acids content is more likely a result of shifts in primary and protein metabolism rather than a redistribution of nitrogen from CGs. In contrast, amino acid profiling revealed higher levels of several amino acids in plants grown on ½ MS compared with full-strength MS, particularly at later time points of the experiment. Given the absence of detectable differences in CGs metabolism, this increase in total amino acid content is more likely attributable to changes in primary and protein metabolism rather than to nitrogen redistribution from CGs. Under nutrient-limited conditions such as ½ MS, plant metabolism may be reprioritized toward the maintenance and synthesis of primary metabolites, including specific amino acids, thereby supporting their higher accumulation while secondary metabolic pathways receive reduced resource allocation. As an alternative explanation, the observed increase in amino acid levels may be at least partly apparent

and reflect a growth dilution effect, whereby differences in biomass accumulation influence concentration-based measurements [29]. On full-strength MS, sustained rapid growth may lead to dilution of amino acid pools within expanding tissues, whereas earlier growth deceleration under ½ MS, likely occurring around day 10, could result in relatively higher amino acid concentrations despite unchanged or only moderately altered metabolic fluxes. Thus, the combined effects of metabolic reprioritization and growth dilution likely shape the amino acid profiles observed under reduced nutrient supply. Notably, methionine and lysine did not follow this general pattern. These amino acids are among the least abundant in the plant proteome and are subject to particularly tight regulatory control. Methionine biosynthesis is constrained by feedback regulation via S-adenosylmethionine (SAM)[30], whereas lysine levels are controlled through strong allosteric inhibition of dihydrodipicolinate synthase (DHPS) [31] and may additionally be reduced by enhanced catabolism [32].

Some agronomic observations suggest that sulfur availability may influence cyanogenic potential. In cassava, field studies have reported that improved sulfur supply in soils was associated with reduced HCN concentrations in roots [33]. Consequently, insufficient sulfur availability has been proposed as a factor that may promote higher cyanogenic glucoside accumulation under field conditions. However, evidence from other cyanogenic species indicates that the effect of sulfur alone may be limited. Experiments conducted on sorghum seedlings grown in nutrient solutions with varying mineral compositions showed only modest changes in HCN levels in response to sulfate supply, whereas the form and availability of nitrogen had a much stronger influence on cyanogenic potential [34]. Similarly, studies in field-grown forage sorghum demonstrated that soil-applied sulfate increased plant sulfur status only slightly and did not consistently modify cyanogenic potential. In contrast, nitrogen fertilization increased cyanogenic potential. Together, these observations suggest that although sulfur availability may modulate cyanogenic metabolism under certain conditions, the overall cyanogenic potential of plants is more broadly governed by multinutrient interactions and the balance between key elements such as nitrogen, phosphorus, and sulfur. In this study the potential of sulfur status to constrain cyanide removal and shape CGs dynamics at the metabolite and transcript levels was assessed. Under sulfur deficiency, flax seems to display a safety-oriented response within the CGs pathway that is consistent with and hierarchical regulation of secondary metabolism [35]. At day 20, transcripts of genes involved in CGs degradation and cyanide detoxification increased, whereas expression of the biosynthetic gene CYP79, catalyzing the initial step of CGs biosynthesis, remained largely unchanged at most analyzed sampling stages (Fig. 6A). The induction of degradation and detoxification related genes was restricted to this single sampling stage, what may suggest a response to the immediate physiological status of the plant and may contribute to maintaining metabolic homeostasis. Reviews on cyanogenic plants similarly emphasize that immediate detoxification of cyanide is essential to sustain respiration and redox balance during stress perturbations [6]. The absence of a pronounced increase in CYP79 expression further suggests that sulfur deficiency does not act as a stress signal triggering CGs biosynthesis but may instead impose a metabolic constraint on this pathway. According to the metabolic safety or risk minimization concept, plants may restrict the accumulation of potentially toxic metabolites when detoxification capacity is limited, thereby prioritizing

cellular homeostasis over the production of defensive compounds (Morrison et al., 2024). Despite transcriptional changes, β-CAS activity remained stable under sulfur deficiency and did not reflect transcript abundance (Fig. 6C). This observation is consistent with the concept of substrate-driven control of detoxification enzymes, whereby key cyanide-detoxifying enzymes are regulated predominantly at the post-transcriptional level and respond mainly to substrate availability [3] rather than changes in gene expression. A reduction in cyanogenic potential was observed in sulfur-deficient plants, particularly at days 10 and 20 (Fig. 6E), likely reflecting limited availability of sulfur-containing amino acids such as cysteine, which plays a central role in cyanide detoxification [36]. Reduced sulfur availability may also limit cyanide detoxification capacity, constraining CGs turnover and thereby restricting their accumulation, while free cyanide levels remained unchanged. A similar relationship between sulfur availability and specialized metabolite production has been described for glucosinolates, where sulfur deficiency reduces metabolite accumulation. Although cyanogenic glycosides differ structurally from glucosinolates, both belong to nitrogen-containing defense metabolites whose biosynthesis is tightly coordinated with sulfur metabolism and overall cellular resource status.

In the early phase of the sulfur supplementation experiment transcripts of biosynthetic genes were consistently lowered relative to control plants, followed by moderate induction at days 10–20 and pronounced upregulation at later stages. A comparable temporal pattern was observed for genes associated with CGs degradation. This result in early time points may reflect a developmental asynchrony between plants grown under sulfur supplementation and control conditions during the most dynamic stage of CGs metabolism. Notably, HNL showed a strong induction from day 10 onward, closely resembling its response under severe drought stress. This suggests that HNL is primarily responsive to stress-related perturbations of cellular homeostasis and cyanide turnover, rather than to sulfur availability per se. Elevated sulfur levels may therefore have imposed secondary osmotic or ionic stress, indirectly activating CGs metabolism rather than acting solely as a nutritional signal. Free cyanide levels followed a pattern similar to that observed under mannitol-induced drought stress, which further reinforces the similarity in response patterns between sulfur supplementation and osmotic drought stress. Sulfur toxicity in plants is generally rare, and even relatively high sulfur concentrations typically do not induce pronounced stress responses, although excessive sulfate may interfere with the uptake or utilization of other nutrients, such as nitrogen, thereby indirectly altering plant metabolic status. In contrast to the pronounced transcriptional changes observed for biosynthetic and degradation genes, transcripts of detoxification related genes showed only minor or no induction. Nevertheless, β-CAS enzymatic activity was significantly increased at day 5, corresponding to the developmental window closest to the peak of CGs metabolic activity. This apparent uncoupling between transcript levels and enzyme activity further supports the concept of substrate-driven control of detoxification enzymes, whereby key cyanide-detoxifying enzymes are regulated predominantly at the post-transcriptional level and respond mainly to substrate availability, rather than transcriptional induction. Importantly, a comparable decrease in β-CAS activity was not observed under sulfur deficiency, suggesting that a minimum level of β-CAS activity is maintained regardless of sulfur status. This observation aligns with the concept of homeostatic buffering of essential detoxification enzymes, which are preserved at a

constitutive activity level to prevent autotoxicity [10], while modulation of cyanide risk is instead achieved through regulation of CGs pool size.

CGs intersect sulfur metabolism via cyanide detoxification and are also tightly integrated with the plant nitrogen economy. They mainly act as a remobilizable reservoir of reduced nitrogen returned to primary metabolism through endogenous turnover [37]. In contrast to sulfur-related treatments, changes in the transcript levels of genes involved in CGs metabolism under nitrogen deficiency and supplementation in relation to plants grown on ½ MS medium were generally less pronounced. Both N- and N+ conditions resulted in comparatively moderate and gene-specific transcriptional responses, indicating that nitrogen availability does not trigger a strong or coordinated transcriptional reprogramming of CGs metabolism under the experimental conditions applied.

Under nitrogen deficiency, a transient reduction in transcript levels was observed at day 5, coinciding with the developmental stage characterized by maximal CGs metabolic activity. This response may reflect a nitrogen-conserving strategy, whereby limited reduced form nitrogen is preferentially allocated to essential primary metabolism rather than to the synthesis or storage of nitrogen-containing secondary metabolites. This result may contrasts with observations reported, that flax can accumulate nitrogen in CGs even under nitrogen deficiency, suggesting that CGs may serve as a temporary nitrogen reservoir for later amino acid synthesis [38]. The discrepancy likely results from differences in the timing of nitrogen limitation. In the present study nitrogen deficiency was imposed from germination, whereas in the study by Siegień et al. nitrogen restriction was introduced after timepoint when plants had already passed the developmental maximum of CGs metabolic activity and CGs accumulation had likely already occurred.

Despite these gene-specific responses, nitrogen deficiency did not induce a coordinated or sustained transcriptional activation of the CGs metabolic pathway during later stages of plant development, further indicating that the metabolic flux through the CGs pathway remains limited under conditions of nitrogen deficiency. Changes observed at the transcript level under nitrogen deficiency were partially reflected at the enzymatic and metabolic levels with a temporal delay. Reduced β-CAS activity and decreased cyanogenic potential were detected at day 10, following the transcriptional repression of all CGs metabolism analyzed genes observed at day 5. This temporal shift suggests that transcriptional changes contribute to downstream metabolic effects. Importantly, the absence of changes in free cyanide content under N- conditions may indicate that cyanide homeostasis was maintained.

Under nitrogen supplementation, transcript levels of CGs metabolism genes remained largely comparable to those observed in control plants grown on ½ MS medium. Nevertheless, increased transcript levels were detected for CYP79 at days 30 and 40 and for UGT85 at day 30, resembling patterns previously observed under sulfur supplementation and drought stress. This similarity suggests that nitrogen supplementation imposed a mild osmotic or ionic stress, rather than acted solely as a nutritional signal. However, the lack of a concomitant increase in free cyanide content indicates that factor was insufficient to induce a detectable imbalance in cyanide production or turnover. In contrast to sulfur availability, nitrogen does not appear to directly constrain cyanide detoxification capacity, and its

effects on CGs metabolism are primarily manifested through transient enzymatic responses and metabolic buffering rather than through coordinated transcriptional control.

Conclusions

The results demonstrate that cyanogenic glycoside metabolism in flax is governed by a complex, multi-level regulatory network integrating nitrogen and sulfur status, developmental stage, and environmental stress conditions. Across all treatments, the observed partial decoupling between transcript levels, enzyme activities, and metabolite pools indicates that CGs metabolism is not controlled by a single regulatory layer but rather reflects dynamic metabolic buffering and post-transcriptional regulation. Sulfur availability appears to play a more direct role in constraining cyanide detoxification and shaping CGs pool size, whereas nitrogen primarily modulates the timing and intensity of pathway activity rather than its overall outcome. Under stress conditions such as drought, CGs likely fulfill multiple roles, including defense readiness and participation in cyanide-mediated signaling linked to hormonal and redox responses. Together, these findings support a model in which CGs metabolism in flax is developmentally programmed and environmentally responsive, with its functional significance emerging from the balance between biosynthesis, turnover, and detoxification rather than from changes in any single component.

Abbreviations

CGs -Cyanogenic glycosides ; β-CAS - β-cyanoalanine synthase ; cv.-cultivar; MS - Murashige and Skoog; ½MS -half-strength MS; FW -fresh weight; DW- dry weight ; GC-FID – gas chromatography system equipped with a flame ionization detector ;HNL - O-hydroxynitrile lyase ; β-CAN – β-cyanoalanine hydratase; SAT – serine O-acetyltransferase; OASTL – O-acetylserine (thiol) lyase; ASG – asparaginase; MetS – methionine synthase; ACS – ACC synthase; ACO – ACC oxidase; DHDPS – dihydrodipicolinate synthase; LDC – L-lysine decarboxylase; LKR – lysine-ketoglutarate reductase; SDH – saccharopine dehydrogenase; P5CS – pyrroline-5-carboxylate synthase; OAT – ornithine aminotransferase; ODC – ornithine decarboxylase; SPDS – spermidine synthase; OTC – ornithine transcarbamylase; ASS – argininosuccinate synthetase; ASL – argininosuccinate lyase; OAH-OAS - O-acetylhomoserine-O-acetylserine

Declarations

Ethics approval and consent to participate - Not applicable

Consent for publication -- Not applicable

Availability of data and materials -The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing Interests -The authors declare that they have no competing interests

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Authors' contributions: -Conceptualization- A.S. and M Z ; performing research – AS and PP, analyze data – AS ,Writing – original draft AS ; Writing – review & editing- MZ. Supervision- MZ

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Figures

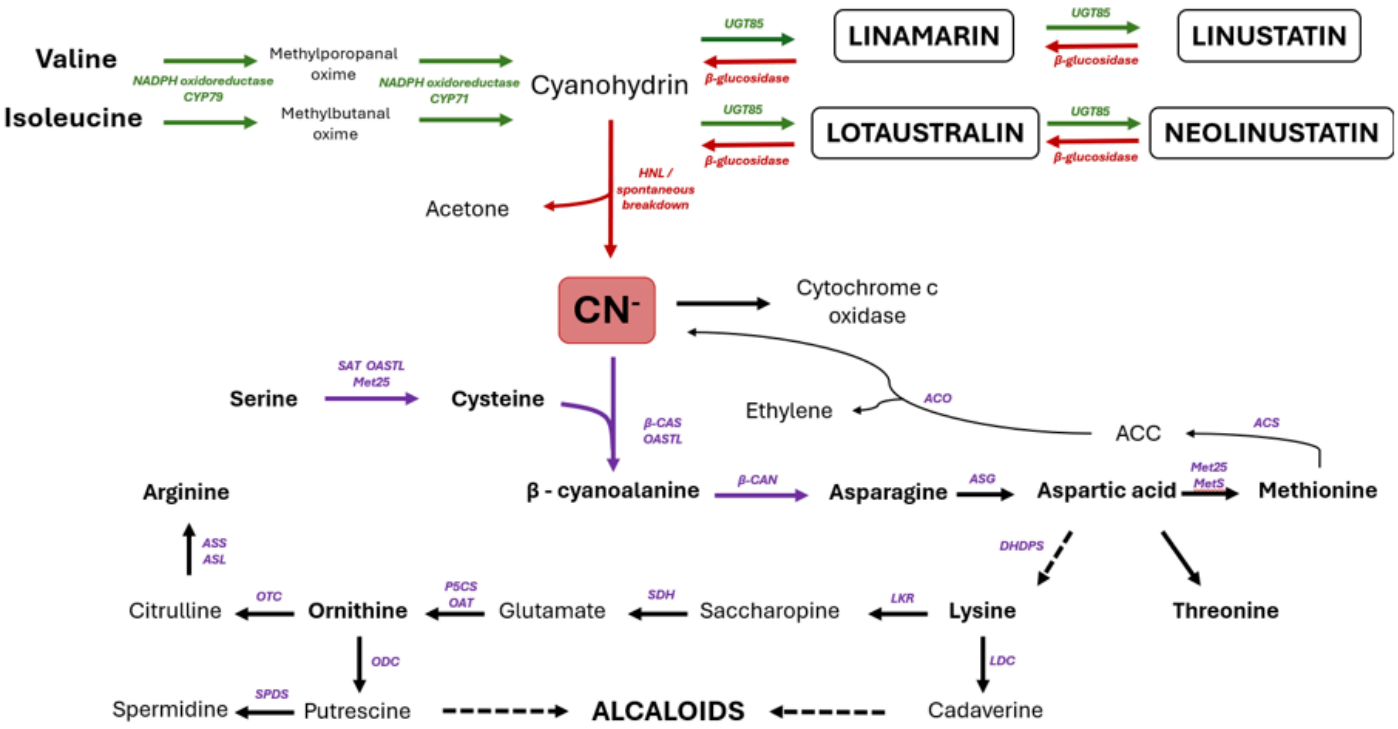

Figure 1

Metabolism of cyanogenic glucosides

The biosynthetic pathway of cyanogenic glycosides is marked in green (green arrows and corresponding enzymes), whereas their degradation is indicated in red, and cyanide detoxification is represented in violet. Other interconnected metabolic pathways are shown in black. Enzymes on the scheme: biosynthesis : NADPH oxidoreductases (CYP79, CYP71), UDP-glucosyl-transferase (UGT85); catabolism: β-glucosidase, O-hydroxynitrile lyase (HNL); cyanide detoxification: β-CAS – β-cyanoalanine synthase, β-CAN – β-cyanoalanine hydratase, SAT – serine O-acetyltransferase, OASTL – O-acetylserine (thiol) lyase, ASG – asparaginase, MetS – methionine synthase, ACS – ACC synthase, ACO – ACC oxidase, DHDPS – dihydrodipicolinate synthase, LDC – L-lysine decarboxylase, LKR – lysine-ketoglutarate reductase, SDH – saccharopine dehydrogenase, P5CS – pyrroline-5-carboxylate synthase, OAT – ornithine aminotransferase, ODC – ornithine decarboxylase, SPDS – spermidine synthase, OTC – ornithine transcarbamylase, ASS – argininosuccinate synthetase, ASL – argininosuccinate lyase. Exogenous gene Met25 from Saccharomyces cerevisiae encodes O-acetylhomoserine-O-acetylserine (OAH-OAS) sulfhydrylase and is involved in the biosynthesis of sulfur-containing amino acids.

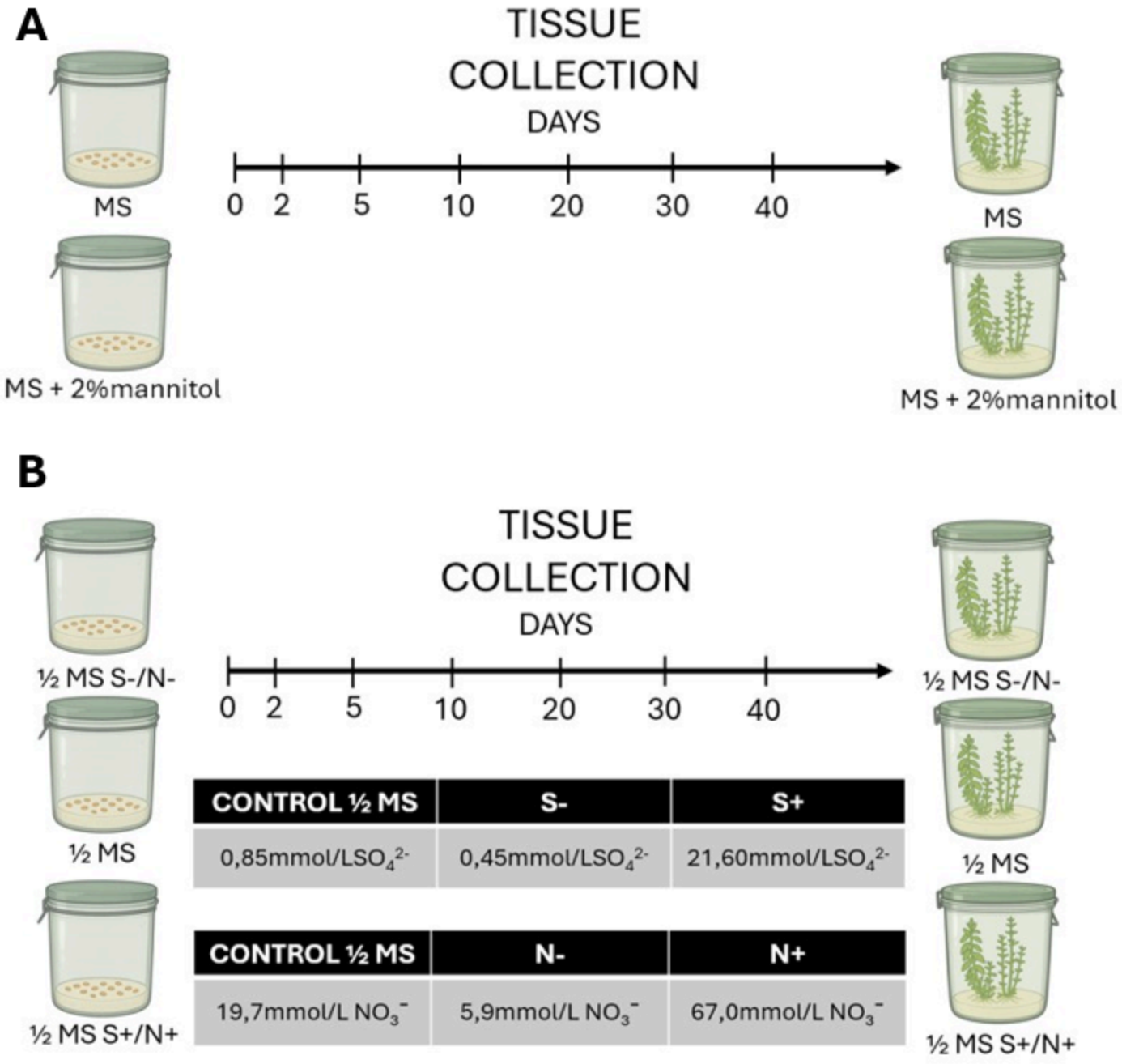

Figure 2

Experimental design of abiotic stress treatments and sampling scheme

(A) Osmotic stress treatment: plants were cultivated on Murashige and Skoog (MS) medium supplemented with 2% (w/v) mannitol, with tissue collection performed at defined developmental time points (days 0–40). Control plants were grown on standard MS medium. (B) Nutrient stress treatments: plants were cultivated on half-strength MS (½ MS) medium with modified sulfur (S−, S+) or nitrogen (N−,

N+) availability. Changes in sulfate (SO_4^{2-}) and nitrate (NO_3^-) concentrations were applied relative to the ½ MS control. Tissue collection was performed across the same developmental time course.

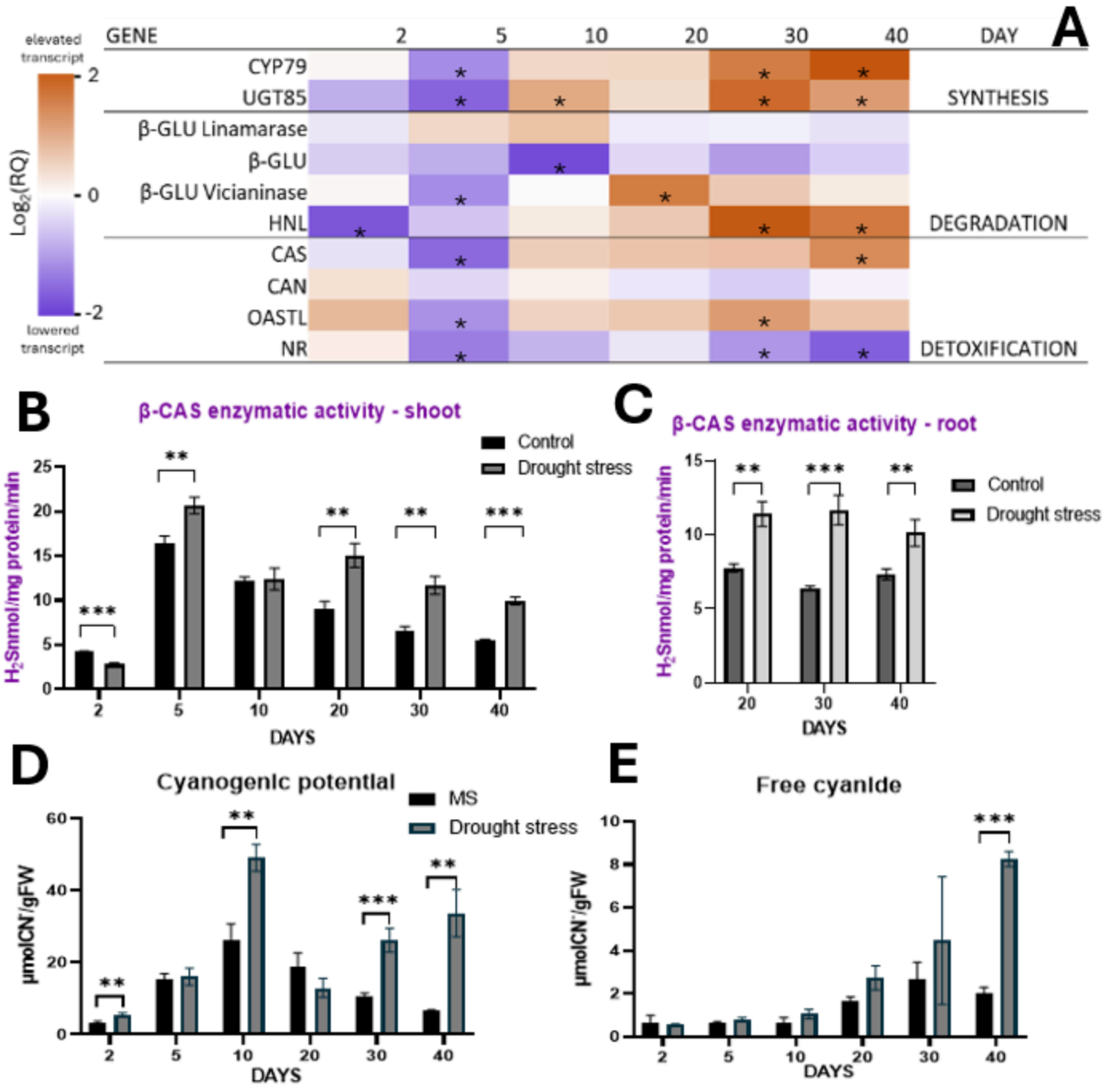

Figure 3

Effect of drought stress on cyanogenic glycoside metabolism in flax plants.

(A) Heat map showing relative transcript levels of genes involved in cyanogenic glycoside biosynthesis (CYP79, UGT85), degradation (β-GLU isoforms, HNL), and cyanide detoxification (CAS, CAN, OASTL, NR) in flax shoots at days 2, 5, 10, 20, 30, and 40 under drought stress, compared with control plants grown

on MS medium. Transcript levels were determined by real-time RT-PCR, normalized to flax actin, and expressed as $\log_2$ fold changes relative to the corresponding control plants. Heat map boxes represent mean values from three biological replicates. The color scale indicates relative changes in transcript abundance (purple, downregulation; orange, upregulation). Statistical significance was assessed using two-way ANOVA followed by Tukey's post hoc test, with asterisks indicating significant differences compared to the corresponding control at the same time point (*P < 0.05). Only changes meeting the criterion of biological relevance (≥2-fold induction or ≤0.5-fold repression relative to control) were considered biologically significant. (B) β-CAS enzymatic activity in flax shoots under drought stress, compared with control plants grown on MS medium. (C) β-CAS enzymatic activity in flax roots under drought stress, compared with control plants grown on MS medium. (D) Cyanogenic potential in flax shoots under drought stress, compared with control plants. (E) Free cyanide content in flax shoots under drought stress, compared with control plants. For panels B, D, and E, measurements were performed at days 2, 5, 10, 20, 30, and 40, whereas for panel C analyses were conducted at days 20, 30, and 40. For panels B–E, bars represent the mean ± SD from three independent biological replicates. Statistical significance was assessed using two-way ANOVA followed by Tukey's post hoc test. Asterisks indicate significant differences compared to the corresponding control (MS) at the same time point (*P < 0.05).

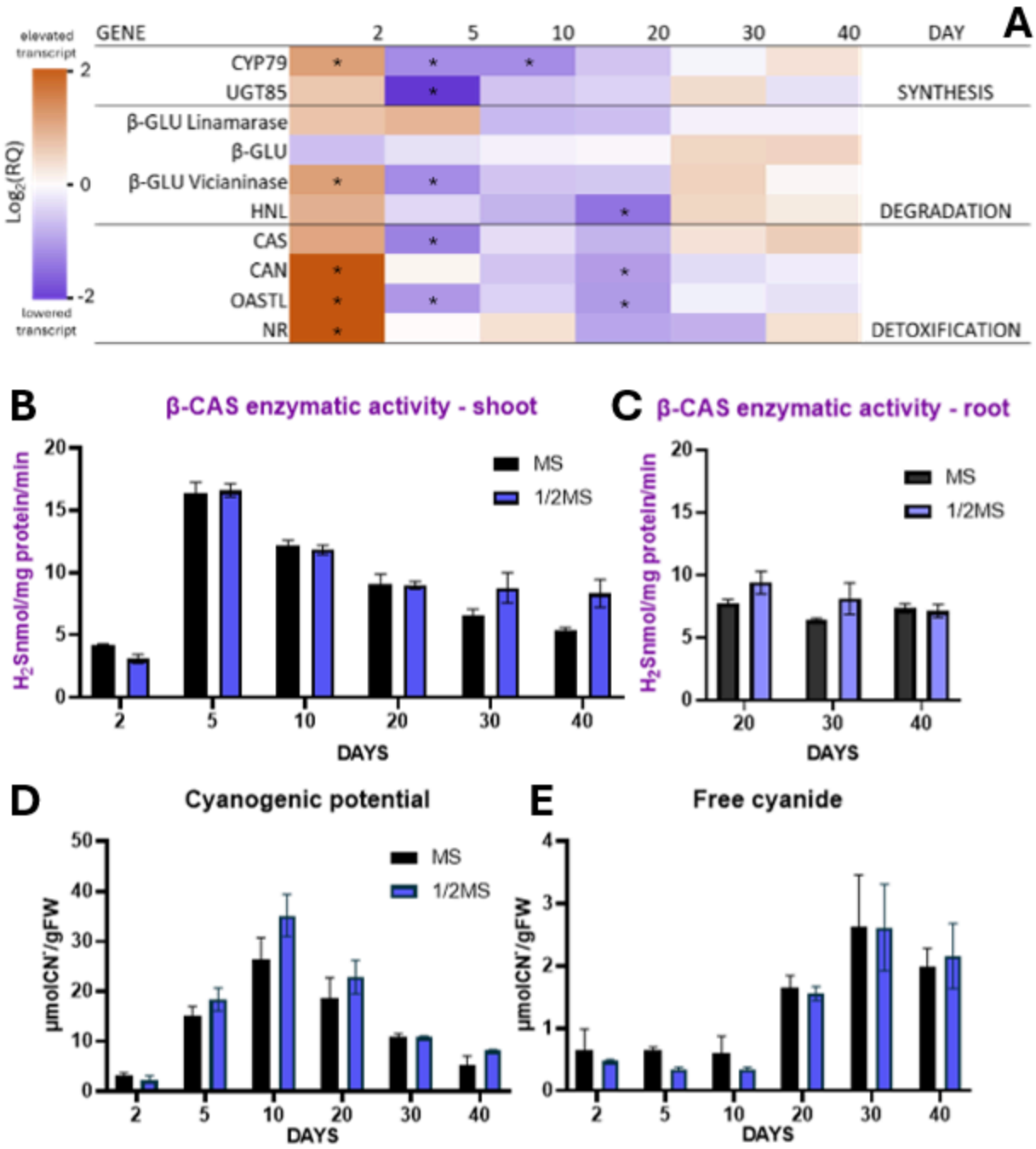

Figure 4

Effect of nutrient availability (MS vs ½ MS) on cyanogenic glycoside metabolism in flax plants.

Heat map showing relative transcript levels of genes involved in cyanogenic glycoside biosynthesis (CYP79, UGT85), degradation (β-GLU isoforms, HNL), and cyanide detoxification (CAS, CAN, OASTL, NR) in flax shoots at days 2, 5, 10, 20, 30, and 40 in plants grown on full-strength MS medium compared with plants cultivated on half-strength MS (½ MS) medium. Transcript levels were determined by real-time RT-PCR, normalized to flax actin, and expressed as $\log_2$ fold changes relative to the corresponding MS-

grown control plants. Heat map boxes represent mean values from three biological replicates. Only changes meeting the criterion of biological relevance (≥2-fold induction or ≤0.5-fold repression relative to control) were considered biologically significant. The color scale indicates relative changes in transcript abundance (purple, downregulation; orange, upregulation). Statistical significance was assessed using two-way ANOVA followed by Tukey's post hoc test, with asterisks indicating significant differences compared to MS-grown plants at the same time point (*P < 0.05). (B) β-CAS enzymatic activity in flax shoots grown on MS and ½ MS media. (C) β-CAS enzymatic activity in flax roots grown on MS and ½ MS media. (D) Cyanogenic potential in flax shoots of plants grown on MS and ½ MS media. (E) Free cyanide content in flax shoots of plants grown on MS and ½ MS media. For panels B, D, and E, measurements were performed at days 2, 5, 10, 20, 30, and 40, whereas for panel C analyses were conducted at days 20, 30, and 40. For panels B–E, bars represent the mean ± SD from three independent biological replicates. Statistical significance was assessed using two-way ANOVA followed by Tukey's post hoc test. Asterisks indicate significant differences between MS- and ½ MS-grown plants at the same time point (*P < 0.05).

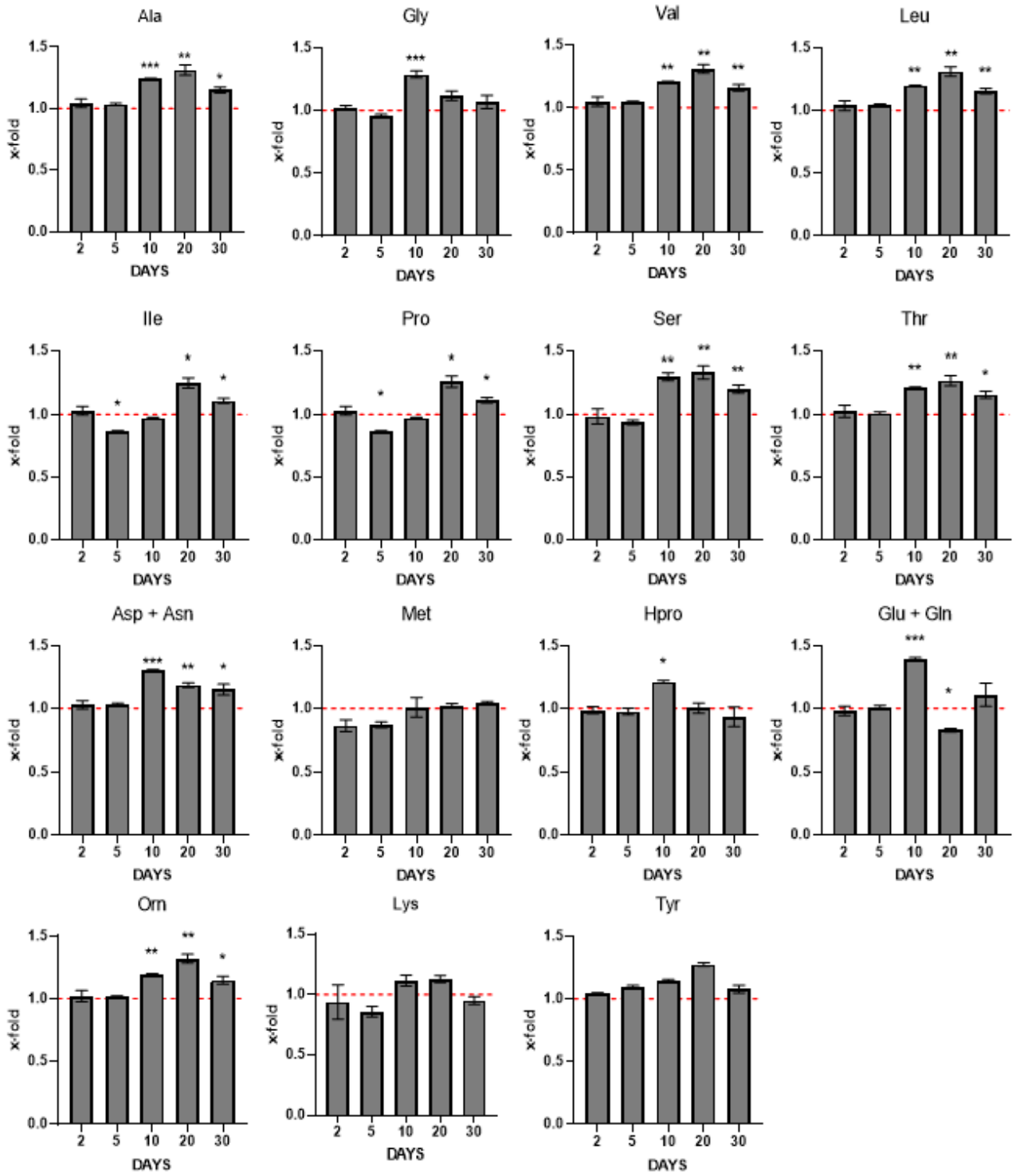

Figure 5

Effect of nutrient availability (MS vs ½ MS) on amino acid content in flax.

The content of individual amino acids was analyzed in flax shoots at days 2, 5, 10, 20, and 30 in plants grown on full-strength MS medium and half-strength MS (½ MS) medium. Amino acid levels were quantified and expressed relative to the corresponding MS control, which was normalized to 1 (indicated

by the red dashed line). Bars represent mean values ± SD from three independent biological replicates. Statistical analysis was performed using two-way ANOVA with growth medium (MS vs ½ MS) and time as fixed factors, followed by Tukey's post hoc test. Differences were considered statistically significant at $P < 0.05$ and are indicated by asterisks.

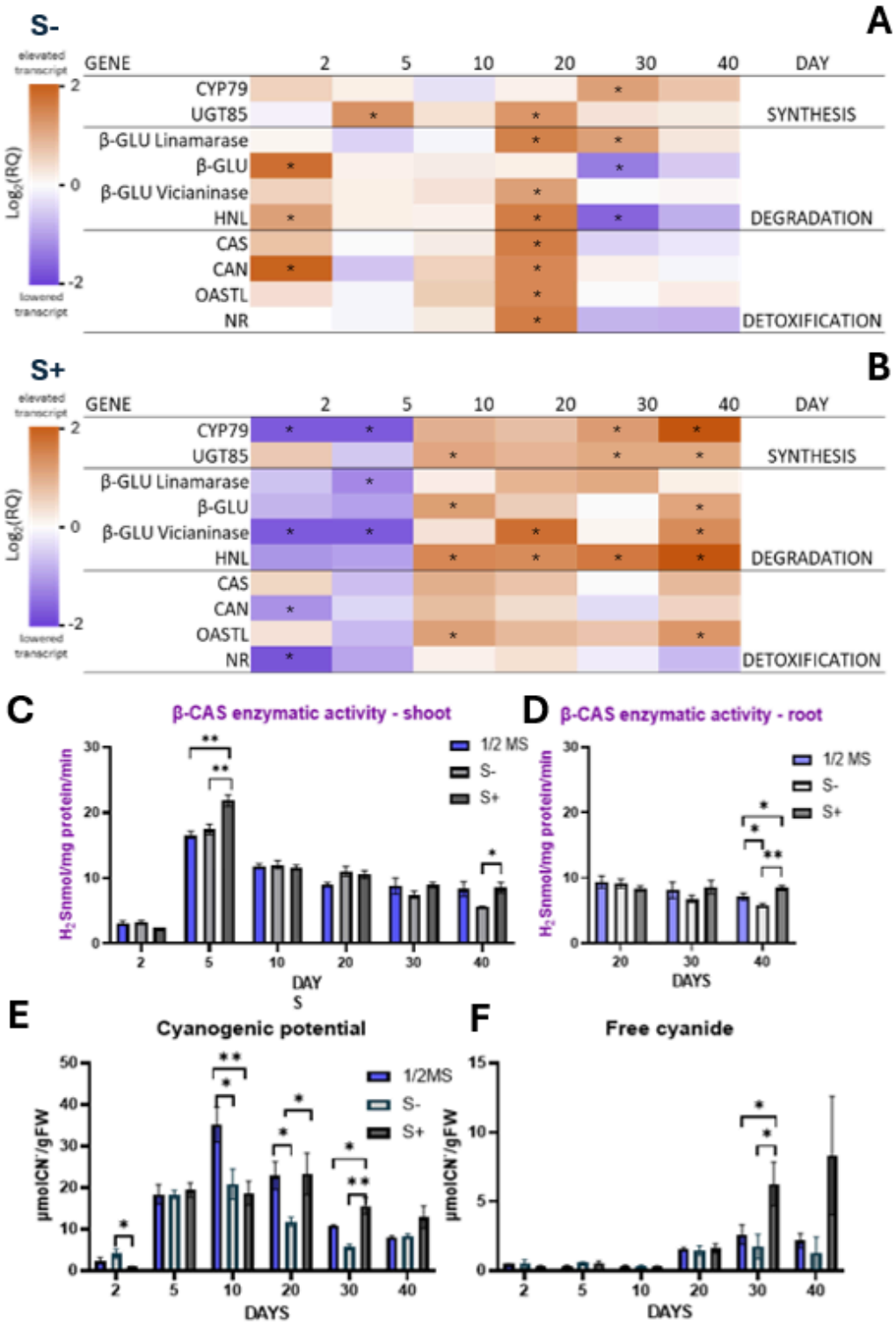

Figure 6

Effect of sulfur deficiency (S^-) and sulfur supplementation (S^+) on cyanogenic glycoside metabolism in flax.

(A) Heat map showing relative transcript levels of genes involved in cyanogenic glycoside biosynthesis (CYP79, UGT85), degradation (β-GLU isoforms, HNL), and cyanide detoxification (CAS, CAN, OASTL, NR) in flax shoots grown under sulfur-deficient conditions (S^-), compared with control plants (½ MS medium).
(B) Heat map showing relative transcript levels of the same set of genes in flax shoots grown under sulfur supplementation (S^+), compared with control plants (½ MS medium). For panels A and B, transcript levels were determined by real-time RT-PCR, normalized to actin, and expressed as $\log_2$ fold changes relative to control plants. Heat map boxes represent mean values from three biological replicates. Only changes meeting the criterion of biological relevance (≥2-fold induction or ≤0.5-fold repression relative to control) were considered biologically significant. The color scale indicates relative changes in transcript abundance (purple, downregulation; orange, upregulation). Statistical significance was assessed using two-way ANOVA followed by Tukey's post hoc test, with asterisks indicating significant differences compared to the corresponding control (*P < 0.05). Measurements for panels A and B were performed at days 2, 5, 10, 20, 30, and 40. β-CAS enzymatic activity in flax shoots(C) and roots (D) grown under S^- and S^+ conditions. (E) Cyanogenic potential in flax shoots grown under S^- and S^+ conditions. (F) Free cyanide content in flax shoots grown under S^- and S^+ conditions. For panels C, E, and F, measurements were performed at days 2, 5, 10, 20, 30, and 40, whereas for panel D analyses were conducted at days 20, 30, and 40. Bars represent the mean ± SD from three independent biological replicates. Statistical significance was assessed using two-way ANOVA followed by Tukey's post hoc test. Asterisks indicate significant differences compared to the corresponding control (½ MS) (*P < 0.05).

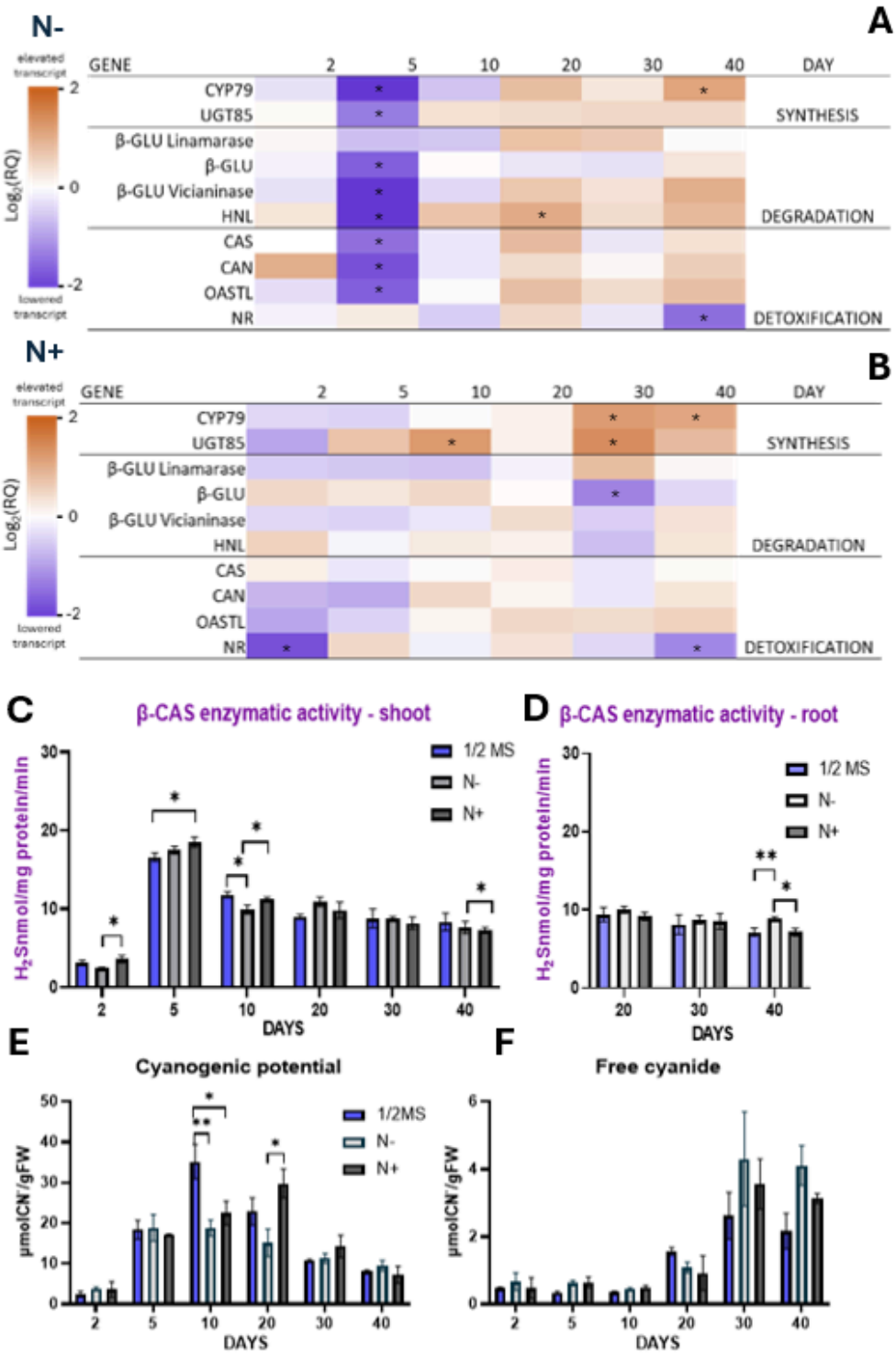

Figure 7

Effect of nitrogen deficiency (N^-) and nitrogen supplementation (N^+) on cyanogenic glycoside metabolism in flax plants.

Genes analyzed include those involved in cyanogenic glycoside biosynthesis (CYP79, UGT85), degradation (β-GLU isoforms, HNL), and cyanide detoxification (CAS, CAN, OASTL, NR). (A) Heat map

showing relative transcript levels of the indicated genes in flax shoots grown under sulfur-deficient conditions (S^-), compared with control plants cultivated on ½ MS medium. (B) Heat map showing relative transcript levels of the same set of genes in flax shoots grown under sulfur supplementation (S^+), compared with control plants cultivated on ½ MS medium. For panels A and B, transcript levels were determined by real-time RT-PCR, normalized to flax actin, and expressed as $\log_2$ fold changes relative to the corresponding control plants. Heat map boxes represent mean values from three biological replicates. Only changes meeting the criterion of biological relevance (≥2-fold induction or ≤0.5-fold repression relative to control) were considered biologically significant. The color scale indicates relative changes in transcript abundance (purple, downregulation; orange, upregulation). Statistical significance was assessed using two-way ANOVA followed by Tukey's post hoc test, with asterisks indicating significant differences compared to the corresponding control at the same time point (*P < 0.05). Measurements for panels A and B were performed at days 2, 5, 10, 20, 30, and 40. (C) β-CAS enzymatic activity in flax shoots grown under S^- and S^+ conditions. (D) β-CAS enzymatic activity in flax roots grown under S^- and S^+ conditions. (E) Cyanogenic potential in flax shoots grown under S^- and S^+ conditions. (F) Free cyanide content in flax shoots grown under S^- and S^+ conditions. For panels C, E, and F, measurements were performed at days 2, 5, 10, 20, 30, and 40, whereas for panel D analyses were conducted at days 20, 30, and 40. Bars represent the mean ± SD from three independent biological replicates. Statistical significance was assessed using two-way ANOVA followed by Tukey's post hoc test. Asterisks indicate significant differences compared to the corresponding control (½ MS) at the same time point (*P < 0.05).

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