TITLE

A comparative analysis of salt stress adaptiveness between two flax (*Linum usitatissimum* L.) genotypes, Flanders and Astella, having contrasting lignan contents

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ABSTRACT

On the verge of the United Nations' predicted upcoming food scarcity, research regarding plants' potential to withstand environmental stresses to provide better yield is important. In this context, plant lignans have great potential and possess multifunctional roles towards different organisms including plants and humans. Lignans accumulate in plants and primarily act as defence substances during abiotic stresses. Flax (*Linum usitatissimum* L.) is a rich source of lignans. However, the lignan contents are dependent on plant genotypes. Therefore, this report searched for the answer to the question – "under salinity stress, how much better can a high lignan-containing flaxseed genotype adapt than a low lignan-containing flaxseed genotype?". We applied 100 mM NaCl stress for one week on two chosen flaxseed genotypes, Flanders and Astella. Astella has a higher lignans (SDG) content than Flanders. Morphologic analyses revealed an unaffected shoot and facilitated root growth Astella than Flander under salinity. Salt stress created higher ROS production and associated cell damage in Flanders than in Astella. In biochemical stress marker assays, we found comparatively upregulated enzymatic antioxidants, osmoprotection machinery, and lignan-related miRNAs in Astella, showing its higher ROS scavenging and cellular protection capabilities than Flanders. However, multivariate analysis could not provide evidence of lignan involvement in stress adaptation. Although the lignan-related miRNA expressions were higher in Astella over Flanders, through multivariate analysis we hypothesised miRNAs as a pleiotropic tool in salinity adaptation and lignan content as positive salt stress markers. The results depict the superiority of Astella in salt stress mitigation over Flanders.

Keywords: flax; salinity stress; lignan; morphological analysis; biochemical analysis; miRNA

ABBREVIATIONS:

ANOVA = One-way analysis of variance

APX = Ascorbate peroxidase

CAE = Caffeic acid

CAT = Catalase

DPPH = 2,2-Diphenyl-1-picrylhydrazyl

DMRT = Duncan's multiple range test

EIF5A = Eukaryotic transcription initiation factor 5A

FDA = Fluorescein diacetate

GAE = Gallic acid

GPOX = Guaiacol peroxidase

H_2O_2 = Hydrogen peroxide

LN = Leaf Number

LRWC = Leaf relative water content

miRNA = MicroRNA

MDA = Malondialdehyde

MYB = Myeloblastosis

PA = Phenolic acids

PC = Principal component

PCA = Principal component analysis

PCC = Pearson's correlation coefficient

PI = Propidium iodide

PP = Polyphenols

RD = Root diameter

RDW = Root dry weight

RF = Root fork

RFW = Root fresh weight

RL = Root length

RMD = Root mass density

ROS = Reactive oxygen species

RRWC = Root Relative water contents

RT = Root tip

RV = Root volume

SDW = Shoot dry weight

SFW = Shoot fresh weights

SECO = Secoisolariciresinol

SFW = Shoot fresh weight

SL = Shoot length

SOD = Superoxide dismutase

SRWC = Shoot Relative water contents

TEAC = 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

TF = Transcription factor

TT-PCR = Two-tailed qPCR

INTRODUCTION

The rapid climatic change-mediated abiotic stresses cause the annual loss of 51–82% of crop yield in world agriculture (Onyekachi et al. 2019; Oshunsanya et al. 2019). Salinity, one of the major abiotic stresses, is a soil condition denoted by a high concentration of soluble salts. Soils are considered saline when the ECe is 4 dS/m or more, equivalent to approximately 40 mM of NaCl and generating an osmotic pressure of approximately 0.2 MPa (Munns and Tester 2008). Presently, salinity affects around 20% of total cultivated and 33% of irrigated lands including more than 424 million hectares of topsoil (0-30 cm) and 833 million hectares of subsoil (30-100 cm) spanning 73% of the global land area (FAO 2021). Around 195.1 million, 6.1 million, 6.73 million and 70 thousand hectares of soil are salinity-affected in Asia and the Pacific and Australia, Europe, India, and Slovakia, respectively (Tractor Junction 2022; FAO 2025a; Stehlo and Ková 2025). Soil salinity reduces around 1.5 million hectares of farmland annually (FAO 2025b). By 2050, around 50% of total arable land may be affected by salinity (FAO 2021). This alarming situation demands research to produce salinity-stress-resilient crops (UN 2025).

High concentration of salts in soil limits a plant's water and nutrient uptake capability creating a physiologic drought condition. To balance this soil solute potential to favour stable water flux, plants channelise photosynthates in synthesising and accumulating osmolytes or compatible solutes such as proline and glycine betaine (Türkan and Demiral 2009). The diversion of cellular resources from the major physiologic process such as photosynthesis results in drastic growth rate reduction of a salinity-affected plant. Growth arrest under stress is considered a plant adaptive mechanism to preserve carbohydrates for sustained metabolism, prolonged energy supply and better recovery after stress relief (Bartels and Sunkar 2005).

Salinity stress disrupts the cellular balance between energy absorption and consumption. Consequently, electrons start leaking from electron transport chains of photosynthesis (chloroplast), respiration (mitochondria), and photorespiration (peroxisomes). These leaked electrons oxidise oxygen molecules creating a large amount of reactive oxygen species (ROS) that damages molecular and cellular components by oxidation of biomolecules (lipids, carbohydrates, proteins, enzymes, and DNA) and cause plant death (Hasanuzzaman et al. 2019; Alaei et al. 2020; Mekawy et al. 2020; Sachdev et al. 2021). To combat this fatal situation, plant cells have a well-established ROS-scavenging antioxidant defence mechanism consisting of enzymatic components such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), glutathione S-transferase (GST), glutathione peroxidase (GPX) and guaiacol peroxidase (GPOX) along with non-enzymatic components such as ascorbic acid (AsA), dehydroascorbate (DHA), Glutathione (GSH), alkaloids, α-tocopherol, tocotrienols, carotenoids, polyphenols, quercetin, kaempferol-glycosides, cytochromes, polyamines, proteins carrying redox-active S-groups (thioredoxins, peroxiredoxins and sulfiredoxins) and non-protein amino acids like proline (Smirnoff and Cumbes 1989; Abogadallah 2010; Gill and Tuteja 2010; Hasanuzzaman et al. 2019). Multiple hydroxyl groups also make polyphenols (phenolic acids, flavonoids, stilbenoids and lignans) powerful ROS scavengers (Šamec et al. 2021).

Lignans are secondary metabolites belonging to the group of dimeric phenylpropanoids which naturally occur in superior plants where they primarily act in plant defence. Phenylpropanoids are phenylpropane derivatives whose skeleton consists of a propane moiety (a three-carbon chain) attached to a phenyl group. Phenylpropanoid dimers where two phenylpropane units are linked by their carbon 8 (β-β' link) represent the simplest lignan. Naturally occurring plant lignans have varying degrees of substitution in the phenylpropane skeletons (Sainvitu et al. 2012; Teponno et al. 2016;

Markulin et al. 2019; Ražná et al. 2021). Lignans are found in most plants. They are present in different plant organs such as flowers, seeds, leaves, roots, fruits, and wooden parts (Ražná et al. 2021).

Lignans are involved in plant defence mechanisms through their antioxidant activity (Ghotbzadeh Kermani et al. 2019). Synthesis of several polyphenols including lignans increases during abiotic stresses such as drought, extreme temperatures, salinity, heavy metal, and UV radiations (Sharma et al. 2019; Ražná et al. 2021). Under drought stress, lignan contents of sesame change in a genotype-specific manner. Moreover, the drought-tolerant sesame genotypes contain a high level of lignans such as sesamin and/or sesamolin (Ghotbzadeh Kermani et al. 2019). Heavy metal stress-tolerant rapeseed (*Brassica napus* L.) genotypes accumulated more lignans under the stress of Cadmium heavy metal than the susceptible genotypes (Mwamba et al. 2020). Lignans also possess antibacterial (Cespedes et al. 2006; Gaafar et al. 2013; Mahendra Kumar and Singh 2015), antifungal (Cespedes et al. 2006; Akiyama et al. 2007; Cho et al. 2007) and antifeedant (Kraus and Spiteller 1997) properties.

MicroRNAs (miRNAs) are a class of non-coding, 19-24 nucleotide long RNA molecules that negatively regulate gene expression at the post-transcriptional level. In plants, a miRNA is loaded into the RNA-induced silencing complex (RISC) that involves argonaute (AGO) protein. The miRNA-RISC complex then binds to its target mRNA and degrades it using AGO, thereby repressing the associated gene expression (Li et al. 2018). Reinhart et al. (2002) detected the first-ever miRNA in plants. Besides regulating numerous physiological processes, miRNAs also impact plants' tolerance toward various abiotic stresses including salinity stress. Several miRNAs such as miR156, miR159, miR160, miR164, miR166, miR167, miR168, miR169, miR172, miR319, miR394, miR395, miR396, miR397, miR399 and miR408 are considered as conserved for salt stress in plants (Gao et al. 2022). Studies depict that miRNAs impact salt stress tolerance by regulating either hormonal pathways (Sunkar et al. 2012; Baek et al. 2016; Liu et al. 2019; Makkar et al. 2021) or antioxidant systems of a plant (Noman et al. 2017; Yang and Guo 2018; Parmar et al. 2020; Cheng et al. 2021).

Several secondary metabolites of plants, including lignans, are biosynthesised via the phenylpropanoid pathway. Several miRNAs regulate this pathway by targeting either biosynthesis-related genes, transcription factors (TFs) or noncoding RNAs (ncRNAs) (Ražná et al. 2021). In a recent study, Tu et al. (2022) depicted that 23 miRNAs regulate 80 genes of the phenylpropanoid pathway belonging to 14 gene families of *Liriodendron chinense*. This study revealed the direct involvement of many novel miRNAs in this regulation process besides some of the known miRNAs such as lch-miR165a-5p, lch-miR168a-5p, lch-miR169a-3p and lch-miR393a-5p. The authors also found indirect regulation of the phenylpropanoid pathway by miRNAs and long non-coding RNAs (lncRNAs). They target some key myeloblastosis (MYB) TFs that either act as repressors (MYB4, MYB39, MYB44 and MYB308) or activators (MYB2, MYB5, MYB12, MYB330 and MYB340) of this pathway. Phenylalanine ammonia lyase (PAL) performs the first and committed step of the phenylpropanoid pathway converting phenylalanine to cinnamic acid. Few studies reveal the involvement of miR172, miR399, miR408, miR477, miR830, miR7729 and miR1873 regulating PAL (Singh et al. 2016; Yan et al. 2019). In this regard, Biswas et al. (2016) reported that miR172i, a member of miR172 family, regulates 4-coumarate-CoA ligase that catalyses subsequent steps of phenylpropanoid pathway of converting 4-coumarate and other 4-hydroxycinnamates to their corresponding thiol esters. Several members of the cytochromes P450 gene superfamily exert important steps in the phenylpropanoid pathway such as cinnamic acid 4-hydroxylase (C4H; CYP73A), coumarate 3-hydroxylase (C3H; CYP98A) and ferulic acid 5-hydroxylase (F5H; CYP84A) (Reinprecht et al. 2017). In tea, both miR5251 and miR7777-5P.1 downregulate C4H (Sun et al. 2017). A miR319-based artificial miRNA (AMR) downregulates both

C3H and F5H genes in jute (Shafrin et al. 2015). Barvkar et al. (2013) reported that lus-miR168b regulates CYP450 genes in flax. Dimerization of monolignols to pinoresinol by the joint action of a dirigent protein (DIR) and a laccase is an important step of lignan biosynthesis (Zoclanclounon et al. 2022). Three abiotic stress-responsive Indica rice miRNAs regulate DIR expression: ath-mirf10317-akr, cre-mir910, and osa-mirf10761-akr (Singh et al. 2020). Cotton miRNA396 targets a dirigent-like protein (Zhang et al. 2007). Multiple studies report that both miR397 and miR408 are involved in laccase gene regulation in different plants including flax (Le Roy et al. 2017; Carrió-Seguí et al. 2019; Li et al. 2019; Ražná et al. 2022; Guo et al. 2023). Further downstream of the lignan biosynthesis pathway, UDP-glycosyltransferases (UGTs) glycosylate secoisolariciresinol (SECO) into secoisolariciresinol diglucoside (SDG), which is a lignan. A recent article reports a 41 miRNAs-derived regulation of 74 CsUGT genes in cucumber (Yuan et al. 2024).

Being a food, oilseed and fibre crop, flax (*Linum usitatissimum*, family *Linaceae*), commonly known as flaxseed or linseed, is considered a multipurpose crop. Flaxseed is one of the richest sources of lignans. Major lignans found in flax are secoisolariciresinol diglucoside (294-2410 mg/100 gm), secoisolariciresinol (257.6 mg/ 100 gm), lariciresinol (3.04-11.46 mg/100 gm), matairesinol (0.55-6.68 mg/100 gm) and pinoresinol (3.32 mg/100 gm) (Goyal et al. 2014; Rodríguez-García et al. 2019). Flax is susceptible to multiple abiotic stresses such as drought, salinity and heat that endanger the plant's life and reduce its overall productivity (Yadav et al. 2022). Being both a great reservoir of lignans and susceptible to salinity stress, we choose flax as the ideal study plant to draw a correlation between these two factors.

Lignans accumulate in plants and primarily act as defence substances during abiotic stresses. However, the lignan contents are dependent on plant genotypes. Therefore, we searched for the answer to the question – "under salinity stress, how much better can a high lignan-containing flaxseed genotype adapt than a low lignan-containing flaxseed genotype?". Various morphological, biochemical, microscopic, and genomic expression assays were applied to determine the salinity stress-responsiveness between the two chosen flaxseed genotypes, Flanders and Astella, having contrasting lignan content. To our knowledge, this is the first report on lignan-related miRNA-mediated salt stress tolerance study for the multi-utilitarian crop flax.

MATERIALS AND METHODS

Plant materials and salt treatment

Seeds of flax cv. Flanders and Astella were kindly provided by Agritec Plant Research Ltd., Šumperk, Czech Republic. According to our previous laboratory estimation, the lignan (Secoisolariciresinol diclucoside, SDG) content of Astella (13.07 mg/g) is much higher than Flanders (7.75 mg/g) (Harenčár 2023). Healthy and uniform seeds were placed on tap water-moistened filter paper and incubated in a growth chamber (Climatic Chamber KK 400, Pol-Eko-Aparatura, Poland) at 23±2°C in the dark for germination. One-week-old, healthy seedlings having uniform height were transferred to TS2 white peat potting substrate (Klasmann-Deilmann GMBH, Germany) and kept in square pots (8 cm x 8 cm x 9.5 cm, length x breadth x height). Every pot had 5 seedlings. These pots were incubated in a growth chamber (Climatic Chamber KK 1200, Pol-Eko-Aparatura, Poland) at 23±2°C under a 16/8 hours photoperiod having a light intensity of 5000 lux using fluorescent tube lights and 50% humidity. The substrate was irrigated thrice a week (Monday, Wednesday, and Friday) with a watering solution (Grandiol Universal Liquid Plant Food, ASB Grünland Helmut Aurenz GmbH, Germany, 1:20 v/v dilution with distilled water) to a reference weight (pot + substrate + watering

solution) of 272 grams that represented 80% of the maximum soil water-holding capacity (MSWC) of the substrate measured gravimetrically (Junker et al. 2015). The seedlings were allowed to grow for two weeks more.

Experimental treatments were arranged in a randomised complete block design. A 100 mM NaCl (Sigma-Aldrich Chemie GmbH, Germany) solution prepared in distilled water was used for salt stress application. Three-week-old, randomly selected plants of both cultivars were subjected to salinity stress treatment using the NaCl solution. The watering solution was used for control treatment. The substrate was irrigated thrice (Monday, Wednesday, and Friday) to its 80% MSWC for both treatments. After one week of stress treatment (next Monday), fresh plant samples were harvested for morphological and microscopic analyses, and the rest of the samples were stored at -50°C for biochemical and genomic analysis.

Morphological assays

Various shoot, root, and leaf parameters were recorded in this assay. Due to the differential growth rates of Flanders and Astella, we processed the data in two steps to obtain an unbiased result as suggested previously (Julkowska and Testerink 2015). Firstly, we estimated the relative changes by subtracting the values obtained before stress from values obtained after stress until otherwise mentioned. Secondly, the data were normalised against the control treatments. We plotted the normalised relative data into graphs. The percentage (%) change of salt stress treatment data was calculated against the respective controls.

Shoot length (SL) was measured using a ruler scale. Root length (RL), diameter (RD), volume (RV), and tip (RT) and fork (RF) numbers were measured using an Epson Expression 11000XL scanner coupled with WinRHIZO Pro 2013e software (Regent Instruments Inc., Canada). The scanner was calibrated for Regent Instruments Software. The RL/SL values were calculated. Relative change of leaf number (LN) under salt stress was also counted.

Shoot and root fresh weights (SFW and RFW) were measured separately using a digital balance (Explorer Analytical, Ohaus Corporation, USA). For the dry weight (SDW and RDW) measurement, freshly harvested shoot and root samples of plants were dried in Venticell LSIS-B2V/VC55 (MMM Group, USA) drying oven at 105°C for one hour and at 80°C for another four hours; weights were measured afterwards using the digital balance. Relative water contents of shoot (SRWC) and root (RRWC) were calculated according to Yan et al. (2023) using the following formula:

$$SRWC \text{ or } RRWC = [(FW - DW) / FW] \times 100\%$$

The RFW/SFW, RDW/SDW, and RRWC/SRWC values were calculated.

Leaf relative water content (LRWC) was measured according to Turner (1986). The first fifteen leaves of a plant were removed using a fine-tipped scissor and fresh weight (FW) was recorded and floated on distilled water at 4°C. After 24 hours, leaf turgid weight (TW) was recorded. The leaves were dried using the abovementioned drying procedure and dry weight (DW) was recorded. LRWC was calculated using the following formula:

$$LRWC = [(FW - DW) / (TW - DW)] \times 100\%$$

Reactive Oxygen Species (ROS) generation assay

Hydrogen peroxide (H_2O_2) concentration was determined according to Sinha (1972) to assess the extent of reactive oxygen species (ROS) generation. A mixture of 1 g of leaf sample, 100 mg activated charcoal and 10 ml 5% trichloroacetic acid (TCA) was centrifuged at 12000 rpm for 10 min. The supernatant (0.5 ml) was mixed with 0.01 M phosphate buffer, pH 7.0 to make 1 ml volume and a 3 ml mixture of potassium dichromate and glacial acetic acid (1:3 v/v) was added. The absorbance was quantified spectrophotometrically at 570 nm and the H_2O_2 concentration was estimated against standards of 10-160 µmoles.

Lipid peroxidation assays

Malondialdehyde (MDA) assay was used as a membrane lipid peroxidation marker under salt stress-derived oxidative stress damage. MDA contents were measured using thiobarbituric (TBA) method according to Dhindsa et al. (1981). Briefly, a mixture of 0.1 g of leaf sample, 0.5% of TBA and 20% of trichloroacetic acid was heated in a hot water bath at 90°C for 30 min and then cooled in an ice bath. The supernatant was then extracted by centrifuging at 12000 rpm for 20 min. The absorbance for MDA was estimated at 532 nm spectrophotometrically and MDA contents were calculated using an extinction coefficient of 155/mM/cm.

Enzymatic antioxidants analyses

Sample preparation

Freshly harvested leaves were homogenised in 0.1 M potassium phosphate buffer, pH 7.8 in cold conditions. The homogenates were centrifuged at 4°C, 12000 rpm (Hettich, Germany) for 20 min. The supernatant obtained was stored at −80 °C to analyse the enzymatic activities.

SOD

SOD activity was measured based on the protocol of Giannopolitis and Ries (1977). The 3.7 ml reaction mixture contained 100 ml plant supernatant sample, 0.5 mM nitroblue tetrazolium (NBT), 17 mM methionine, 0.1 mM EDTA, 60 µm riboflavin, 0.1 M potassium phosphate buffer, pH 7.8. The one unit of SOD activity was defined as the quantity of enzyme required to inhibit photolytic reduction of nitroblue tetrazolium (NBT) by 50 %.

CAT

CAT activity was assayed by the Aebi (1984) method. The 1 ml reaction mixture contained 20 µl plant supernatant sample, 10 mM H_2O_2, and 0.05 M potassium phosphate buffer, pH 7.0. The decomposition of H_2O_2 by catalase is recorded by the decrease in absorbance per unit time at 240 nm.

APX

The Nakano and Asada (1987) method was used to estimate APX activity. The 1 ml reaction mixture contained 100 ml plant supernatant sample, 1.5 mM H_2O_2, 0.5 mM ascorbic acid, 0.1 mM EDTA, and 0.05 M potassium phosphate buffer, pH 7.0. The enzyme activity was measured by the rate of oxidation of ascorbic acid, which is directly followed by the decrease in absorbance at 290 nm per minute.

GPOX

GPOX activity was measured according to Pütter (1974). The reaction mixture contained 100 µl plant supernatant sample, 50 µl guaiacol solution, 30 µl H_2O_2, and 3 ml phosphate buffer, pH 7.0. The increase in absorbance was read at 436 nm, and GPOX activity was calculated using an extinction coefficient of 6.39 cm^2/µmol.

Non-enzymatic antioxidants analyses

Sample preparation

A 0.10 g sample was extracted with 10 ml of 80% ethanol for 2 hours. After centrifugation at 4000 g (Rotofix 32 A, Hettich, Germany) for 10 min, the supernatant was used for the following experiments.

Total polyphenol content

Total polyphenol content was measured according to Singleton and Rossi (1965) using the Folin-Ciocalteu reagent. A 0.1 ml sample was mixed with 0.1 mL of the Folin-Ciocalteu reagent, 1 mL of 20% (w/v) sodium carbonate and 8.8 ml of distilled water, and left in darkness for 30 min. The absorbance at 700 nm was measured spectrophotometrically. Gallic acid (25-300 mg/l; R^2=0.998) was used as a standard and the results were expressed in mg/l of gallic acid equivalent. Analysis was carried out in triplicate.

Total phenolic acid content

Total phenolic acid content was determined using the method of Jain et al. (2017). A 0.5 ml of sample extract was mixed with 0.5 ml of 0.5 M hydrochloric acid, 0.5 ml Arnova reagent (10% $NaNO_2$ +10% Na_2MoO_4), 0.5 ml of 1 M sodium hydroxide (w/v) and 0.5 ml of water. Absorbance at 490 nm was measured spectrophotometrically. Caffeic acid (1 – 200 mg/l, R^2 = 0.999) was used as a standard and the results were expressed in mg/g caffeic acid equivalents. Analysis was carried out in triplicate.

Radical scavenging activity

The radical scavenging activity of samples was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sanchéz-Moreno et al. 1998). The sample (0.4 ml) was mixed with 3.6 ml DPPH solution

(0.025 g DPPH in 100 ml ethanol). The absorbance of the reaction mixture was determined spectrophotometrically at 515 nm. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (10-100 mg/l; R^2=0.989) was used as the standard and the results were expressed in mg/g Trolox equivalents. Analysis was carried out in triplicate.

All the chemicals used in non-enzymatic antioxidants analyses were analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA) and CentralChem (Bratislava, Slovak Republic). The analyses were carried out in triplicate. The absorbances were measured using a Jenway spectrophotometer (6405 UV/Vis, England).

Proline content analysis

The proline content (µg/g FW of tissue) was determined by the procedure of Bates et al. (1973). Leaf tissues were homogenised in sulphosalicylic acid (3% w/v) and reacted with an equal volume of acid ninhydrin and glacial acetic acid for an hour at 95 °C in a water bath. The reaction mixture was further transferred to an ice bath to terminate the reaction. After 10 min, toluene was added to each tube, shaken, and the proline concentration was estimated spectrophotometrically by measuring the absorbance at 520 nm.

RNA isolation

Total RNA was isolated from 100 mg of shoot sample using the NucleoSpin RNA Plant mini kit (Macherey-Nagel, Germany) according to the kit-specified protocol. The isolated RNA was eluted in 50 µL kit-supplied RNase-free water. The quantity and purity of total RNA were estimated in NanoPhotometer P 360 (Implen GmbH, Germany) spectrophotometer.

Two-tailed qPCR (TT-PCR) assay

Expression analysis of miRNA genes was performed using the Two-Tailed qPCR Assay kit (BioVendor – Laboratorni medicina a.s., Czech Republic) adapting protocol described by Androvic et al. (2017). The protocol of the kit is biphasic. Primer sets for three miRNA genes, miR168a, miR399g and miR828a for both phases were designed by BioVendor and supplied with the kit. In the first phase, cDNA was synthesised from total RNA using the C1000 thermal cycler with a dual 48/48 fast reaction module (Bio-Rad, USA). An equal volume of total RNA (326 ng) was used in all the reactions. In the second phase, qPCR was performed using the C1000 thermal cycler with CFX96 Real-Time System optical reaction module (Bio-Rad, USA). Expressions of cyclophilin and eukaryotic transcription initiation factor 5A (EIF5A) (primers, Table S1) were used to normalise the miRNA expression levels. The specificity of qPCR products was determined based on melting curve (Cq) analysis using CFX Maestro 2.0 version 5.0.021.0616 (Bio-Rad, USA). The data was processed according to Livak and Schmittgen (2001) and expressed as fold change.

Viability assay of flax roots

The viability of flax root cells was determined by co-staining with fluorescein diacetate (FDA; Thermo Fisher Scientific) and propidium iodide (PI; Sigma Aldrich) according to a protocol of Jones et al (2016) with a few modifications. PI as a membrane-impermeable stain was used to visualise dead cells (red fluorescence) and FDA for viable cells (green fluorescence). To prepare stock solutions, five milligrams of FDA and one mg of PI were dissolved in one ml of acetone and one ml of distilled water, respectively, and stored at -20°C. The working staining solution was freshly prepared by dissolving four µl of FDA stock and ten µl of PI stock in distilled water and the volume was made up to one ml. Root samples were dipped in the stained solution and incubated for five minutes in the dark. Samples were observed using a Leica DM5500B fluorescence microscope equipped with a Leica DFC450 C digital camera (Leica Microsystems). The Leica filter set L5 (BP 480/40, DM 505, BP 527/30) was used to detect FDA staining and the N2.1 filter set (BP 515-560, DM 580, LP 590) was used for PI. The images were acquired with the LAS version 4.6 software (Leica Microsystems) and processed with the ImageJ version 1.54d software (Schneider et al. 2012).

Statistical analysis

All the experiments had three biological and three technical replicates. Data were analysed by one-way analysis of variance (ANOVA) to calculate statistical significance. The significant differences in treatment data (mean ± standard error) were estimated through Duncan's multiple range test (DMRT) at $p < 0.05$ significance level (Duncan 1955). Both analyses were done in SPSS version 19 (IBM Corporation, USA). Graphs were prepared in OriginPro 2021 version 9.8.0.200 (OriginLab Corporation, Northampton, MA, USA). Independent variable data were logarithmically transformed, standardised, and normalised for multivariate analysis using Pearson's correlation coefficient analysis (PCC), principal component analysis (PCA), and heatmap generation. Multivariate analysis was executed in R version 4.4.2 (R Core Team 2024) coupled with RStudio version 2024.09.1 Build 394 (Posit Team 2024) software.

RESULTS

Effect of salinity stress on plant morphology

Our applied 100 mM NaCl salt stress was not lethal to both flax genotypes. However, their differential response to salt stress was evident from several morphological assessments (Fig. 1 a-c, e-g, and S1; Table 1, and S2) and visual (Fig. 1d) perspectives.

Salt stress arrested the shoot growth and affected the overall root morphology of both genotypes. Stress-treated shoots of Flanders were significantly shorter than the control, but this change was non-significant in Astella. Compared to controls, the root length of stressed Flanders did not change significantly; a significant length increase was noted in Astella roots. Although the relative shoot length (SL) difference between the two genotypes was non-significant, the relative root length (RL) change of stressed Astella was significantly higher than that of Flanders indicating elongated Astella roots under salt stress (Fig. 1a; Table S2). The higher RL/SL ratio also showed that the salt-stressed roots grew much faster than the shoots of Astella compared to Flanders (Table 1). Salinity decreased root diameter (RD) and root volume (RV) in both genotypes, however, RD changed in Flanders non-significantly. Moreover, on the one hand, the reduction of stress-mediated relative root diameter (RD) of Astella was higher than the control compared to Flanders (Fig. 1a; Table S2). On the other hand, stress decreased the relative root volume (RV) of Astella less than Flanders (Fig. 1b; Table

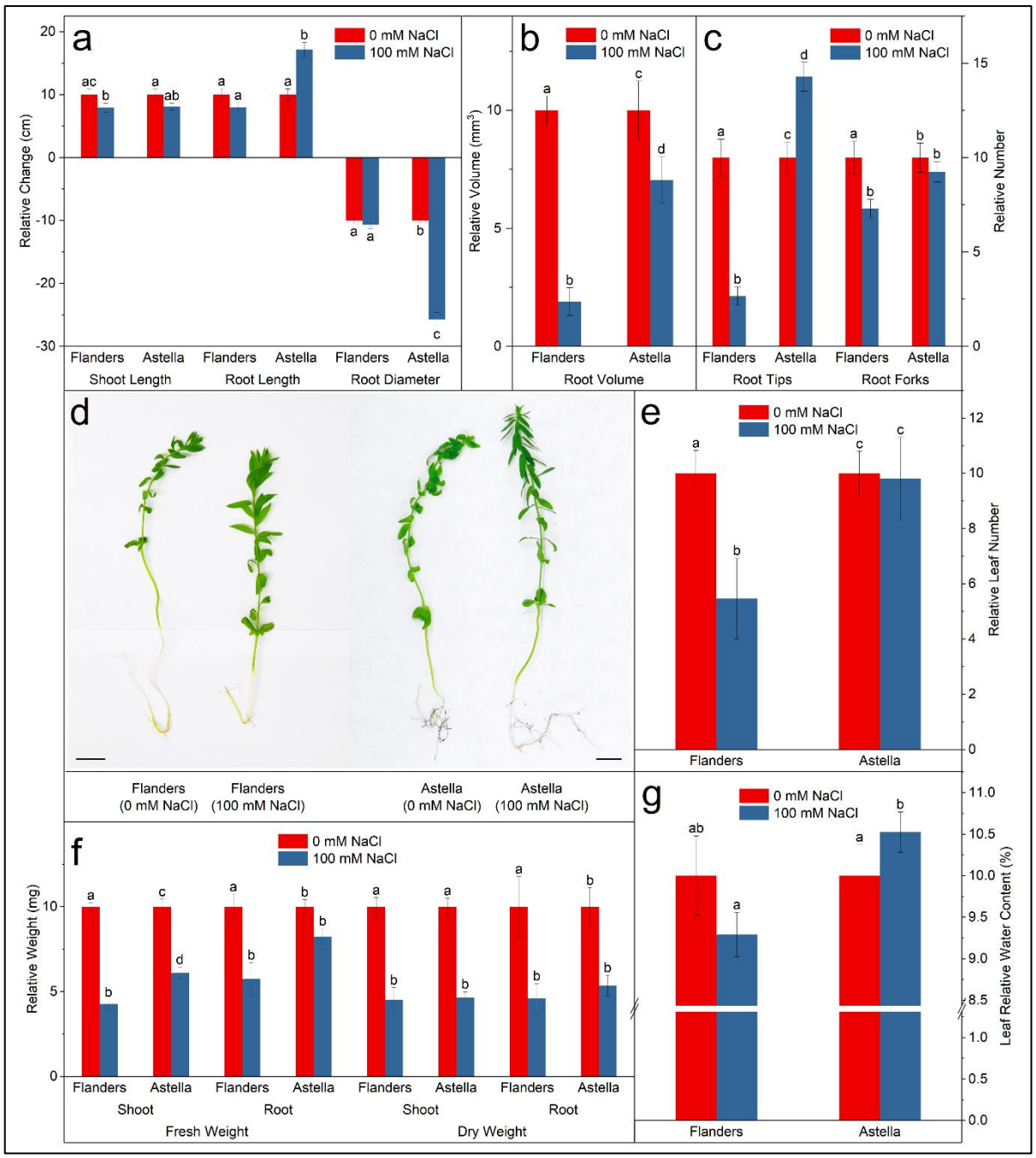

Fig. 1 Changes of several morphological parameters of two flax varieties, Flanders and Astella, under one week of 100 mM NaCl stress. Data represent relative changes during the stress period, except for leaf relative water content at the end of NaCl stress. Data were normalised to the control treatment. **a** Shoot length, root length and diameter; **b** root volume; **c** root tips and forks; **d** a photograph depicting comparative morphology between Flanders and Astella in control and stressed conditions (scale bar = 10 mm); **e** leaf number; **f** relative water contents of shoot and root; **g** leaf relative water content; **h** fresh and dry weights of shoots and roots; **i** root density. Data represent mean ± standard errors (n = 3). Different letters followed by the means represent statistical differences at $p < 0.05$ (one-way ANOVA with DMRT post hoc test). Refer to the abbreviation list for the full forms

Table 1 Ratio of different shoot and root parameters of two flax genotypes Flanders and Astella under 100 mM NaCl treatment

Genotype	RL/SL	RFW/SFW	RDW/SDW	RRWC/SRWC
Flanders	1.01	1.35	1.02	1.03
Astella	2.12	1.35	1.16	1.01

The data were estimated as relative changes under the stress period, normalised against control treatment. The ratios of normalised relative values are represented in this table. The ratios of control treatment values are omitted. Refer to the abbreviation list for the full forms

S2). As a result, the Astella root system became significantly thinner but more voluminous than Flanders during stress treatment. The root relative tip (RT) and fork (RF) numbers indicate root branching (RB) status. Salt stress reduced Flanders' RT and RF. Although the RT of Astella increased under stress, the RF decreased. Among the two genotypes, higher RT and RF of Astella depicted a highly branched root system architecture (RSA) under stress than Flanders (Fig. 1c; Table S2).

We tested relative changes in fresh and dry weights (FW and DW) of shoots (SFW and SDW) and roots (RFW and RDW) in Flanders and Astella to understand plant biomass change under stress. Stress significantly decreased biomass except for Astella RFW and RDW, indicating salinity affected Astella roots less (Fig. 1f; Table S2). The equal RFW/SFW ratio between the two genotypes indicated that their stress-mediated FW change was similar. Also, a higher RDW/SDW ratio in Astella showed a steep RDW increase than in Flanders under stress (Table 1). These findings collectively suggested that Astella RDW change was less affected by salinity and the change was higher than Flanders. Moreover, the salt stress-mediated non-significant shoot and root relative water content (SRWC and RRWC) change (Fig. S1) and a nearly equal RRWC/SRWC ratio (Table S2) of Flanders and Astella represented a similar water content change in both shoots and roots of these plants under salinity. Interestingly, leaf relative water content (LRWC), a vital marker of salt stress, was significantly increased in Astella compared to Flanders under stress (Fig. 1g; Table S2). Stress decreased relative leaf numbers (LN) in both genotypes. However, Astella's relative LN was significantly higher than Flanders's (Fig. 1e; Table S2).

The overall morphological analyses represented that despite having thinner roots, Astella maintained more elongated, branched, and voluminous RSA, biomass was less affected, held more leaf water, and developed more leaves than Flanders under 100 mM NaCl stress (Fig. 1d).

the Flanders root system became significantly thicker but less voluminous than Astella ($p < 0.05$). The root relative tip (RT) and fork (RF) numbers indicate root branching (RB) status. Salt stress reduced

Effect of salinity stress on ROS generation and cell damage

The cellular extent of salt stress-derived ROS generation and subsequent damage can be estimated by H_2O_2 and lipid peroxidation assays, respectively. Malondialdehyde (MDA) is the final product of lipid peroxidation and is widely considered an oxidative stress-mediated cell membrane damage indicator. Cumulatively, H_2O_2 and MDA are salt stress markers (Soltabayeva et al. 2021). Stress significantly increased the H_2O_2 and MDA generation in the treatment plants and the contents were higher in stress-treated Astella than in Flanders (Fig. 2; Table S3). The results suggested that Flanders was experiencing more salt toxicity than Flanders.

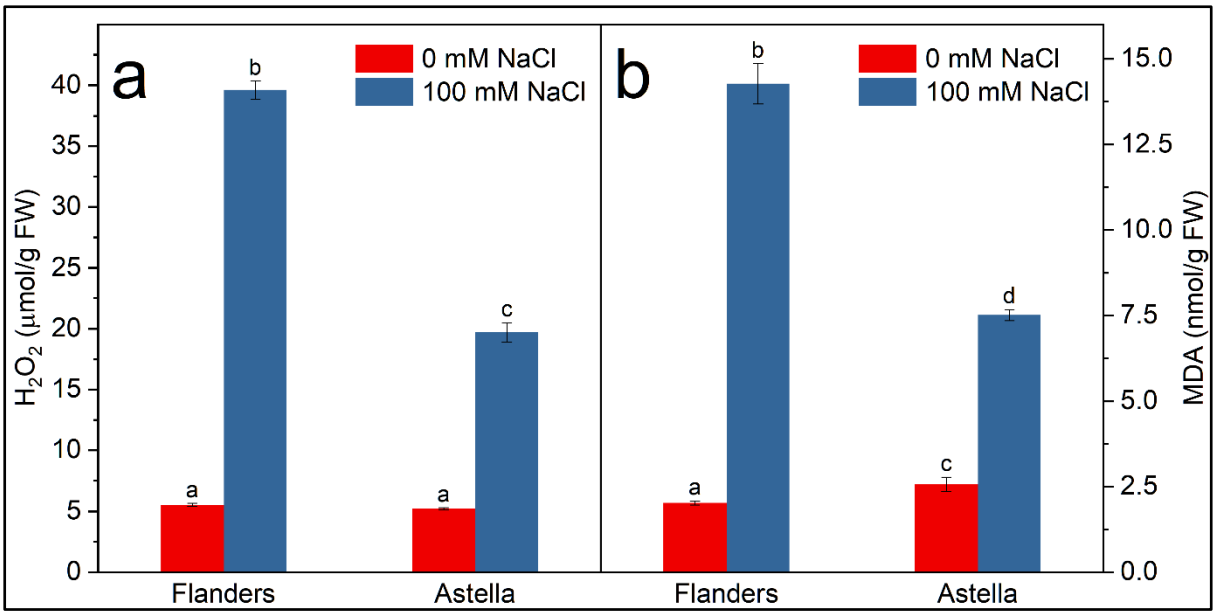

Fig. 2 Biochemical estimation of ROS and associated cell damages in two flax varieties Flanders and Astella after one week of 100 mM NaCl stress. In our study, we represented **a** H_2O_2 as the ROS marker and, **b** MDA content as the cell damage marker. ROS is a regular byproduct of cellular metabolism. Antioxidants scavenge ROS molecules to create a neutral redox potential inside the cell. ROS overproduction during salt stress disturbs the redox state of the affected cells, generating the "oxidative burst" situation. Excessive ROS molecules damage lipid bilayer membranes by lipid oxidation generating MDA as the end-product. Data represent mean ± standard errors (n = 3). Different letters followed by the means represent statistical differences at $p < 0.05$ (one-way ANOVA with DMRT post hoc test). Refer to the abbreviation list for the full forms

Effect of salinity stress on antioxidant system

Cellular stress activates plant antioxidant enzymes. We found several enzymatic and non-enzymatic antioxidant markers were significantly hyperactivated in Flanders and Astella upon NaCl stress treatment. However, the stress-mediated responses were genotype-dependent.

We observed significantly elevated levels of important enzymatic antioxidant markers, SOD, CAT, APX, and GPOX and two vital non-enzymatic antioxidant markers, polyphenols (PP) and phenolic acids (PA), in stress-treated Astella compared to Flanders (Fig. 3, 4a, b; Table S3). The DPPH assay-estimated total radical scavenging activity of antioxidants in Astella was significantly higher than in Flanders (Fig. 4c; Table S3).

One vital component of the cellular stress mitigation system is proline, which contributes to osmoregulation and antioxidant activity. Treatment plants of both genotypes had significantly higher proline accumulation than the respective controls. Also, salinity significantly elevated proline content in Astella more than in Flanders (Fig. 4d; Table S3).

All these biochemical tests to assess antioxidant activity revealed that the antioxidant system of both genotypes was heightened under stress. The salt stress-mediated antioxidant hyperactivity of Flanders than Astella was also notable.

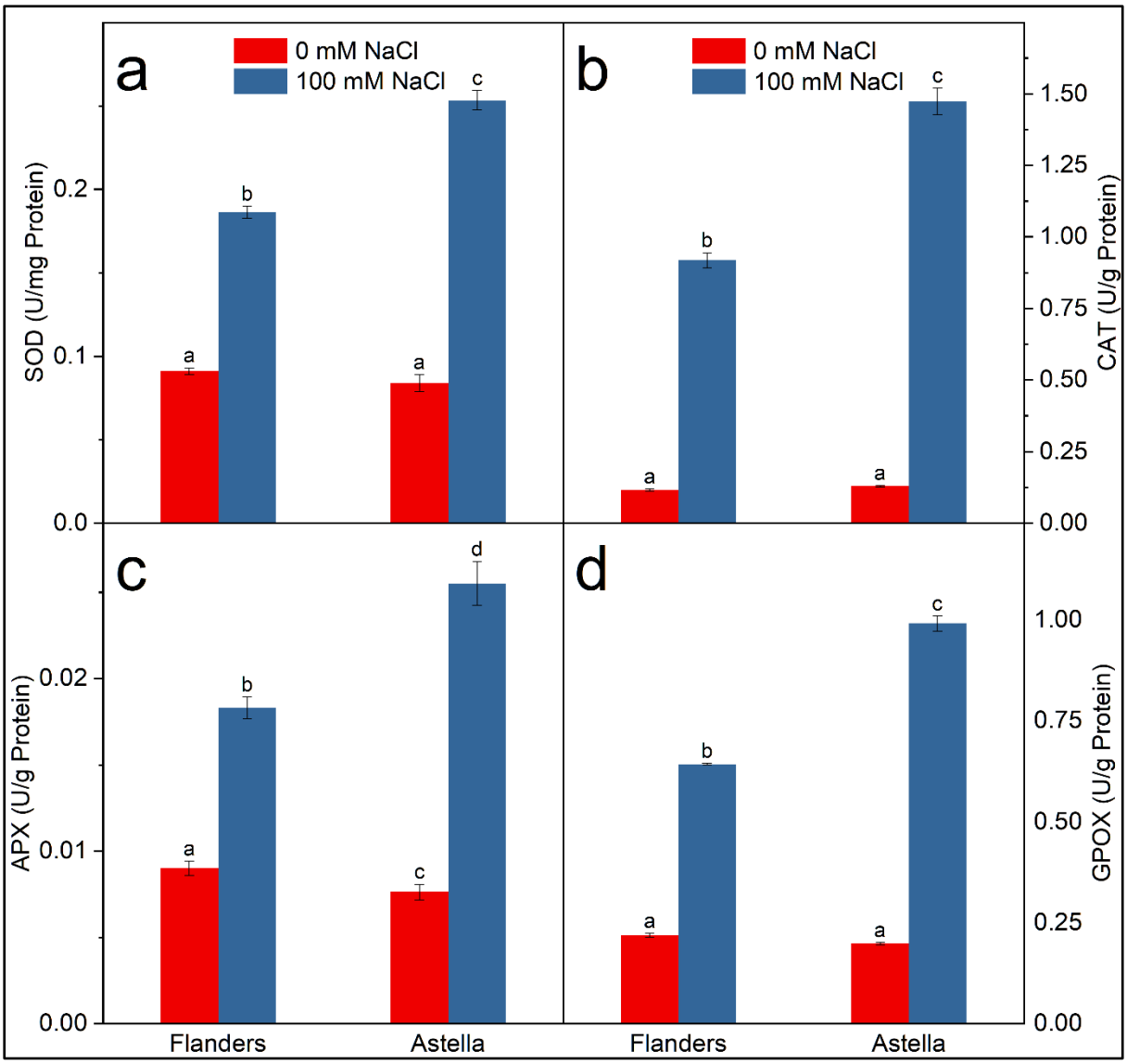

Fig. 3 Biochemical estimation of enzymatic antioxidants in two flax varieties Flanders and Astella after one week of 100 mM NaCl stress. In response to the salt stress-mediated "oxidative burst", plants activate a well-synchronised antioxidant system for ROS scavenging. **a** The frontline defence is provided by SOD by converting superoxide ($O_2^{\bullet-}$) to H_2O_2; **b** CAT, **c** APX, and **d** GPOX converts H_2O_2 to H_2O and O_2. Data represent mean ± standard errors (n = 3). Different letters followed by the means represent statistical differences at $p < 0.05$ (one-way ANOVA with DMRT post hoc test). Refer to the abbreviation list for the full forms

Effect of salinity stress Lignan-related miRNAs

In this experiment, we monitored the expression of three miRNA molecules, miR168a, miR399g, and miR828a using the two-tailed qPCR (TT-PCR) assay. We found that all these molecules were upregulated under NaCl stress in both genotypes. Expressions of these miRNAs were significantly higher in salt-stressed Astella than in Flanders (Fig. 5).

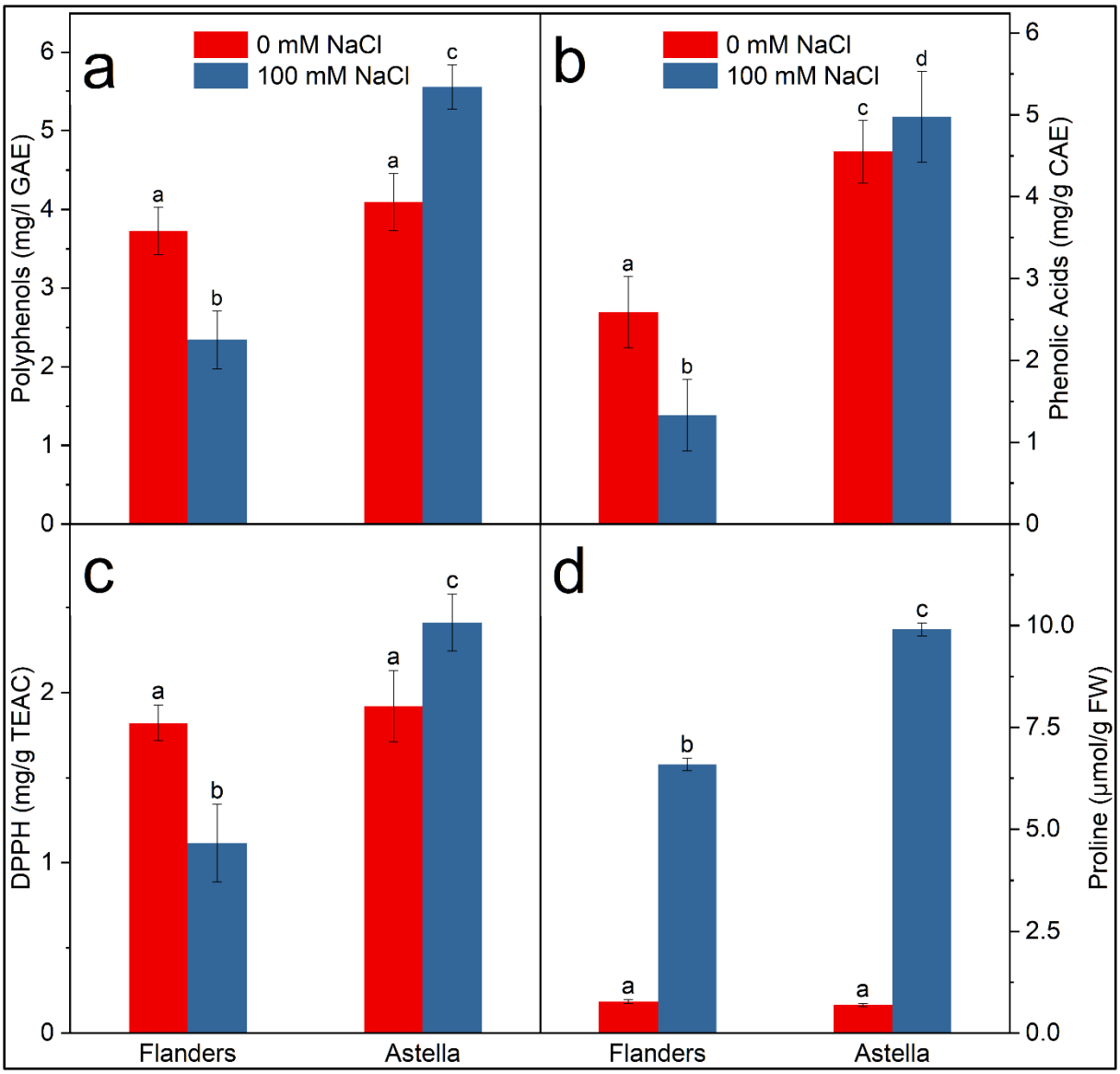

Fig. 4 Biochemical estimation of non-enzymatic antioxidants, total radical scavenging activity, and proline content in two flax varieties Flanders and Astella after one week of 100 mM NaCl stress. The non-enzymatic antioxidants **a** polyphenols and **b** phenolic acids are excellent antioxidants due to the presence of multiple hydroxyl groups; the total radical scavenging activity was measured using **c** DPPH assay; **d** proline provides cellular protection against salt stress in numerous ways. Data represent mean ± standard errors (n = 3). Different letters followed by the means represent statistical differences at $p < 0.05$ (one-way ANOVA with DMRT post hoc test). Refer to the abbreviation list for the full form

Effect of salt stress on roots cell viability

We observed the viability of NaCl stress-affected roots under the microscope. Alive and dead cells appeared green and red respectively, through differential FDA-PI staining. In both genotypes, root tips (Fig. 6a, b) and lateral root buds (Fig. 6c, d) were alive; the epidermis of the rest of the roots was dead. We found a sporadic pattern of live cells in the root maturation zone (Fig. 6e, f). Root cross-sectional views depicted thriving root tissues despite the damaged epidermis layers (Fig. 6g, h).

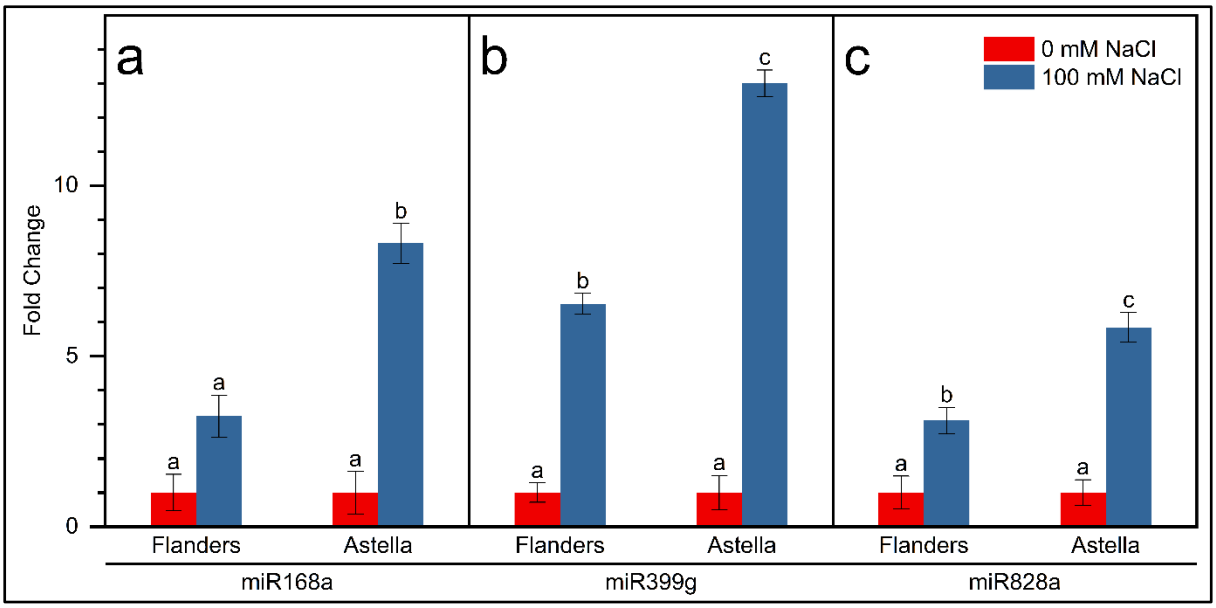

Fig. 5 Genomic expression analysis of lignan-miRNAs in two flax varieties Flanders and Astella after one week of 100 mM NaCl stress. The miRNAs were isolated from treatment plants and the relative expressions of **a** miR168a, **b** miR399g, and **c** miR828a were measured using the two-tailed qPCR assay kit using cyklophilin and EIF5A as internal controls for normalisation. Data represent mean ± standard errors (n = 3). Different letters followed by the means represent statistical differences at $p < 0.05$ (one-way ANOVA with DMRT post hoc test). Refer to the abbreviation list for the full forms

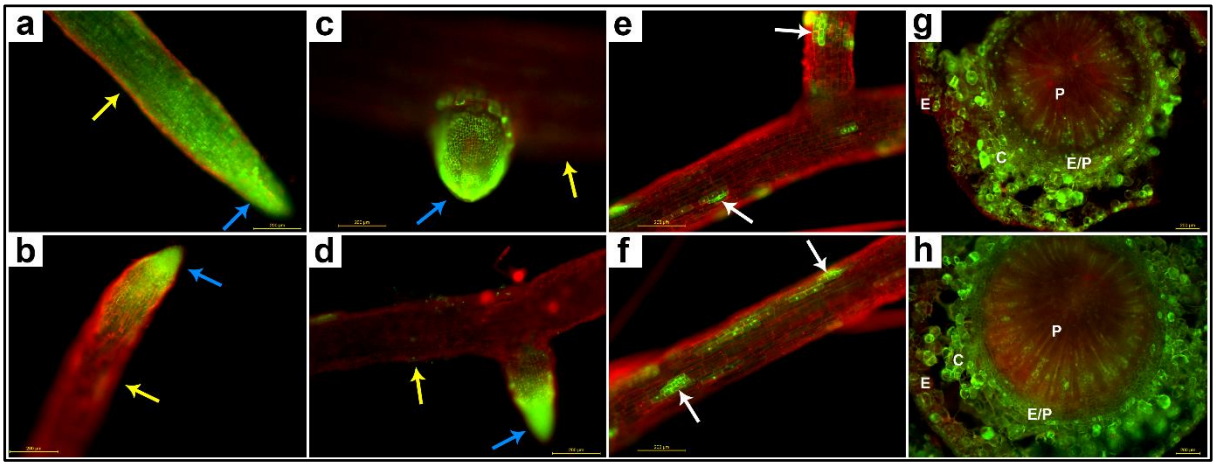

Fig. 6 Fluorescence microscopic study of root cell viability of flax varieties Flanders and Astella using FDA-PI differential staining after one week of 100 mM NaCl stress. Alive cells were green and dead cells were red. **a**, **c**, **e**, and **g** are Flanders roots; **b**, **d**, **f**, and **h** are Astella roots. Root tips (blue arrows in Figures A and b) and lateral root buds (blue arrows in Figures C and d) were alive in both genotypes. The root epidermal layer was mostly dead (yellow arrows in Figures A, b, c, and d). A sporadic pattern of live cells was found in the root maturation zone (white arrows in Figures E and f). Cross-sectional views also depicted the dead epidermal layer (E in Figures G and h). *C* cortex, *E* epidermis, *E/P* endodermis/pericycle, *P* pith

Multivariate analysis

Data of 26 independent variables were linearised by logarithmic transformation, standardised to keep the means of each treatment at 0 with a standard deviation of 1, and normalised to create an evenly distributed data matrix. The heatmap showed the abundance of the tested variables on the treatment individuals. Although a similar abundance pattern of the variables was visible among the treatments without stress (C.F and C.A), it differed between the salt-treated samples (S.F and S.A; Fig. 7a). Salt-stress markers H_2O_2 and MDA were more abundant in salt-treated Flanders (S.F) than in Astella (S.A). Relative to these stress markers, the abundance of proline, enzymatic antioxidants (SOD, CAT, APX, GPOX), non-enzymatic antioxidants (PP, PA), DPPH, and miRNAs (miR168a, miR399g, and miR828a) were respectively higher and lower in S.A and S.F. Moreover, the heatmap pattern depicted the negative effect of salinity on morphological markers, except RL and LRWC, relative to H_2O_2 and MDA in S.A but not in S.F.

Pearson's correlation coefficient (PCC) showed the overall correlation of the variables in our experiment. We considered different correlation coefficient (r) thresholds according to Ratner (2009). We observed that H_2O_2 and MDA had a high positive ($r \geq 0.7$) correlation with proline, enzymatic antioxidants and miRNAs, and a high negative ($r \leq -0.7$) correlation with morphological markers such as LN, SDW, RV, SL, SFW, and RFW (Fig. 7b; Table 2). These findings complemented the data obtained in the heatmap analysis. Moreover, we considered these variables the most important for the study. The moderately negative ($-0.7 \leq r \leq -0.3$) correlation of LRWC, PP, RF, DPPH, PA, RT, and RDW with salinity markers suggested their lesser importance in our study.

We verified the PCC findings with principal component analysis (PCA). In the PCA plot, proline, SOD, CAT, APX, GPOX, miR168a, miR399g, and miR828a were clustered together with H_2O_2 and MDA confirming their positive correlation with salinity stress. Morphology-related variables except RT, LRWC, and RL were nearly opposite to this cluster showing their inverse relationship with salinity stress. RT, LRWC, RL, DPPH, PA, and PP were mostly orthogonal to the salinity markers suggesting their minimal contribution (Fig. 7b). We considered principal components (PCs) with eigenvalue > 1 for further analysis. The PC1 and PC2 variances were 62.27% and 28.47%, respectively. Cumulatively, PC1 and PC2 represented the 90.74% variance of our study. PC1 had a 62.27% variance and explained the most contributing variables in this study. The order of loading values explains the magnitude of contributions of variables to their principal component. The loading value of MDA ($|r| = 0.24$) was higher than H_2O_2 in PC1. The higher loading values of CAT, Proline, miR399g, GPOX, SDW, and SFW than MDA ($|r| \geq 0.24$) suggested that they were more strongly correlated with the salinity markers and we considered them as the most contributing salinity stress variables. With a variance of 28.47%, PC2 explained the second most contributing study variables. Here, the loading value of H_2O_2 ($|r| = 0.12$) was higher than MDA. Non-enzymatic antioxidants, miR168a, and morphological variables such as RT, RL, LRWC, LN, RV, RD, RDW, and RF had higher variance than H_2O_2 ($|r| \geq 0.12$). They were the second most contributing salinity stress variables. PC3 had a 2.32% variance. The loading value of H_2O_2 ($|r| = 0.08$) was higher than MDA in this PC. The unique entries of SL, RFW, and APX with higher loading than H_2O_2 ($|r| \geq 0.08$) made them the least important salinity stress variables (Table 3).

Table 2 Variable loadings, eigenvalues, and variances in principal component analysis

Variables	PC1	Variables	PC2	Variables	PC3
		Positive Loadings			
CAT	***0.24953883***	***PP***	***0.37268083***	***PA***	***0.200196516***
Proline	***0.24792412***	***DPPH***	***0.36577779***	***SL***	***0.165937885***
miR399g	***0.24539655***	***RT***	***0.35691304***	***RT***	***0.046761127***
GPOX	***0.24283295***	***PA***	***0.34788717***	*SDW*	*0.007009944*
MDA	***0.24245284***	***RL***	***0.33839559***	*PP*	*0.005151547*
SOD	**0.2417349**	***LRWC***	***0.30173728***	*MDA*	*0.003713172*
miR828a	**0.24023291**	***LN***	***0.22407767***	SFW	0.002088378
H_2O_2	**0.23917004**	***RV***	***0.19080559***		
APX	0.23815336	***miR168a***	***0.1323565***		
miR168a	0.23309862	miR828a	0.10196622		
RL	0.07561718	SFW	0.10055164		
		SL	0.09690029		
		SOD	0.07628466		
		APX	0.07544624		
		GPOX	0.07288533		
		miR399g	0.07217699		
		CAT	0.04852053		
		Proline	0.03513028		
		Negative Loadings			
SDW	***-0.2517897***	***RD***	***-0.2011469***	***RD***	***-0.47730637***
SFW	***-0.2441553***	***RDW***	***-0.1595287***	***RF***	***-0.41671427***
SL	-0.23631946	***RF***	***-0.1180865***	***LRWC***	***-0.35926876***
RV	-0.21340764	***H_2O_2***	***-0.1151618***	***RFW***	***-0.34709459***
RDW	-0.21216207	*MDA*	*-0.1091776*	***RDW***	***-0.22587523***
RFW	-0.20782049	RFW	-0.08271598	***RL***	***-0.20366549***
LN	-0.18933139	SDW	-0.0425856	***LN***	***-0.1911627***
RF	-0.17898057			***APX***	***-0.18179645***
RD	-0.10888689			***SOD***	***-0.14526618***
RT	-0.0753388			***GPOX***	***-0.13814204***
LRWC	-0.05846306			***Proline***	***-0.12173121***
DPPH	-0.05526618			***RV***	***-0.1173679***
PA	-0.05430071			***miR168a***	***-0.11276602***
PP	-0.02717064			***miR828a***	***-0.10908201***
				miR399g	***-0.10480101***
				H_2O_2	***-0.08073587***
				CAT	*-0.07729502*
				DPPH	-0.011045939
		PCA Result			
Eigenvalues	15.60		7.12		2.32
Variance	62.27		28.47		9.26

Only independent variables were analysed. |Values| ≥ |loadings| of H_2O_2 and MDA were marked in bold and italics, respectively. Refer to the abbreviation list for the full forms

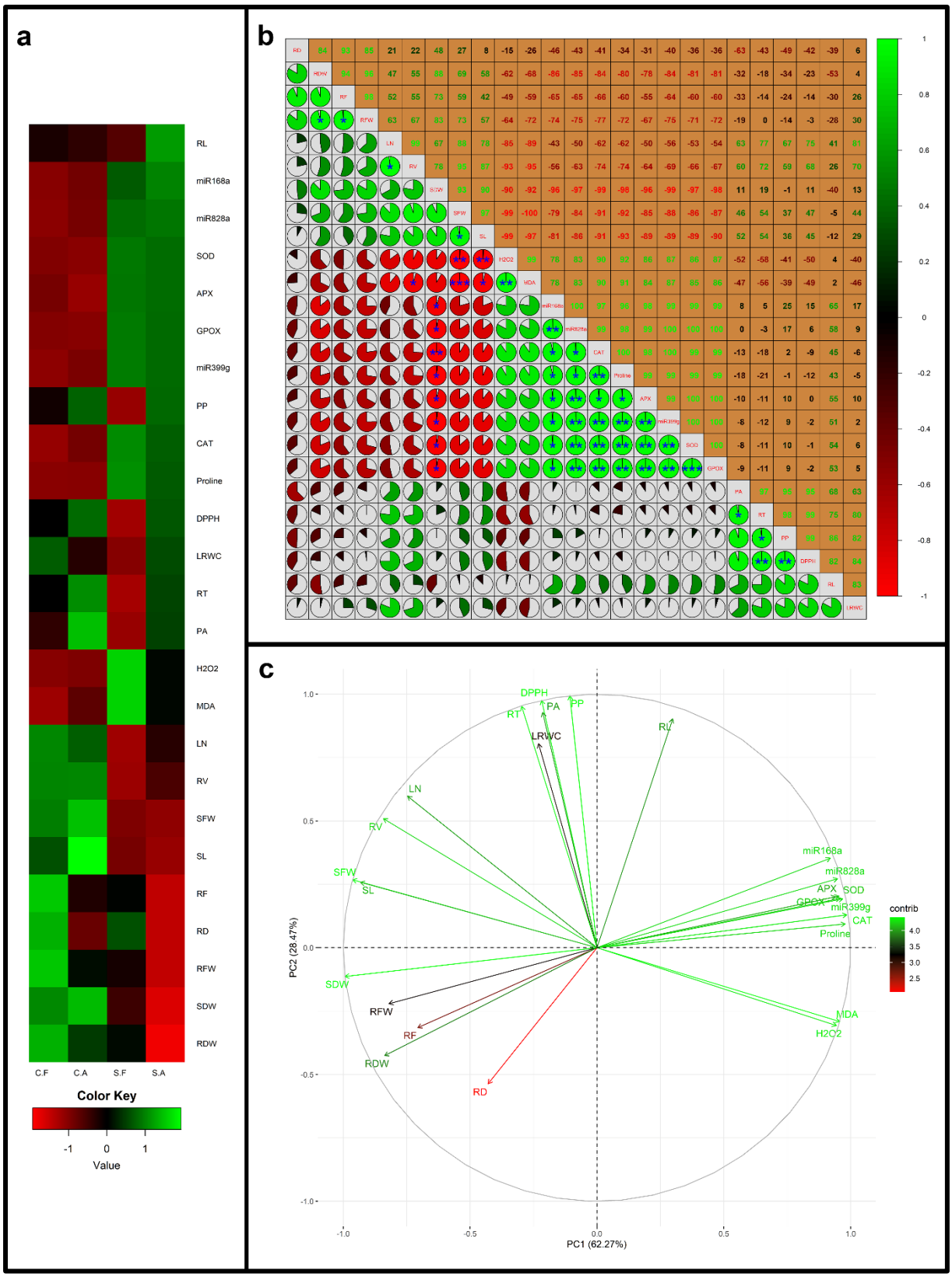
a
RL
miR168a
miR828a
SOD
APX
GPOX
miR399g
PP
CAT
Proline
DPPH
LRWC
RT
PA
H2O2
MDA
LN
RV
SFW
SL
RF
RD
RFW
SDW
RDW
C.F
C.A
S.F
S.A
Color Key
-1
0
1
Value
b
c
DPPH
PA
PP
RT
LRWC
RL
LN
RV
SFW
SL
miR168a
miR828a
APX
SOD
GPOX
miR399g
CAT
Proline
SDW
RFW
RF
RDW
RD
MDA
H2O2
contrib
4.0
3.5
3.0
2.5
PC2 (28.47%)
PC1 (62.27%)

Fig. 7 Multivariate analyses using heatmap, PCC, and PCA using the tested independent variables. Data was obtained after providing 100 mM NaCl stress for one week on two flax varieties Flanders and Astella. Data from 26 independent variables were logarithmically transformed, standardised, and normalised for these analyses. **a** Heatmap analysis represented the abundance of the tested variables on the treatment individuals; **b** PCC analysis represented the correlation constants as pie figures and the percentage of the correlation values as numbers in the plot; **c** PCA plot represents the loading values of different variables measured in this study. PC1 and PC2 described a total of 90.74% variations. Refer to the abbreviation list for the full forms

Table 3 Pearson's correlation of different independent variables with H_2O_2 and MDA

Variables	H_2O_2		MDA	
	Correlation	Annotation	Correlation	Annotation
H_2O_2	++++	Perfect	+++	High
MDA	+++	High	++++	Perfect
Proline	+++	High	+++	High
CAT	+++	High	+++	High
miR399g	+++	High	+++	High
GPOX	+++	High	+++	High
SOD	+++	High	+++	High
APX	+++	High	+++	High
miR828a	+++	High	+++	High
miR168a	+++	High	+++	High
RL	+	Weak	+	Weak
RD	-	Weak	-	Weak
LRWC	--	Moderate	--	Moderate
PP	--	Moderate	--	Moderate
RF	--	Moderate	--	Moderate
DPPH	--	Moderate	--	Moderate
PA	--	Moderate	--	Moderate
RT	--	Moderate	--	Moderate
RDW	--	Moderate	--	Moderate
RFW	--	Moderate	---	High
LN	---	High	---	High
SDW	---	High	---	High
RV	---	High	---	High
SL	---	High	---	High
SFW	---	High	---	High

Only independent variables were analysed. Pearson's correlation coefficients are represented in Table S3. Correlation markings and annotations: $r = 1$, '++++', perfect; $|r| \geq 0.7$, '+++' or '---', high; $0.7 \geq |r| \geq 0.3$, '++' or '--', moderate; $|r| \leq 0.3$, '+' or '-', weak; $r = 0$, '----', unrelated (Ratner 2009). Refer to the abbreviation list for the full forms

DISCUSSION

Multiple abiotic stresses, including salinity stress, reduce the productivity of the lignan-rich muti-utilitarian crop flax (Goyal et al. 2014; Yadav et al. 2022). Flax salt tolerance has been assessed in many studies (Li et al. 2022a, b; Siddiqui et al. 2022; Wang et al. 2022; Hussein 2023; Yaşar 2023; Bayram et al. 2024; Yadav 2024). Lignans have been reported to be useful in abiotic stress mitigation in other plants except flax (Paniagua et al. 2017; Ghotbzadeh Kermani et al. 2019; Sharma et al. 2019; Mwamba et al. 2020; Ražná et al. 2021). Therefore, for the first time, we assessed the unexplored role of lignan in flax salinity stress mitigation in this study. We selected two flax varieties Flanders (SDG: 7.75 mg/g) and Astella (SECO: 13.07 mg/g) of contrasting lignan content and compared their performance under salinity stress. We applied 100 mM NaCl stress as per the previous reports of salinity stress studies on flax (Qayyum et al. 2019; Varga et al. 2020; Hussein and Alshammari 2022; Li et al. 2022a; Wang et al. 2022; Zeki Koçak et al. 2022). We carried out our experiments 21-d post-germination of flax plants because flax undergoes a transition of slower to faster growth at this age (Li et al. 2022b). Salinity initiates two stresses in plants – osmotic and ionic. The osmotic stress mostly affects plant morphology. In ionic stress, physiologic ion and nutrient homeostasis imbalance leads to oxidative stress-mediated cell damage (Munns and Tester 2008). We assayed various morphological and biochemical markers to understand the effect of these two phases. Additionally, we carried out genomic and microscopic assays to comprehend the study in detail.

Salinity stress caused more growth arrest in Flanders than in Astella

Our morphological analyses revealed that salt stress differentially impacted Flanders and Astella. Salinity decreased relative SL, RV, RT, and RF in Flanders while RL and RD were unaffected compared to the control. In Astella, stress did not affect relative SL, increased RL and RT, and decreased RD, RV and RF compared to the control (Fig. 1a, b, c, d; Table S2). Plant roots are the first organ to perceive salinity stress. In saline conditions, less soil water and nutrient availability create physiologic drought in plants, initiating osmotic stress. Plants respond immediately with growth reduction in this phase affecting aerial parts too (Munns and Tester 2008). Salinity-mediated arrest of several shoot and root parameters of Flanders and Astella were thought to occur via gene expression reprogramming and altered hormonal homeostasis of plants under osmotic stress that decreases cell division, cell elongation, and meristem size and activity (West et al. 2005; Bartels and Sunkar 2005). Such growth attenuation under saline conditions was commonly observed in other plants (Ma et al. 2010; Li et al. 2013; Colaneri et al. 2014; Zeeshan et al. 2020; Lu et al. 2023) including flax (Mekawy et al. 2020; Li et al. 2022a, b; Wang et al. 2022). Under stress, Astella had similar SL, increased RL, RV, RT, RF and RL/SL, and decreased RD compared to Flanders. Such a contradictory observation between genotypes was reported previously and explained as the genotype-specificity of stress tolerance (Robin et al. 2016; Saddiq et al. 2021). Roots show remarkable plasticity under stress. Although salinity usually decreases root length, sometimes stress-mediated root length and branching increase due to an attempt of roots to access water further down in the soil profile to avoid the high salt concentration near the surface. While a decrease in RD might seem negative, it can also be part of a plant's adaptive strategy to minimise salt uptake by reducing the root's contact with the saline soil (McLoughlin et al. 2012; Arif et al. 2019; Yu et al. 2022; Hu et al. 2023). Therefore, the improved root parameters designated a better RSA in Astella, and coupled with it, a nearly unchanged stress-mediated shoot growth represented a better salt-stress adaptiveness of Astella over Flanders.

Along with the visual morphology of flax plants, biomass is also altered under salinity stress in our study. Salt stress affected relative shoot biomass (SFW and SDW) of both genotypes and relative root biomass (RFW and RDW) in Flanders but not in Astella (Fig. 1f; Table S2). Salt stress-mediated physiologic drought and cellular nutritional imbalance-derived oxidative stress damage vital photosynthetic enzymes and lead to reduced photosynthesis in affected plants (Munns 2002; Ueda et al. 2003; Mekawy et al. 2020; Yildiz et al. 2020; Zhao et al. 2021). Photosynthesis impairment results in drastic growth rate reduction-derived low plant biomass (De Herralde et al. 1998; Bartels and Sunkar 2005). Despite salinity, the unaffected root biomass of salt-treated Astella represented its better stress management in roots than Flanders, also evidenced by visual morphologic findings. In saline conditions, >1 ratios of RFW/SFW, and RDW/SDW represented increased biomass of roots than shoots in both salt-treated flax genotypes (Table 1). When compared between Flanders and Astella, an equal RFW/SFW ratio evidenced their indifferent root-shoot FW growth rate under stress. However, the higher RDW/SDW ratio of Astella represented that it can maintain root dry weight under salt stress better than Flanders; it could be due to physiological adaptations like deeper root penetration in soil for water uptake, improved RSA, or enhanced ion exclusion mechanisms to lower physiologic salt concentration (Bartels and Sunkar 2005; Acosta-Motos et al. 2017). Therefore, salinity-mediated improved root biomass indicated a better stress management approach for Astella than Flanders. Our results are consistent with previous findings (Sengupta and Majumder 2009; Hu et al. 2012; Wang et al. 2020, 2022).

Moreover, salt stress inhibits leaf initiation possibly by suppressing leaf primordia (Dolatabadian et al. 2011; Farooq et al. 2015). We also found relative LN reduction upon stress treatment (Fig. 1e; Table S2) that possibly contributed to further photosynthesis reduction on a whole plant level. The statistically non-significant minuscule reduction of stressed Astella relative LN from control stated that new leaf initiation was not much affected under stress in this genotype. On the contrary, the significantly higher relative LN of Astella indicated a comparatively lower progressive damage rate and better health of leaf initiation mechanism than Flanders during the stress period as reported previously (Yildrim et al. 2008; Rahneshan et al. 2018; Dikobe et al. 2021).

Relative water contents are important plant traits for salinity stress assessment (Tao et al. 2021; Ahmed et al. 2022). The significantly higher LRWC of salt-treated Astella than Flanders (Fig. 1g; Table S2) and their statistically non-significant change of SRWC and RRWC in all treatments (Fig. S1; Table S2) signified that despite the similar water content of shoot and root, the leaf of Astella was holding more water than Flanders under stress. Higher LRWC often signifies better salinity stress management in various plants (Rodríguez et al. 2005; Esringü et al. 2005; Boussora et al. 2024) including flax (Siddiqui et al. 2022; Li et al. 2022b), indicating better salt stress management of Astella than Flanders in our study.

The results depicted that salt stress did not affect Astella shoot growth. Moreover, compared to Flanders, Astella improved RSA, increased LN, and retained more leaf water under salinity. These are well-known stress-adaptive phenomena.

Salinity stress was causing more harm to Astella

We found a significant increase in ROS (expressed by H_2O_2) and lipid peroxidation (expressed by MDA) in salt-stressed Flanders and Astella. (Fig. 2a, b; Table S3). The production of ROS molecules such as eukaryotic transcription initiation factor 5A (EIF5A)($O_2^{\bullet-}$), hydroxyl ($^{\bullet}OH$), alkoxyl ($RO^{\bullet}$) and peroxyl ($ROO^{\bullet}$) radicals, hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and ozone (O_3) are natural

byproducts of normal cellular metabolic processes. Plant cells generally maintain a dynamic balance of ROS and ROS-scavenging antioxidants to achieve a neutral redox potential state (Hasanuzzaman et al. 2019; Sachdev et al. 2021). The balance alters when salinity stress overwhelms the antioxidant defence and causes electron leakage by disrupting the electron flow in photosynthesis, photorespiration, and electron transport chain apparatuses. These leaked electrons partially reduce O_2 and trigger ROS. It initiates the so-called cellular "oxidative burst". The most dangerous outcome of oxidative stress is ROS-mediated lipid peroxidation which generates MDA. Lipid peroxidation destroys cell membranes causing cell death (Ahmad et al. 2008; Gill and Tuteja 2010). H_2O_2 and MDA have been considered important stress markers in many previous studies with flax (Alaei et al. 2020; Kiani et al. 2020; Hussein and Alshammari 2022; Siddiqui et al. 2022; Wang et al. 2022). Elevated ROS and lipid peroxidation are reported to be associated with salt susceptibility compared to a tolerant variety in many plants such as rice (Chawla et al. 2013; Kaur et al. 2016), *Brassica juncea* (Kumar et al. 2018), wheat and barley (Zeeshan et al. 2020; Ashraf et al. 2023) and safflower (Vijayalakshmi et al. 2016). In our study, the significantly higher ROS and MDA levels in Flanders signified its greater ROS-mediated cell damage and lower salt tolerance than in Astella. It also indicated the existence of a comparatively better ROS scavenging system in Astella compared to Flanders that minimises ROS and associated cell damage.

Salinity stress hyperactivated the antioxidant system in Flanders

Plants activate the antioxidant defence system to mitigate oxidative stress. In our study, enzymatic antioxidants (SOD, CAT, APX, and GPOX) were upregulated upon salt stress in both genotypes (Fig. 3a-d; Table S3). The antioxidant system of Astella was significantly upregulated compared to Flanders after stress application. SOD gives frontline defence against ROS by dismuting $O_2^{\bullet-}$ to H_2O_2 (Gill and Tuteja 2010). H_2O_2 is quickly scavenged by CAT, GPOX, and APX and converted to H_2O and O_2 (Carrasco-Ríos and Pinto 2014; Hasanuzzaman et al. 2019; Mekawy et al. 2020). In plants, H_2O_2 has a dual role – 1) at low concentrations, it acts as a signalling molecule to activate various pathways to alleviate stress; 2) at high concentrations, it acts as a toxic substance and initiates programmed cell death (PCD) (Saxena et al. 2016). In our study, the comparatively lower concentration of H_2O_2 in Astella possibly hyperactivates its antioxidant system than in Flanders under stress.

Various non-enzymatic antioxidants are also excellent ROS scavengers in plants. PP and one of its sub-categories PA are important plant stress markers (Šamec et al. 2021). We found PP and PA were upregulated in Astella and downregulated in Flanders from their respective controls under stress (Fig. 4a, b; Table S3). Salt stress influences the levels of various PP compounds by affecting key enzyme activities of their centralised biosynthesis using the phenylpropanoid pathway (Sharma et al. 2019). According to the previous reports, PP (Kiani et al. 2021; Lungoci et al. 2022; Sarker et al. 2023) and PA (Singh et al. 2013; Ozfidan-Konakci et al. 2015) contents generally increase under salt stress. However, opposite reports do exist. Slama et al. (2017) reported declining PP content at elevated salt concentrations in *Sesuvium portulacastrum*. Total PP content decreased after salt treatment in both salt-sensitive and -tolerant genotypes of Medicago (Salah et al. 2011). In Romaine lettuce, despite the total PP content increase, PA content decreased after 100 mM NaCl stress (Mahmoudi et al. 2010). Telesiński et al. (2008) found that the total PP content of bean plants decreased and enzymatic antioxidants increased under salt stress; according to them, stress-derived nutrient deprivation was the main culprit for antioxidant hypoactivation. In our study, ROS-mediated photosynthetic apparatus damage and a decrease in overall photosynthesis level due to low LN could result in the decline of non-enzymatic antioxidant contents in Flanders over control under salt stress.

Moreover, ROS-derived damage of antioxidant biosynthetic pathway enzymes (Dunn 1998) could have accelerated the injury. Lower radical scavenging activity of Astella than its control could also be correlated with enzyme damage (Fig. 4c; Table S3).

Multiple studies compared salt sensitivity between known tolerant and susceptible genotypes and established the connection between elevated antioxidant activity and salinity stress tolerance in plants (Neto et al. 2006; Chutipaijit et al. 2005; Aktas 2012; Chawla et al. 2013; Vijayalakshmi et al. 2016; Kaur et al. 2016; Kumar et al. 2018; Wang et al. 2019). We also found significant upregulations of various antioxidant components and radical scavenging activity in Astella over Flanders under stress. It signified a better antioxidant system-mediated superior salinity resilience of Astella than Flanders.

Proline was providing multi-dynamic protection to Flanders

We found remarkable upregulations of proline in both genotypes under stress (Fig. 4d; Table S3). Proline has multifaceted roles in salt stress mitigation by – 1) acting as an osmolyte and helping to stabilise the disrupted osmoregulation in salinity-affected cells; 2) scavenging ROS molecules; 3) maintaining Na^+, K^+, Ca^+, and Cl^- homeostasis in affected cells; 4) restoring redox homeostasis of injured cells; 5) providing energy during nutrient deprivation; 6) maintaining metabolic balance by crosslinking multiple pathways; 7) upregulating antioxidant synthesis; 8) acting as chaperon to protect antioxidant enzymes (Smirnoff and Cumbes 1989; Cuin and Shabala 2007; Kaul et al. 2008; Liang et al. 2013; Kaur and Asthir 2015; Mansour and Ali 2017; Truong et al. 2023). Due to the wide array of stress-adaptive functions, proline is generally upregulated during abiotic stresses and is considered a crucial salinity stress index (Mansour and Ali 2017). Salt treatment-mediated proline upregulation in tolerant genotypes has already been reported in different plants (Kumar et al. 2018; Nguyen et al. 2021; Vasilakoglou et al. 2021) including flax (Li et al. 2022b). Therefore, the comparatively increased proline accumulation in stressed Astella further supported its better salt stress mitigation capability than Flanders. Moreover, lower proline accumulation in stressed Flanders signified its lesser degree of protection against salt stress and could also explain its elevated ROS level and decreased antioxidant activity under stress.

Lignan-related miRNAs were upregulated under salt stress

We analysed the expressions of three lignan biosynthesis-related miRNAs - miR168a, miR399g, and miR828a, using a kit-based two-tailed qPCR (TT-PCR) assay. Originally designed by Androvic et al. (2017), very few reports of the TT-PCR assay to measure plant microRNA expression exist to date (Anna et al. 2019; Barczak-Brzyżek et al. 2022; Kong et al. 2022; Pawełkowicz et al. 2022). We are reporting a kit-based two-tailed qPCR (TT-PCR) assay for the first time. Our tested miRNAs were upregulated in both genotypes under salt stress (Fig. 5a-c).

The miR168 family containing miR168a and miR168b is well-known to express under salt stress in various plants (Li et al. 2012; Xia et al. 2023) including flax (Barvkar et al. 2013; Yu et al. 2016). Under salt stress, miR168 primarily affects AGO1 of RISC via feedback downregulation (Ding et al. 2009). Since AGO is a core component of the RISC complex that guides miRNAs to cleave complementary target-mRNAs, the downregulation could impact global miRNA action by potentially adjusting metabolic processes in response to salt stress (Carnavale Bottino et al. 2013). Studies have also shown that miR168 affects the expression of CYP450 in flax (Barvkar et al. 2013) and rice (Wei

and Chen 2018). CYP450 is a critical controller of the phenylpropanoid pathway that produces lignan. Therefore, under salt stress, miR168 can affect lignan biosynthesis by affecting the phenylpropanoid pathway via deregulation of CYP450 (Reinprecht et al. 2017; Zhang et al. 2022).

The role of miR399 plant phosphate starvation is well established; little is known about its function in salt stress. The miR399 family consists of six members, from miR399a to miR399f, each member has a different role in salt stress. Upregulation of Arabidopsis miR399f during salt treatment has been reported (Bari et al. 2006; Baek et al. 2016). Upon salt treatment, Lu et al. (2011) observed upregulation of TAmiR399 in wheat and Lelandais-Briere (2009) observed downregulation of Mtr-miR399 in *Medicago truncatula*. Possibly these two groups observed different miR399 family members and therefore obtained contrasting results. Islam et al. (2022) proposed that miR399 is involved in the ABA metabolic pathway during salt stress. In an *in-silico* analysis, Yan et al. (2019) reported that ata-miR399a-5p regulates the JrPAL3 gene of the phenylpropanoid pathway of lignan biosynthesis.

Reports of miR828 involvement in salt stress are also meagre. Shuai et al. (2016) reported that miR828 is expressed under abiotic stress. Under salt stress, miR828 can either directly upregulate various MYB transcription factors (TFs) (Deng and Lu 2017), or it can upregulate TAS4 family phased secondary small interfering RNAs (phasiRNAs) (Fei et al. 2013; Shuai et al. 2016; Liu et al. 2020a; Mittal et al. 2023; Panchal 2023; Xie et al. 2023). The phasiRNAs can upregulate various MYB TFs (Luo et al. 2012; Zhang 2015). MYB TFs can promote lignan biosynthesis (Chiang et al. 2019; Markulin et al. 2019; Pratyusha and Sarada 2022; Ražná et al. 2022; Xie et al. 2023).

Studies related to the role of lignan-related miRNAs for salt stress mitigation are minuscule, and according to the available reports, our results connect miRNAs to lignan via some mediators. These mediators mostly affect the phenylpropanoid pathway, which produces many (secondary) metabolites, including lignan. Nevertheless, most miRNAs regulate multiple target sequences across numerous biological processes. It is a densely interconnected regulatory network and its pleiotropic effect is obvious (Ražná et al. 2023; Wang et al. 2024). Therefore, our study provides indicative, rather than direct evidence of lignan involvement in flax salt stress mitigation.

Salt stress affected root cell viability

Our FDA and PI differential staining revealed alive root tips and lateral root buds stained green. The dead epidermis of the rest of the roots was stained red (Fig. 6a-h). Roots are the primary organ of plants that are affected by salt stress (AbdElgawad et al. 2016). Since soil salinity creates physiologic drought in plants, they respond similarly under drought and salt stress. Previously, Duan et al. (2010) reported salt stress-mediated ROS generation damaged the root cells of the meristematic and elongation zone ceasing the overall root growth. Such stress-mediated root damage could have minimised root growth more in Flanders than in Astella in our study. Moreover, direct exposure of root epidermis cells, the outmost layer of roots, to the high soil salt concentration must have induced a high level of oxidative burst that damaged them the most.

Multivariate analysis correlated study variables and coined the most vital salt stress-responsive variables

We observed the abundance of our measured variables on treatment individuals using heatmap analysis. We further correlated the variables using PCC and PCA to understand their

cumulative effect on stress in this study. Heatmap analysis showed that despite the similar abundance of variables in non-stressed treatments, the abundance pattern between the salt-treated samples was entirely different (Fig. 7a). This phenomenon suggested a genotype-dependent differential salt-stress response regulation, corroborated the previous findings of the study, and was well-documented in other reports (Kaashyap et al. 2018; Nefissi Ouertani et al. 2022; Khan et al. 2023).

The salt stress markers H_2O_2 and MDA represent the extent of salinity-derived ROS generation and associated cell damage, respectively. We correlated salt stress markers with our tested variables using PCC (Fig. 7b; Table S4). Our tested morphological trait variables were negatively correlated with H_2O_2 and MDA suggesting an opposite correlation between them; meaning that suppressed stress could represent improved morphological traits. We found an improved morphology of Astella with suppressed stress markers compared to Flanders where growth arrest could have been due to the elevated ROS and cell damage (Fig. 1). Studies reported that plants often show improved morphology under stress as their adaptive response (Arif et al. 2019; Hu et al. 2023). ROS generation was positively correlated with enzymatic antioxidants, proline, and miRNAs signifying their simultaneous activation under salt stress. The co-upregulation and salinity-ameliorate role of antioxidants and proline to minimise ROS has already been established (Hasanuzzaman et al. 2019; Li et al. 2022b) and is also reflected in our study (Fig. 3a-d, 4a-d). The strong positive correlations between our studied miRNAs with ROS explained the salinity-mediated upregulation of these miRNAs (Fig. 5a-c) and supported the connection of these miRNAs with salt stress mitigation, however, the mechanism is not well understood yet. The respective negative and positive correlations of non-enzymatic antioxidants and lignan-related miRNAs with salt stress markers were contradictory and unusual. Plant miRNAs often have pleiotropic effects (Ražná et al. 2023; Wang et al. 2024). Plant developmental and nutrient-deficiency stress-responsive roles of miR168 (Xian et al. 2014; Liu et al. 2020b), nutrient stress and homeostasis-related roles of miR399 (Pant et al. 2008; Hu et al. 2015), and developmental roles of miR828 (Rock 2020; Yamagishi et al. 2020) are reported. Therefore, stress-mediated upregulation of miRNA(s) could have more elaborative roles other than lignan biosynthesis in salinity response in our study. Since higher lignan-containing Astella showed better salt adaptiveness than Flanders in this study, we hypothesise that lignan content could be a positive marker for salt stress tolerance.

PCA confirmed the findings of PCC analysis and depicted SFW, SDW, CAT, GPOX, Proline, and miR399g as the most influencing salt stress-responsive variables in our study. Among these variables, CAT, GPOX, Proline and miR399g were upregulated, and SFW and SDW were lowered during stress treatment.

CONCLUSION

This paper reports the salt stress adaptive differential response of two flax genotypes, Flanders and Astella, having contrasting lignan contents. Under stress, we found unaffected shoot and facilitated root growth, lower ROS generation, lower cell damage, higher activities of antioxidant system components, higher amount of proline, and upregulated lignan biosynthesis-related miRNAs in Astella over Flanders. This study is the first report on kit-based TT-PCR assay. Root viability assay using differential staining depicted the existence of viable root tips despite ROS-mediated cell damage. Multivariate analyses divulged important salinity-responsive variables and depicted lignan as a positive salt stress marker. Our results established Astella as the superior flax genotype over Flanders for salt stress adaptation.

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AUTHOR CONTRIBUTIONS

AJD and KR: conceptualisation, methodology, and fellowship; AJD, ĽH, MatK, MarK, EI, and VM: investigation; AJD and JG: data curation and formal analysis; AJD: visualisation, and writing – original draft preparation; KR: funding acquisition, project administration, resources, supervision, and validation; AJD, ĽH, MatK, MarK, EI, VM, JG, KR: writing – review and editing.

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STATEMENTS AND DECLARATIONS

The authors disclose no financial or non-financial interests directly or indirectly related to the work submitted for publication. They also have no relevant competing interests to declare to the content of this article.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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