Proline Exogenously Supplied or Endogenously Overproduced Induces Different Nutritional, Metabolic, and Antioxidative Responses in Transgenic Tobacco Exposed to Cadmium

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Abstract

Proline plays adaptive roles in plant tolerance to cadmium (Cd)-induced stress, but many gaps remain to be elucidated as the responses triggered by exogenously supplied proline or endogenously overproduction are not well known. Thus, we assayed the nutritional status, metabolite profiling, and antioxidative responses in wild type and transgenic tobacco (*Nicotiana tabacum* L.) containing the *P5CSF129A* gene under control of the cauliflower mosaic virus (CaMV35S) or stress inducible rd29A promoters. The plants were exposed or unexposed to Cd (0 and 50 μmol L^{-1} CdCl$_2$·H$_2$O) for 24 and 72 h. The wild type plants were also treated with or without exogenous proline (1 mmol L^{-1}). Plants supplied with exogenous proline exhibited lower Cd translocation from roots to leaves than plants overproducing proline, avoiding oxidative damages in the leaves of these plants. Meanwhile, tobacco overproducing proline was less susceptible to Cd-induced nutritional changes than wild type plants and presented better metabolic adjustment under Cd exposure compared to plants supplied with exogenous proline. Plants overproducing proline increased the synthesis of sugars and organic acids under Cd exposure, which contributed to absence of oxidative stress, since both superoxide dismutase and catalase were not active against Cd-induced oxidative stress in these genotypes. Plants overproducing proline under the control of rd29A presented higher proline concentration in comparison to the CaMV35S promoter. With exception of rd29A plants that presented high proline and reduced glutathione (GSH) concentrations, the other plants presented an inverse correlation between proline and GSH synthesis after 72 h of Cd exposure.

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Introduction

Cadmium (Cd) can induce several damages to plants, such as nutritional imbalance, photosynthesis impairment, and oxidative stress (Gallego et al. 2012; Lavres et al. 2019; Carvalho et al. 2020). Oxidative stress has been discussed as a primary effect of Cd exposure (Clemens 2006). Once formed, reactive oxygen species (ROS) must be detoxified as efficiently as possible by enzymatic and non-enzymatic antioxidants to minimize eventual damages in plants (Soares et al. 2019). Superoxide dismutase (SOD, EC 1.15.1.1) which dismutates superoxide ($O_2^{·-}$) into hydrogen peroxide (H_2O_2) and H_2O, and catalase (CAT, EC 1.11.1.6) that reduces H_2O_2 into H_2O are between the most important enzymes involved in antioxidative defense (Gratão et al. 2005). However, Iannone et al. (2015) suggested that CAT did not play a crucial role in protection against Cd toxicity in tobacco (*Nicotiana tabacum* L.) plants, since this species is able to activate alternative defense mechanisms such as ameliorated synthesis of proline and glutathione.

The importance of proline for tobacco tolerance to Cd was displayed by Islam et al. (2009), who described that exogenous proline supply decreased lipid peroxidation in tobacco cells exposed to Cd. Exogenous proline supply also contributed to adjusting the nutritional status of olive (*Olea europaea* L.) under Cd exposure (Zouari et al. 2016). Nevertheless, the role of proline in modulating these responses is unknown. Also endogenous proline accumulation is believed to play adaptive roles in plant tolerance against Cd-induced toxicity (Islam et al. 2009; Zouari et al. 2016). Under Cd exposure, proline is synthesized mainly from glutamate that is converted to proline by two successive reductions catalyzed by Δ^1-pyrroline-5-carboxylate synthetase (P5CS, EC 2.7.2.11) and pyrroline-5-carboxylate reductase (P5CR, EC 1.5.1.2), respectively (Verbruggen and Hermans 2008; Repkina et al. 2019). However, proline synthesis can be limited by P5CS activity that is subject to feedback inhibition by the product proline (Hong et al. 2000). Thus, the use of transgenic plants containing the mutated enzyme P5CSF129A (EC 2.7.2.11/1.2.1.41) that presents twice more proline accumulation than wild type plants containing the enzyme P5CS can be an alternative to understand the role of proline in tolerance mechanisms against abiotic stress (Hong et al. 2000).

Siripornadulsil et al. (2002) pointed out that microalga *Chlamydomonas reinhardtii* P. A. Dang mutated to express free proline was more tolerant to Cd than the wild type. However, this increased tolerance was attributed to a higher proline-induced glutathione synthesis. Glutathione (GSH, γ-Glu-Cys-Gly) can be oxidized to GSSG during ROS scavenging, which contributes to preventing the oxidative damages in cells (Yadav 2010). Once oxidized, glutathione reductase (GR, EC 1.6.4.2) catalyzes the reduction of GSSG into GSH (Gratão et al. 2005). Clemens (2006) stated that in plants exposed to Cd, symptoms of oxidative stress such as lipid peroxidation often are a consequence of GSH depletion due to the binding of Cd^{2+} to GSH and/or its use as substrate for the synthesis of phytochelatins [PCs, (γ-Glu-Cys)$_n$-Gly, with $n = 2$–11]. Phytochelatins are involved in Cd chelation and its transport from the cytosol to the vacuole (Yadav 2010). It is known that GSH and PCs synthesis are strongly induced by Cd, but there is no available information about the synthesis of these thiol compounds under Cd exposure in plants overproducing proline. Therefore, we do not know if there could be competition between proline and GSH, since both need glutamate to be synthesized (Verbruggen and Hermans 2008; Yadav 2010).

The action of enzymatic and non-enzymatic antioxidants in ROS scavenging depends on the plant's ability to reconfigure its metabolic network to allow both the maintenance of metabolic homeostasis and the production of compounds that ameliorate the stress (Obata and Fernie 2012). Sun et al. (2010) reported that the concentrations of proline, serine, sucrose, and other metabolites with compatible properties to these increased in *Arabdopsis thaliana* L. exposed to 50 μmol L^{-1} Cd compared to control, attenuating the Cd-induced stress. Like proline and GSH, organic acids, amino acids, sugars, and other related metabolites also can attenuate the Cd-induced oxidative stress by acting as chelators, antioxidants, and osmoprotectants (Sharma and Dietz 2006). However, there is no information on metabolic adjustment under Cd-induced stress in plants overexpressing proline or plants supplied with exogenous proline. Thus, our aim with this study was to better understand how exogenous proline supply or proline overproduction could affect the nutritional status, metabolite profile, activity of antioxidant enzymes, concentrations of proline and glutathione, and transcript levels of genes related to the metabolism of proline and glutathione and contribute to attenuate the Cd-induced stress in tobacco (notably in the leaves).

Materials and Methods

Plant Material and Experimental Design

The study was conducted in a greenhouse with controlled conditions: 12 h/22 °C and 12 h/18 °C, 12/12 h light/dark, photosynthetic active radiation of 170 μmol m^{-2} s^{-1} at the leaf level delivered by a combination of blue and red Philips® Green-Power LED modules, and 65% relative humidity. Seeds of wild type (Petite Havana SR1) and

transgenic (T3) tobacco (*N. tabacum* L.) containing the *P5CSF129A* mutated gene under control of the cauliflower mosaic virus (CaMV35S; (called 35S from this point)) or stress inducible rd29A promoters were sown in trays containing vermiculite as substrate, with a daily supply of deionized water. Eleven days after sowing, the nutrient solution of Hoagland and Arnon (1950) at 15% of the ionic strength was supplied, for 14 days. Then, the seedlings were placed in a styrofoam support and transferred to hydroponics composed of plastic trays containing 12 L of nutrient solution of Hoagland and Arnon (1950) at 30% of the ionic strength. From this point, the ionic strength of the solution was gradually increased until it reached 100% at 43 days after sowing. The undiluted nutrient solution was composed of 6 mmol L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$, 6 mmol L^{-1} KNO_3, 2.5 mmol L^{-1} $MgSO_4 \cdot 7H_2O$, 1 mmol L^{-1} KH_2PO_4, 100 µmol L^{-1} H_3BO_3, 100 µmol L^{-1} $MnSO_4 \cdot 4H_2O$, 30 µmol L^{-1} $ZnSO_4 \cdot 7H_2O$, 0.1 µmol L^{-1} $CuSO_4 \cdot 5H_2O$, 1 µmol L^{-1} $Na_2MoO_4 \cdot 4H_2O$, and 75 µmol L^{-1} FeNa-EDTA. Solutions were replaced weekly and remained constantly aerated throughout the entire experiment through plastic tubes connected to an air compressor.

Wild type (WT) and transgenic tobacco plants (35S or rd29A) were either unexposed or exposed to Cd (50 µmol L^{-1} $CdCl_2 \cdot H_2O$), for 24 and 72 h. In addition, we evaluated the effect of exogenous proline supply (1 mmol L^{-1}) on WT plants either unexposed or exposed to 50 µmol L^{-1} $CdCl_2 \cdot H_2O$, for 24 and 72 h. The exposure times were chosen considering the fact that processes like gene expression involved on plant adjustment to stress Cd-induced tends to occur more often in short-term (Hendrix et al. 2020b; Zdunek-Zastocka et al. 2021). On the 44th day after sowing proline was added to the nutrient solution of WT plants, and on the 45th day after sowing Cd was added to the nutrient solution of all genotypes assayed. Moreover, WT, WT receiving exogenous proline (WT + Pro), and transgenic tobacco (35S and rd29A) collected before Cd supply (time 0 h) were used as control treatments. The trays used to grow the tobacco plants were distributed in completely randomized design with four replicates per condition, and ten plants by replicate. At the moment of the harvest, plants were separated into shoot and roots. The plant material collected to determine the biomass production, nutrients and Cd concentrations was dried in a forced ventilation oven at 60 °C for 72 h, whereas the plant material collected to perform the other analyses was snap frozen in liquid N and stored at – 80 °C until the analyses were carried out.

Determination of Nutrients and Cd Concentrations in Roots and Leaves

After drying in an oven at 60 °C for 72 h, the plant material was ground in a Wiley type mill (Model 4, Thomas Scientific, Swedesboro, USA) and digested with 70–71% HNO_3 in a heat block (Cuypers et al. 2002). The concentrations of P, K, Ca, Mg, S, Cu, Fe, Mn, Zn, and Cd were determined by inductively coupled plasma optical emission spectrometry (ICP-OES, Agilent Technologies, 700 Series, Belgium). Blank reagent samples were used in the digestion for quality control. Standard reference material (NIST 1570a—spinach) was also used to assure the accuracy and precision of the analytical methods. From Cd concentrations, we calculated the Cd translocation factor by dividing the Cd concentration in the leaves by the Cd concentration in the roots.

Determination of Metabolite Profiling in the Leaves

Metabolites were extracted from 100 mg of frozen leaves tissue in 0.5 mL of cold extraction solution [isopropanol/ acetonitrile/water (3:2:2, v/v/v)] containing succinic acid as internal standard (1 mg mL^{-1}), as described by Zhao et al. (2015, 2016). Tungsten magnetic beads were added to the mixture, and then, the samples were subjected to agitation in a Vibration Mill (Retsch GmbH & Co., KG, Haan, Germany) for 30 s and 20 Hz. Beads were removed and samples centrifuged for 16,000×*g* for 10 min at 4 °C. The supernatant was filtered (Millex 0.22 µM filter, Millipore) and stored at – 80 °C.

For derivatization of the samples, 30 µL of methoxyamine (15 µg $µL^{-1}$ in pyridine) were added to 100 µL of the filtered extract previously lyophilized. The samples were vortex-mixed for 1 min and incubated at room temperature in dark for 16 h. After this step, 30 µL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) with 1% of trimethylchlorosilane (TMCS) were added and the samples were incubated in the dark for 1 h. Then, 30 µL of heptane were added to the samples, vortex-mixed, and injected in a gas chromatography mass spectrometer (GC–MS) (Pegasus 4D GCxGC-TOFMS, Leco Corporation, St. Joseph, USA). At this stage, control samples (blanks) and a series of alkanes (C_{12}–C_{40}) were used, which it made possible to calculate the retention indices (Schauer et al. 2005). After derivatization, the samples were injected into the 7890A gas chromatograph (Agilent Technologies, Santa Clara, USA) coupled to a Comb-xt automatic processor (Leap Technologies, Carrboro, USA).

The temperature of sample injection was 280 °C and the septum purge flow was 20 mL min^{-1} for 60 s. Helium gas flow was constant through the column with a flow rate of 1 mL min^{-1}. The column temperature was maintained at 80 °C for 2 min and then increased by 15 °C every minute until reaching 305 °C for 10 min. The column effluent was inserted into GCxGC-TOFMS ionization source (Pegasus 4D, Leco Corp.) equipped with two fused silica columns: the first-dimension column

(Agilent DB-5) with 20 m length (0.18 mm inner diameter × 0.18 µm film) and the second-dimension column of 0.96 m (RXT-170.10 mm inner diameter × 0.10 µm film). The transfer line and ion source temperatures were 280 and 250 °C, respectively. The ions were generated by an electron beam of 70 eV in an ionization flow of 2.0 mA, and 10 spectra s^{-1} in a mass range of m/z 45–800. ChromaTOF v. 4.51 software (Leco Corp.) was used to perform baseline correction and export all MS files in NetCDF. Peak detection, retention time alignment, and library matching were carried out using the TargetSearch package (Cuadros-Inostroza et al. 2009). For the identification of the metabolites, retention indices, spectra with similarity (score) > 600, and metabolites with at least 3 fragments (mass count) were compared with data stored in the database Golm-Metabolome—GMD—(available at http://gmd.mpimp-golm.mpg.de/) (Kopka et al. 2005). The intensity of each metabolite was normalized by the fresh weight (mg) of the corresponding sample and the total ion current (TIC) of each sample.

Determination of H_2O_2 Concentration and Lipid Peroxidation in the Leaves

Concentrations of H_2O_2 were determined as described by Alexieva et al. (2001), with modifications. Firstly, 0.2 g of frozen samples were macerated in 2 mL of 0.1% (w/v) trichloroacetic acid (TCA) in the presence of 20% (w/w) of polyvinyl polypyrrolidone (PVPP). After complete homogenization, 2 mL of extract was centrifuged at 10,000×g for 10 min at 4 °C. An aliquot of 0.2 mL was taken from the supernatant and then an aliquot of 0.2 mL of 100 mmol L^{-1} potassium phosphate buffer (pH 7.0) and 0.8 mL of 1 mol L^{-1} potassium iodide was added to the mixture. The solution was left for 1 h on ice in darkness to stabilize the reaction. The readings were made in a spectrophotometer at 390 nm (Genesys 10S UV–VIS, Thermo Fisher Scientific, Waltham, USA).

The lipid peroxidation was determined estimating the malondialdehyde (MDA) concentration from 2-thiobarbituric acid (TBA) reactive compounds (Heath and Packer 1968). The initial procedures for MDA measurements were the same as described for H_2O_2. Following centrifugation, 0.25 mL of sample supernatant was added to 1 mL of 20% (w/v) TCA containing 0.5% TBA. The samples were incubated for 60 min at 95 °C, and then, cooled in an ice bath for 1 min to stop the reaction. Subsequently, the samples were centrifuged at 10,000×g for 10 min to separate the residues formed during heating and to clarify the samples. The absorbance was measured at 535 and 600 nm by using a spectrophotometer (Genesys 10S UV–VIS).

Protein Extraction and Enzymatic Activities Determination in the Leaves

Proteins were extracted from 250 mg of leaves samples that were homogenized with a mortar and pestle in 100 mmol L^{-1} potassium phosphate buffer (pH 7.5) containing 1 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA), 3 mmol L^{-1} dithiothreitol (DTT), and 4% (w/v) PVPP (Azevedo et al. 1998). The resulting homogenate was centrifuged at 10,000×g for 30 min at 4 °C, and the supernatant was stored at − 80 °C for determination of the activities of antioxidative enzymes. Total soluble protein concentrations were determined by the method of Bradford (1976), using bovine serum albumin—BSA (Protein Standard, Sigma-Aldrich) as standard.

Total SOD activity was determined using a spectrophotometer (Giannopolitis and Ries 1977). The assays contained 1.79 mL of 50 mmol L^{-1} sodium phosphate (pH 7.8), 225 µL of 1 mmol L^{-1} ρ-nitro blue tetrazolium chloride (NBT), 780 µL of 50 mmol L^{-1} methionine, 30 µL of 10 mmol L^{-1} EDTA, 150 µL of 0.1 mmol L^{-1} riboflavin, and 25 µL of protein extract. The reaction mixture was exposed to light for 5 min and measured at 560 nm.

Total CAT activity was determined using a spectrophotometer following the method described by Kraus et al. (1995), with modifications by Azevedo et al. (1998). The reaction medium was composed of 1 mL of 100 mmol L^{-1} potassium phosphate buffer (pH 7.5) and 25 µL of 0.25% H_2O_2 solution. The reaction started after adding 25 µL of plant extract, and CAT activity was determined following the decomposition of H_2O_2 at 10 s intervals for 1 min at 240 nm.

Total GR activity was determined following the method described by Smith et al. (1988), with modifications by Azevedo et al. (1998). The reaction medium (1 mL) was composed of 100 mmol L^{-1} potassium phosphate buffer (pH 7.5), 100 µL of 1 mmol L^{-1} GSSG, 100 µL of 0.1 mmol L^{-1} NADPH, 500 µL of 1 mmol L^{-1} 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), and 35 µL of protein extract. The activity was estimated by GSSG reduction accompanied by monitoring the change in absorbance at 412 nm for 1 min.

Determination of Proline Concentration in the Leaves

The determination of proline concentration was performed by using 100 mg of leaf tissue homogenized in 3% sulfosalicylic acid. After centrifugation, the supernatant was taken for determination of the proline concentration at 520 nm, according to Bates et al. (1973).

Determination of GSH and GSSG Concentrations in the Leaves

The concentrations of GSH and GSSG were determined using spectrophotometry as described by Anderson (1985), with modifications by Borges et al. (2018). Fresh leaves tissue (200 mg) was homogenized in 1 mL of 50 mmol L^{-1} sulfosalicylic acid and centrifuged at 10,000×g for 20 min at 4 °C. Then, 0.2 mL of supernatant were added to 1.8 mL of 100 mmol L^{-1} potassium phosphate buffer (pH 7.0) containing 0.5 mmol L^{-1} EDTA and 100 µL of DTNB. The mixture was kept in the dark for 5 min and then taken to spectrophotometer (Genesys 10S UV–VIS) at 412 nm to measure GSH concentration. Then, 100 µL of 0.4 mmol L^{-1} NADPH and 2 µL of GR (1 U/reaction) were added to the mixture, which was kept in the dark for 20 min and read again in the spectrophotometer at 412 nm to measure the concentrations of GSH + GSSG. The concentrations of GSSG were obtained by the difference between the concentrations of GSH + GSSG and GSH. The redox state of glutathione was also calculated as GSH/GSSG (Jozefczak et al. 2015).

Gene Expression Analysis in the Leaves

The expression of genes related to the synthesis and degradation of proline (*P5CSF129A*, *P5CS-1*, *P5CS-2*, *P5CR*, *PDH1*, and *P5CDH*), glutathione (*GSH*, *GSH2*, and *GR1*), phytochelatins (*PCS1*), and metal transporters (*MRP3* and *PDR8*) plants was measured in the leaves of tobacco by real-time reverse transcription PCR (RT-qPCR), as described by Keunen et al. (2015). Total RNA was extracted using the RNAqueous® Total RNA Isolation Kit (Ambion, Life Technologies, Merelbeke, Belgium), followed by a DNAse treatment (DNAse I Kit, Invitrogen, Thermo Fisher Scientific). Then, extracted RNA was quantified in RNAse-free PCR tubes using the QuantiFluor RNA System (Promega Corp., Madison, USA) and 1 µL of RNA samples using a portable fluorometer (Quantus Fluorometer, Promega Corp., Madison, USA). Random primers and SuperScript III RT Kit (Invitrogen) were used to convert RNA (1 µg) into cDNA according to the manufacturer. After this step, cDNA was diluted 10 times in 1/10 diluted Tris–EDTA (TE) buffer (Tris–HCl 1 mmol L^{-1}, Na$_2$-EDTA 0.1 mmol L^{-1}, pH 8.0) and subsequently stored at 20 °C.

Real-time PCR quantification was performed in 96-well optical plates using the 7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies, Gent, Belgium) and the Fast SYBR Green Master Mix (Applied Biosystems). Amplification occurred under universal cycling conditions (20 s at 95 °C, 40 cycles of 3 s at 95 °C, and 30 s at 60 °C), followed by the generation of a dissociation curve to verify amplification specificity. Forward and reverse primers (300 nmol L^{-1}) were manually designed using tobacco sequences deposited in GenBank (Table 1), and quality verified using NetPrimer (http://www.premierbiosoft.com/netprimer/) software. Gene expression was calculated using the $2^{-\Delta Cq}$ method in relation to WT expression at the time 0 h (control). All data were normalized by using the expression of two stable reference genes: *EF1-a* (Elongation Factor 1-alpha) (Ye et al. 2016) and *NtEFa* (Elongation Factor a) (Fässler et al. 2011). Primer efficiencies were determined using a standard curve of a two-fold dilution series generated from a pooled sample.

Statistical Analysis

To perform statistical analysis, each combination of genotype (WT, WT + Pro, 35S, and rd29A) and Cd rate (0 and 50 µmol L^{-1} CdCl$_2$·H$_2$O) was considered as a treatment, totalling eight treatments. For the longitudinal analysis, the times 0 (control treatments), 24 and 72 h have been considered as factors (except for metabolite profiling that was only determined at the 24 h and analyzed as described below). Thus, for each treatment, a normal regression model was built to test a possible difference in the mean of the response variable between the different Cd exposure times within the same treatment. To compare the means between genotypes within each Cd rate in a fixed time and to compare the means between Cd rate within each genotype in a fixed time, an analysis of variance (ANOVA) was performed. When the F test was significant in the ANOVA, we applied the post hoc tests (Tukey test) to provide specific information on which means differed from each other. For all tests mentioned above, the p-value was fixed in 5%. All statistical analyses were performed using R software v. 3.0.2 (R Core Team 2019). Graphs were created and plotted with SigmaPlot v. 10.0 (SIGMAPLOT 2006), and the results are expressed as means ± standard error of the mean.

Multivariate (Partial least square discriminant analysis—PLS-DA) and univariate (ANOVA) analyses were performed on the entire metabolomics data set using the MetaboAnalyst 4.0 (Chong et al. 2018). Data were normalized by the median, log-transformed, and scaled by Auto scaling prior to data analysis. Differentially abundant metabolites were identified based on the variable importance in projection (VIP ≥ 1), followed by one-way ANOVA (FDR, adjusted p ≤ 0.05).

Table 1 Forward and reverse primers (300 nmol L^{-1}) designed and optimized for *Nicotiana tabacum* L.

Group	Gene	Gene description	Primers	Sequence (5′–3′)	PCR efficiency	References
Reference genes	*EF1-a*	Elongation Factor 1-alpha	F	ATGATGACGACGATG ATGATA	**0.917**	Ye et al. (2016)
			R	GTAAGCCCTTCTTGC TGAACAC		
	NtEFa	Elongation Factor a	F	TTGGAAATGGATATG CTCCAG	**0.885**	Fässler et al. (2011)
			R	CACCAACAGCAACAG TTTGAC		
Proline synthesis and degradation	*P5CSF129A*	Mutated P5CS	F	TCTCGGGGGTTCATG AAGGA	**1.015**	Primer designed for this study
			R	AGCTCCCAATCTTCC AACCG		
	P5CS-1[a]	Δ^1-Pyrroline-5-carboxylate synthetase	F	CTGGAGGCTCGAGTG TAAATG	**0.822**	Dobrá et al. (2011)
			R	TAGTTGTCCTGCCCT TGTCC		
	P5CS-2[a]	Δ^1-Pyrroline-5-carboxylate synthetase	F	GTGCAGAGGTTGGGA TTAGC	**0.885**	Dobrá et al. (2011)
			R	TCACCGTCAACAATT TGTCC		
	P5CR[a]	Pyrroline-5-carboxylate reductase	F	GGGAGCTTGCACTTG GACTA	**1.025**	Primer designed for this study
			R	CAGCCTTCTCCAACT CGTGA		
	PDH1	Proline dehydrogenase	F	CGTGGCAGAAATGAT CTTGA	**1.032**	Dobrá et al. (2011)
			R	ATTCCCCGAGTATGC TCCTT		
	P5CDH	Pyrroline-5-carboxylate dehydrogenase	F	CAACAGGGGCTCCAC AGAAT	**1.004**	Primer designed for this study
			R	GGCACGGGACCAACA TCATA		
Glutathione synthesis and recovery	*GSH1*	γ-Glutamylcysteine synthetase	F	GAGGATAGGCACTGA ACATGAA	**0.988**	Ye et al. (2016)
			R	TCGCTCGGCAATACC ATTTAG		
	GSH2	Glutathione synthetase	F	GCAGGTCAGAGATAC AGAAAGG	**1.015**	Primer designed for this study
			R	ATTGCTGAAATACAT TGCCCTG		
	GR1	Glutathione reductase	F	CAACTGGTAGTAGGG CTCATC	**1.018**	Primer designed for this study
			R	TTGCACCCATTCCTC GCC		
PCs synthesis	*PCS1*	Phytochelatin synthase	F	GCTATCAAGGAAGAG GTATTG	**0.927**	Primer designed for this study
			R	AACTGCTGTCTGATG CTGCT		
Metal transporters	*PDR8*	Pleiotropic drug resistance 8	F	GGGATCAATGTATGC TGCTG	**1.148**	Fässler et al. (2011)
		(metal transporter)	R	TTCAAATCCAATCAT AGCATAGACA		
	MRP3	Multidrug resistance-associated	F	GAGGATGTTCCTCAG CTTCA	**0.796**	Fässler et al. (2011)
		Protein 3 (metal transporter)	R	AGGTACGGGCCAACA AAAG		

Bold values refer to PCR efficiency

PCs phytochelatins

[a]Two copies in the tobacco genome
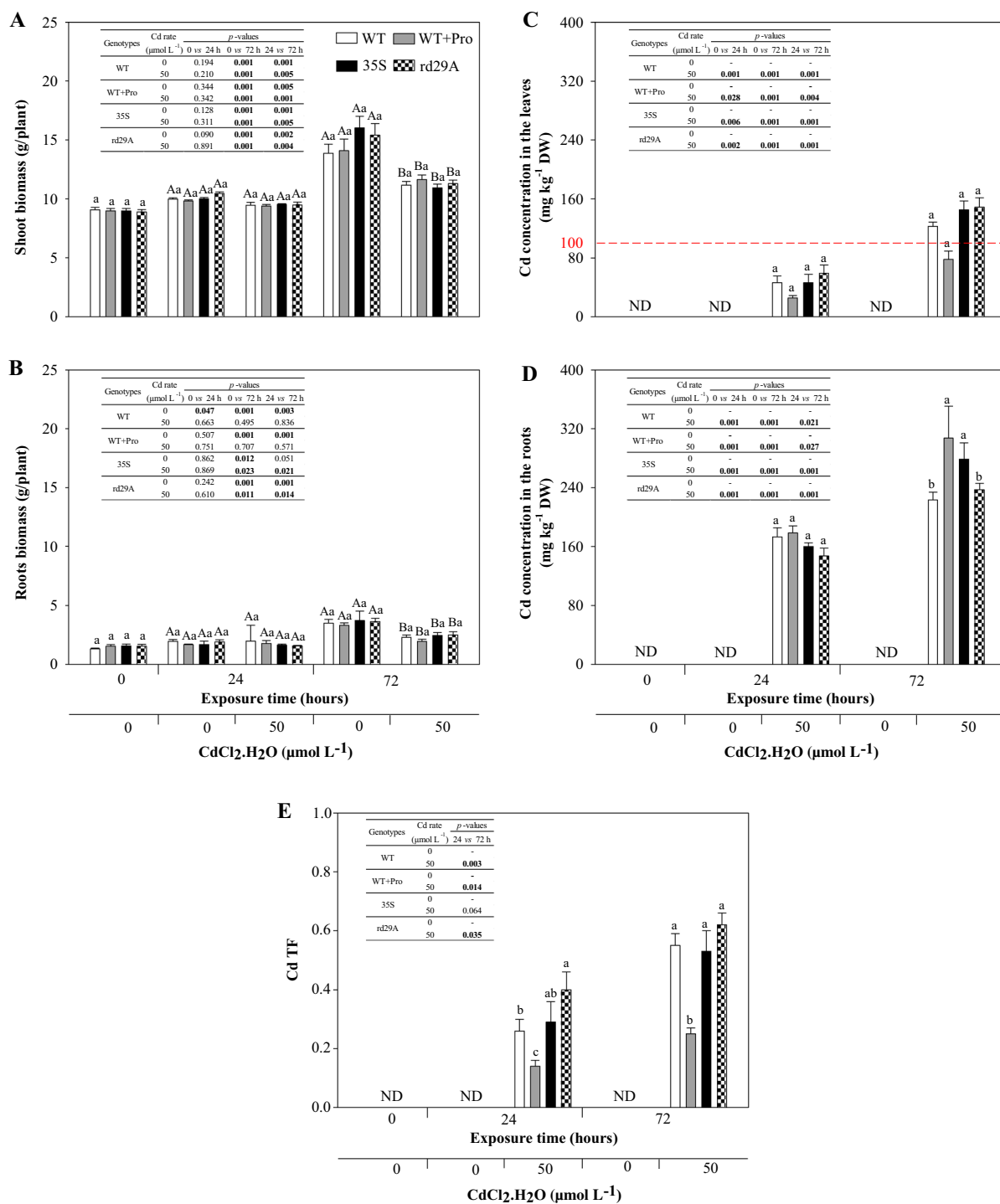
Fig. 1 Biomass production (**A**, **B**), Cd concentrations (**C**, **D**) in the leaves (**A**, **C**) and roots (**B**, **D**), and Cd translocation factor (Cd TF, **E**) in wild type (WT), WT supplied with 1 mmol L^{-1} of exogenous proline 24 h before Cd exposure (WT + Pro), and transgenic tobacco (*Nicotiana tabacum* L.) plants containing the mutated *P5CSF129A* gene under control of the cauliflower mosaic virus CaMV35S (35S) or stress inducible rd29A (rd29A) promoters, exposed to Cd (0 and 50 μmol L^{-1} CdCl$_2$·H$_2$O) for 0 (control), 24 and 72 h. Distinct upper case letters on the bars indicate difference between Cd rate (0 vs 50 μmol L^{-1} CdCl$_2$·H$_2$O) within each genotype (WT, WT + Pro, 35S, and rd29A) over the time, and distinct lower case letters indicate difference between genotypes within each Cd rate for each exposure time (Tukey test, $n = 4$, $p \leq 0.05$). *p*-values in bold in the tables within each figure indicate difference between exposure times within each genotype and Cd rate ($p \leq 0.05$). *ND* non-detected

Results

Proline Overproduction Allowed Continuous Root Biomass Production Even Under Cd Exposure and Exogenous Proline Supply Decreased Root-to-Shoot Cd Translocation in WT Plants

The biomass production of tobacco supplied with exogenous proline or overproducing proline (35S and rd29A) did not differ from that of WT, regardless of Cd rate (Figs. 1A, B). However, all tobacco plants after 72 h exposed to Cd exhibited lower biomass compared to unexposed plants. Analyzing the data over the time, we observed that shoot biomass of all plants continued increasing after 24 or 72 h in both unexposed and Cd exposed conditions (Fig. 1A). However, only the roots biomass of 35S and rd29A plants continued increasing after 24 or 72 h of Cd exposure (Fig. 1B).

Cadmium concentration in the leaves of WT did not differ from that of tobacco supplied with exogenous proline or overproducing proline, regardless of Cd exposure time (Fig. 1C). Meanwhile, Cd concentration in the roots of 35S and WT supplied with exogenous proline was higher compared to the other plants after 72 h of Cd exposure (Fig. 1D). Cadmium concentrations in the leaves and roots of all plants increased over time due to Cd exposure (Figs. 1C, D). Interestingly, Cd translocation from roots to shoots in WT supplied with exogenous proline was lower compared to WT grown without exogenous proline supply and to 35S and rd29A after 24 or 72 h of Cd exposure (Fig. 1E). Cadmium translocation presented a tend to increase over the time in all genotypes (Fig. 1E).

Cadmium Exposure and Exogenous Proline Supply or Proline Overproduction Induced Changes in Nutrients Concentrations in the Leaves and Roots of Tobacco Plants

The concentrations of P, K, Mg, S, Cu, Fe, Mn, and Zn in the leaves of plants supplied with exogenous proline or overproducing proline did not differ from WT collected before Cd supply (control) (Tables 2 and 3). Meanwhile, both plants overproducing proline tended to present higher K concentrations in their roots in relation to WT (Table 2), while WT supplied with exogenous proline tended to present lower Mn and Zn concentrations in their roots compared to other plants (Table 3). 24 h after the beginning of the study, rd29A contained the highest P and Cu concentrations in the leaves under Cd exposure. There was no difference in nutrient concentrations in the leaves and roots between the genotypes after 72 h, regardless of Cd rate (Tables 2 and 3).

Cadmium exposure after 24 h increased P concentration in the leaves of rd29A (Table 2) and decreased Mn and Zn concentrations in the leaves of WT grown without or with exogenous proline supply (Table 3). In general, there was no effect of Cd exposure on nutrient concentrations in the leaves of plants after 72 h, as well as there was no effect of Cd exposure after 24 or 72 h on macronutrient concentrations in the roots of plants (Table 2), regardless of exogenous proline supply or proline overproduction. Nevertheless, 24 h of Cd exposure decreased Fe concentrations in the roots of 35S, whereas 72 h of Cd exposure decreased Mn concentrations in the roots of genotypes overproducing proline (Table 3). Iron concentrations increased after 72 h of Cd exposure in the roots of WT supplied with exogenous proline (Table 3).

Analyzing the nutrient concentrations over the time, we observed that Mg concentration in the leaves of plants tended to decrease, especially under Cd exposure. For the other nutrients there was no pattern as a result of exogenous proline supply, proline overproduction, or Cd exposure (Tables 2 and 3). Nutrient concentrations in the roots of plants were less affected over time than nutrient concentrations in the leaves, with exception of Fe concentration that decreased over time, especially in plants unexposed to Cd (Tables 2 and 3). There was no significant effect of genotypes, Cd rate or exposure time on Ca concentrations (results not shown), which ranged from 19.35 to 29.61 g kg^{-1} DW in the leaves and from 13.12 to 22.68 g kg^{-1} DW in the roots.

The Metabolite Profiling of Plants Overproducing Proline Was Much More Differentiated Than in WT Plants Receiving Exogenous Proline Supply in Order to Adjust to Cd-Induced Stress

To assay the response of plants to Cd exposure we compared WT with WT that received exogenous proline supply and with plants overproducing proline, in the absence and presence of Cd. In total, 163 metabolites with known structures were identified by GC-TOFMS and classified as sugars and sugar acids (21.5%), amino acids and their analogues (12.9%), lipids (12.3%), organic acids (12.3%), steroids (6.7%), alkanes (4.3%), alkaloids (3%), nucleic acids (2.4%), benzenoids (1.8%), and others (19.1%) (Supplementary Table l). To reduce the data dimensionality and visualize the response of tobacco to Cd, we performed a supervised PLS-DA obtaining score plots (Fig. 2A–C). In the models obtained for WT supplied with exogenous proline and for rd29A (Fig. 2A and C), the samples coming from plants exposed to Cd were not grouped with the correspondent ones unexposed to Cd, which indicates that the metabolites' response under Cd exposure can be distinguished from the other ones. Based on the parameter VIP (≥ 1) and ANOVA (FDR, adjusted $p \leq 0.05$), 3, 18 , and

Table 2 Concentrations of P, K, Mg, and S in the leaves and roots of wild type (WT), WT supplied with 1 mmol L⁻¹ of exogenous proline 24 h before Cd exposure (WT+Pro), and transgenic tobacco (*Nicotiana tabacum* L.) plants containing the mutated *P5CSF129A* gene under control of the cauliflower mosaic virus CaMV35S (35S) or stress inducible rd29A (rd29A) promoters, exposed to Cd (0 and 50 μmol L⁻¹ CdCl₂·H₂O) for 0 (control), 24 and 72 h

Leaves

Genotypes	Cd rate (μmol L⁻¹)	Exposure time (h) 0	24	72	p-values 0 vs 24 h	0 vs 72 h	24 vs 72 h
P concentration in the leaves (g kg⁻¹ DW)							
	50		6.87±0.72 Ab	6.84±0.50 Aa	0.448	0.464	0.974
WT+Pro	0	6.66±0.54 a	7.41±0.74 Aa	7.41±0.23 Aa	0.355	0.359	0.995
	50		5.77±0.43 Ab	7.52±0.24 Aa	0.707	0.082	**0.011**
35S	0	5.40±1.50 a	7.72±0.83 Aa	8.56±1.20 Aa	0.207	0.097	0.587
	50		7.45±0.59 Ab	9.20±0.81 Aa	0.345	**0.024**	0.129
rd29A	0	7.93±0.48 a	7.21±0.26 Ba	8.20±0.08 Aa	0.144	0.571	**0.011**
	50		11.32±3.35 Aa	7.97±0.37 Aa	0.266	0.986	0.359
K concentration in the leaves (g kg⁻¹ DW)							
WT	0	21.82±2.26 a	24.85±1.04 Aa	15.78±0.90 Aa	0.194	**0.020**	**0.001**
	50		22.31±4.13 Aa	15.35±1.38 Aa	0.835	0.134	0.161
WT+Pro	0	19.12±1.83 a	22.69±0.80 Aab	14.18±0.38 Aa	0.060	**0.015**	**0.001**
	50		18.39±1.75 Aa	14.27±0.79 Aa	0.685	0.052	0.076
35S	0	19.04±2.68 a	24.29±1.31 Aab	16.13±1.60 Aa	0.090	0.319	**0.007**
	50		23.41±1.17 Aa	14.67±0.41 Aa	**0.037**	**0.047**	**0.001**
rd29A	0	16.50±1.15 a	16.84±2.09 Ab	14.86±0.24 Aa	0.865	0.426	0.384
	50		15.60±1.24 Aa	15.42±0.45 Aa	0.665	0.566	0.898
Mg concentration in the leaves (g kg⁻¹ DW)							
WT	0	5.44±0.09 a	5.76±0.68 Aa	3.94±0.32 Aa	0.614	**0.038**	0.052
	50		5.05±0.57 Aa	3.48±0.30 Aa	0.602	**0.045**	0.051
WT+Pro	0	5.00±0.27 a	4.69±0.55 Aa	3.65±0.18 Aa	0.565	**0.029**	0.122
	50		3.61±0.20 Aa	4.32±0.64 Aa	0.159	0.512	0.329
35S	0	5.33±0.53 a	4.55±0.45 Aa	3.36±0.08 Aa	0.203	**0.007**	**0.040**
	50		4.72±0.52 Aa	3.61±0.15 Aa	0.245	0.445	0.085
rd29A	0	4.53±0.83 a	5.25±0.67 Aa	3.56±0.05 Aa	0.432	0.294	**0.045**
	50		4.81±0.27 Aa	3.60±0.13 Aa	**0.019**	**0.001**	**0.007**
S concentration in the leaves (g kg⁻¹ DW)							
WT	0	2.03±0.09 a	2.15±0.21 Aa	2.67±0.22 Aa	0.652	**0.034**	0.135
	50		1.98±0.11 Aa	2.45±0.14 Aa	0.726	**0.039**	**0.036**
WT+Pro	0	2.04±0.23 a	2.44±0.42 Aa	2.44±0.15 Aa	0.349	0.346	0.999
	50		2.51±0.39 Aa	2.35±0.03 Aa	**0.040**	0.086	0.690

Roots

Genotypes	Cd rate (μmol L⁻¹)	Exposure time (h) 0	24	72	p-values 0 vs 24 h	0 vs 72 h	24 vs 72 h
P concentration in the roots (g kg⁻¹ DW)							
	50		4.16±0.66 Aa	4.20±0.42 Aa	0.892	0.849	0.963
WT+Pro	0	3.06±0.31 a	5.28±0.21 Aa	3.38±0.36 Aa	**0.001**	0.470	**0.003**
	50		4.32±0.33 Aa	4.71±0.62 Aa	0.072	**0.026**	0.600
35S	0	4.34±0.56 a	4.76±0.52 Aa	3.68±0.13 Aa	0.518	0.319	0.087
	50		4.38±0.76 Aa	3.88±0.22 Aa	0.427	0.952	0.548
rd29A	0	4.01±0.72 a	4.23±0.68 Aa	3.04±0.51 Aa	0.812	0.313	0.209
	50		4.35±0.46 Aa	3.41±0.45 Aa	0.712	0.275	0.195
K concentration in the roots (g kg⁻¹ DW)							
WT	0	12.75±1.86 b	10.69±0.61 Ba	14.59±0.83 Aa	0.266	0.316	**0.009**
	50		16.67±2.65 Aa	14.03±1.91 Aa	0.156	**0.036**	0.449
WT+Pro	0	13.26±1.51 b	11.67±2.28 Aa	10.13±1.28 Aa	0.535	0.236	0.575
	50		11.97±2.12 Aa	12.91±0.90 Aa	**0.002**	**0.004**	0.696
35S	0	21.70±2.21 a	9.76±1.00 Aa	13.39±1.61 Aa	**0.001**	**0.006**	0.103
	50		13.86±2.07 Aa	13.16±1.06 Aa	0.662	0.525	0.774
rd29A	0	15.69±2.67 ab	14.96±2.31 Aa	10.29±0.86 Aa	0.812	0.103	0.107
	50		16.91±2.55 Aa	14.42±1.62 Aa	0.620	0.873	0.441
Mg concentration in the roots (g kg⁻¹ DW)							
WT	0	15.35±2.16 a	10.95±0.63 Aa	19.40±2.68 Aa	0.157	0.190	**0.021**
	50		15.29±0.86 Aa	14.73±1.17 Aa	0.984	0.791	0.711
WT+Pro	0	14.98±2.69 a	11.41±1.36 Aa	14.85±2.22 Aa	0.272	0.966	0.234
	50		12.23±2.38 Aa	13.66±1.18 Aa	0.703	0.812	0.609
35S	0	12.52±0.95 a	14.00±1.04 Aa	17.72±2.62 Aa	0.557	0.060	0.234
	50		16.34±0.93 Aa	15.94±1.81 Aa	0.526	0.640	0.849
rd29A	0	12.09±1.81 a	13.24±2.24 Aa	15.60±1.40 Aa	0.669	0.212	0.406
	50		11.44±0.64 Aa	14.69±1.92 Aa	0.767	0.090	0.160
S concentration in the roots (g kg⁻¹ DW)							
WT	0	5.37±0.49 a	5.77±0.76 Aa	6.33±0.55 Aa	0.656	0.296	0.572
	50		6.06±0.76 Aa	4.62±0.59 Aa	0.054	0.642	0.186
WT+Pro	0	5.45±0.22 a	5.94±0.90 Aa	5.38±0.86 Aa	0.647	0.944	0.666
	50		5.98±0.67 Aa	4.40±1.21 Aa	0.257	0.493	0.688

Table 2 (continued)

		S concentration in the leaves (g kg⁻¹ DW)						S concentration in the roots (g kg⁻¹ DW)					
35S	0	2.19±0.32 a	1.99±0.36 Aa	3.14±0.30Aa	0.667		0.071	4.51±0.41 a	4.86±0.65 Aa	5.80±0.88 Aa	0.724	0.210	0.423
	50		1.85±0.11 Aa	2.17±0.33 Ba	0.962		0.295		5.32±0.57 Aa	4.25±0.44 Aa	0.875	0.184	0.191
rd29A	0	2.34±0.24 a	2.49±0.13 Aa	2.70±0.01 Aa	0.512		0.143	4.94±0.46 a	5.39±0.08 Aa	4.13±0.35 Aa	0.369	0.124	**0.013**
	50		2.54±0.27 Aa	3.22±0.19 Aa	0.202		**0.012**		5.17±0.28 Aa	4.87±0.35 Aa	0.480	0.277	0.536

Means±SEM followed by distinct upper case letters indicate difference between Cd rate (0 vs 50 µmol L⁻¹ CdCl₂·H₂O) within each genotype (WT, WT+Pro, 35S, and rd29A) over the time, and distinct lower case letters indicate difference between genotypes within each Cd rate for each exposure time (Tukey test, $n=4$, $p \leq 0.05$)

p-values in bold indicate difference between Cd exposure times within each genotype and Cd exposure condition ($p \leq 0.05$)

34 responsive metabolites were identified in WT supplied with exogenous proline and in 35S and rd29A, respectively. Responsive metabolites were composed mainly of amino acids and derivatives, organic acids, and sugars (Supplementary Table 2). The differentially abundant metabolites for each comparison were plotted as heatmaps with hierarchical clustering (Fig. 2D–F).

From the heatmaps we observed that each genotype adjusted to Cd exposure using different metabolic strategies. Only 3 metabolites were significantly abundant in WT supplied with exogenous proline (Fig. 2D). Ornithine was up-regulated in WT, while erythronic acid was up-regulated in WT supplied with exogenous proline and threonine was up-regulated after Cd exposure in WT supplied with exogenous proline (Fig. 2D). Hierarchical clustering distinguished 5 and 6 groups of metabolites for the comparisons related to 35S and rd29A, respectively (Figs. 2E, F). Metabolites such as galactose-6-phosphate, *N*-tetradecanoyl-homoserine lactone, and erythronic acid were down-regulated in 35S compared to WT unexposed to Cd, but other metabolites such as lactulose and hydroquinone were up-regulated in 35S compared to WT exposed to Cd (Fig. 2E). Cadmium exposure led to accumulation of *N*-tetradecanoyl-homoserine lactone and *N*-(3-oxohexanoyl)-homoserine lactone in WT, and lactulose, hydroquinone, and glyceric acid in 35S (Fig. 2E). Comparing WT and rd29A we observed that metabolites such as sucrose and galactose-6-phosphate were more accumulated in rd29A compared to WT unexposed and exposed to Cd (Fig. 2F). Cadmium exposure induced the accumulation of several metabolites in WT (e.g., raffinose and mannitol) and rd29A (e.g., sucrose and cysteine) (Fig. 2F).

There Was No Lipid Peroxidation and Reduction on Protein Concentration Cd-Induced in the Leaves of Tobacco Plants, Despite Decreased SOD Activity Over Time in WT Plants Exposed to Cd

The H_2O_2 concentration in the leaves of tobacco supplied with exogenous proline or tobacco overproducing proline did not differ from WT, regardless of Cd rate (Fig. 3A). Only the 35S presented higher H_2O_2 concentration in their leaves after Cd exposure for 24 h compared to unexposed plants. From the analysis of the data over time, we can see that there were only small variations on H_2O_2 concentrations in the leaves of plants, regardless of genotypes or Cd rate (Fig. 3A).

In general, there was no relation between the concentrations of H_2O_2, MDA, and soluble protein (Fig. 3A–C). The MDA concentrations in the leaves were very similar to each other in the control treatments and after 24 h, but after 72 h, plants unexposed to Cd that received exogenous proline supply presented higher MDA concentrations

Table 3 Concentrations of Cu, Fe, Mn, and Zn in the leaves and roots of wild type (WT), WT supplied with 1 mmol L⁻¹ of exogenous proline 24 h before Cd exposure (WT+Pro), and transgenic tobacco (*Nicotiana tabacum* L.) plants containing the mutated *P5CSF129A* gene under control of the cauliflower mosaic virus CaMV35S (35S) or stress inducible rd29A (rd29A) promoters, exposed to Cd (0 and 50 μmol L⁻¹ CdCl₂·H₂O) for 0 (control), 24 and 72 h

Genotypes	Cd rate (μmol L⁻¹)	Exposure time (h) — leaves			p-values — leaves			Exposure time (h) — roots			p-values — roots		
		0	24	72	0 vs 24 h	0 vs 72 h	24 vs 72 h	0	24	72	0 vs 24 h	0 vs 72 h	24 vs 72 h
		Cu concentration in the leaves (mg kg⁻¹ DW)						Cu concentration in the roots (mg kg⁻¹ DW)					
WT	0	6.62±0.90 a	8.18±1.31 Aa	5.88±0.19 Aa	0.263	0.586	0.132	33.88±5.27 a	23.86±5.37 Aa	30.99±4.44 Aa	0.193	0.695	0.345
	50		7.73±1.34 Aab	4.90±0.36 Aa	0.438	0.197	0.085		30.67±6.43 Aa	25.57±5.39 Aa	0.759	0.341	0.565
WT+Pro	0	5.78±0.47 a	5.99±0.30 Aa	4.75±0.40 Aa	0.717	0.097	**0.047**	30.37±9.21 a	25.51±3.07 Aa	20.46±3.69 Aa	0.579	0.272	0.333
	50		4.95±0.55 Ab	4.27±0.21 Aa	0.223	0.073	0.293		20.80±3.24 Aa	25.46±4.97 Aa	0.439	0.931	0.462
35S	0	8.59±1.34 a	8.66±0.90 Aa	6.14±0.99 Aa	0.966	0.147	0.109	30.63±2.79 a	28.97±3.07 Aa	16.92±5.08 Aa	0.762	**0.030**	0.088
	50		7.01±1.40 Aab	6.02±0.86 Aa	0.812	0.699	0.569		28.60±7.69 Aa	20.51±3.44 Aa	0.974	0.323	0.374
rd29A	0	6.48±0.15 a	7.29±0.23 Aa	5.57±0.13 Aa	**0.008**	**0.004**	**0.001**	25.01±1.54 a	30.09±5.22 Aa	14.61±2.68 Aa	0.332	0.065	**0.038**
	50		10.25±4.32 Aa	4.49±0.27 Aa	0.483	0.446	0.231		22.62±3.29 Aa	18.12±3.29 Aa	0.516	0.154	0.371
		Fe concentration in the leaves (mg kg⁻¹ DW)						Fe concentration in the roots (mg kg⁻¹ DW)					
WT	0	279.43±50.88 a	341.52±100.18 Aa	141.72±22.37 Aa	0.523	0.175	0.099	234.95±39.42 a	218.12±41.15 Aab	137.06±14.88 Aa	0.734	0.072	0.113
	50		233.45±37.65 Aa	217.43±59.28 Aa	0.665	0.508	0.827		240.73±36.80 Aa	157.34±23.19 Aa	0.712	0.091	0.103
WT+Pro	0	311.49±79.04 a	303.67±40.87 Aa	177.10±23.08 Aa	0.912	0.106	**0.035**	164.37±28.23 a	275.34±16.02 Aab	126.13±14.67 Ba	**0.004**	0.220	**0.001**
	50		208.22±22.23 Aa	209.75±71.14 Aa	0.585	0.597	0.984		216.82±35.85 Aa	220.74±34.62 Aa	0.051	0.058	0.939
35S	0	238.53±58.94 a	264.42±59.80 Aa	139.62±23.78 Aa	0.724	0.198	0.100	229.95±43.56 a	316.60±11.26 Aa	146.31±9.13 Aa	**0.046**	0.052	**0.001**
	50		282.15±48.51 Aa	244.06±69.58 Aa	0.671	0.968	0.669		223.30±60.49 Ba	186.90±34.30 Aa	0.928	0.501	0.619
rd29A	0	191.00±21.62 a	210.49±40.62 Aa	167.66±31.94 Aa	0.679	0.622	0.438	244.73±56.93 a	197.23±32.47 Ab	94.10±5.15 Aa	0.399	**0.020**	**0.020**
	50		146.70±21.58 Aa	117.64±12.85 Aa	0.097	**0.033**	0.291		189.66±23.86 Aa	159.49±13.52 Aa	0.583	0.178	0.313
		Mn concentration in the leaves (mg kg⁻¹ DW)						Mn concentration in the roots (mg kg⁻¹ DW)					
WT	0	53.30±3.38 a	73.63±9.15 Aa	50.01±1.58 Aa	**0.032**	0.693	**0.043**	87.98±2.17 a	49.17±9.46 Ba	81.11±4.17 Aa	**0.001**	0.446	**0.021**
	50		45.17±6.04 Ba	42.00±4.10 Aa	0.830	0.477	0.679		72.87±2.61 Aa	79.93±6.19 Aa	0.917	0.355	0.334
WT+Pro	0	48.58±6.69 a	55.49±4.63 Ab	53.84±1.91 Aa	0.337	0.461	0.752	59.81±6.29 b	57.07±6.27 Aa	73.72±6.11 Aa	0.762	0.149	0.106
	50		40.04±4.97 Ba	42.56±3.26 Aa	0.348	0.646	0.687		70.36±8.75 Aa	74.45±7.36 Aa	0.700	0.425	0.732
35S	0	65.01±7.00 a	54.85±2.27 Ab	51.51±1.48 Aa	0.132	0.055	0.264	68.90±8.53 ab	71.92±7.46 Aa	89.36±10.46 Aa	0.816	0.139	0.223

Table 3 (continued)

Mn concentration in the leaves (mg kg⁻¹ DW)

Genotype	Cd rate					
	50		47.69±2.64 Aa	54.11±3.23 Aa **0.042**	**0.002**	0.175
rd29A	0	48.30±2.03 a	46.13±1.94 Ab	47.61±0.40 Aa 0.372	0.771	0.483
	50		48.36±2.73 Aa	49.56±2.28 Aa 0.171	0.229	0.748

Mn concentration in the roots (mg kg⁻¹ DW)

Genotype	Cd rate					
	50		72.81±3.44 Aa	61.10±10.48 0.990 Ba	0.286	0.326
rd29A	0	86.85±6.55 a	63.75±5.00 Aa	87.82±7.43 Aa **0.031**	0.917	**0.036**
	50		68.23±3.15 Aa	56.75±3.99 Ba 0.560	0.209	0.064

Zn concentration in the leaves (mg kg⁻¹ DW)

Genotype	Cd rate					
WT	0	42.04±3.10a	57.58±5.51 Aa	37.47±0.75 Aa **0.015**	0.402	0.011
	50		42.98±5.25 Ba	32.84±2.09 Aa 0.248	0.407	0.123
WT+Pro	0	41.54±1.76 a	50.99±6.21 Aab	41.06±2.11 Aa 0.123	0.934	0.181
	50		34.28±1.41 Ba	33.19±4.19 Aa 0.331	0.228	0.813
35S	0	49.10±5.26 a	46.56±1.39 Aab	35.04±1.43 Aa 0.592	**0.013**	**0.001**
	50		43.38±4.43 Aa	35.89±2.61 Aa 0.315	0.556	0.195
rd29A	0	40.95±3.52 a	39.02±1.16 Ab	36.21±1.77 Aa 0.579	0.191	0.231
	50		36.08±0.92 Aa	33.62±0.76 Aa 0.071	**0.027**	0.084

Zn concentration in the roots (mg kg⁻¹ DW)

Genotype	Cd rate					
WT	0	37.64±2.17 ab	47.32±11.99 Aa	50.75±2.60 Aa 0.366	0.229	0.789
	50		47.59±3.21 Aa	57.61±7.27 Aa 0.401	0.059	0.254
WT+Pro	0	28.64±2.03 b	61.79±6.38 Aa	44.01±6.94 Aa **0.002**	0.082	0.108
	50		46.13±3.95 Aa	55.86±4.42 Aa 0.479	0.346	0.152
35S	0	58.07±4.63 a	57.48±4.50 Aa	50.05±3.37 Aa 0.923	0.210	0.234
	50		50.55±9.28 Aa	56.84±16.07 Aa 0.965	0.724	0.746
rd29A	0	49.09±7.80 ab	50.34±2.88 Aa	46.69±3.90 Aa 0.871	0.756	0.479
	50		49.46±6.41 Aa	44.55±5.76 Aa 0.649	0.832	0.589

Means ± SEM followed by distinct upper case letters indicate difference between Cd rate (0 vs 50 µmol L⁻¹ CdCl₂·H₂O) within each genotype (WT, WT+Pro, 35S, and rd29A) over the time, and distinct lower case letters indicate difference between genotypes within each Cd rate for each exposure time (Tukey test, $n=4$, $p \leq 0.05$)

p-values in bold indicate difference between Cd exposure times within each genotype and Cd exposure condition ($p \leq 0.05$)
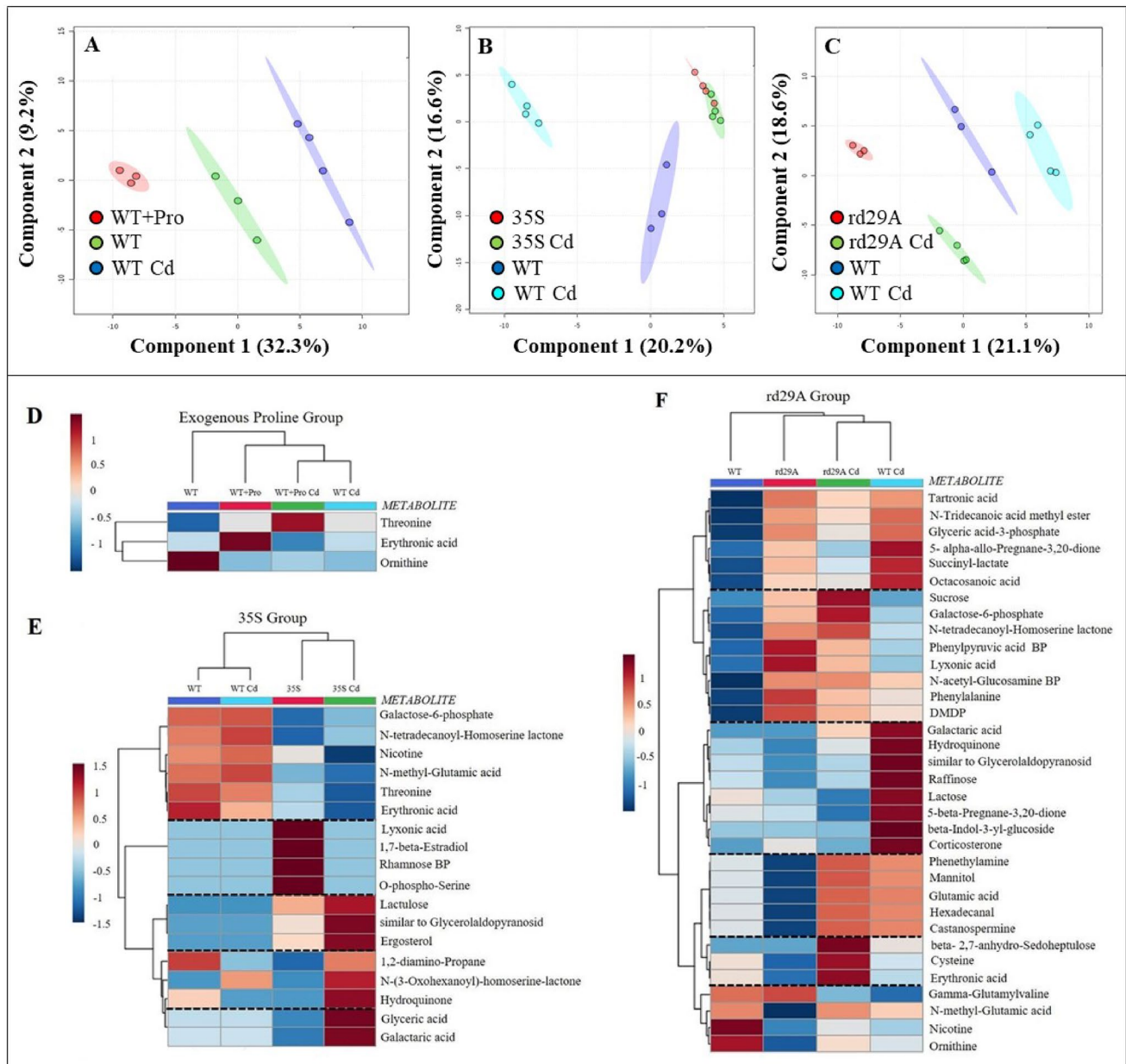
Fig. 2 Partial least squares discriminant analysis (PLS-DA) of the metabolites identified by GC-TOFMS (**A**–**C**) and hierarchical clustering with heatmap showing the relative abundance of the metabolites (**D**, **F**) in the leaves of wild type (WT), WT supplied with 1 mmol L⁻¹ of exogenous proline 24 h before Cd exposure (WT + Pro), and transgenic tobacco (*Nicotiana tabacum* L.) plants containing the mutated *P5CSF129A* gene under control of the cauliflower mosaic virus CaMV35S (35S) or stress inducible rd29A (rd29A) promoters, exposed to Cd (0 and 50 µmol L⁻¹ CdCl₂·H₂O) after 24 h. Score plot model (**A**) and hierarchical clustering (**D**) for WT + Pro − Cd, WT + Pro + Cd, WT − Cd, and WT+Cd; score plot model (**B**) and hierarchical clustering (**E**) for 35S − Cd, 35S + Cd, WT − Cd, and WT+Cd; score plot model (**C**) and hierarchical clustering (**F**) for rd29A − Cd, rd29A+Cd, WT − Cd, and WT+Cd. Outliers (WT + Pro + Cd) were removed from the models (**A**). PLS-DA cross validation parameters: R^2 (correlation analysis: 0.99, 0.99, 0.99), and Q^2 (predictive capability: 0.84, 0.83, 0.84) for the three first principal components from the models **A**, **B** and **C**, respectively. The relative metabolite level is depicted by the color scale (**D**–**F**): red indicates up-regulation and blue indicates down-regulation (Color figure online)

compared to the other plants. Cadmium exposure did not increase MDA concentrations in the leaves, regardless of the genotypes or exposure time. There was also no effect of genotypes nor Cd rate on soluble protein concentrations in the leaves of plants (Fig. 3C). However, we can see that Cd exposure decreased the soluble protein concentration in the leaves of WT over time, differently from which
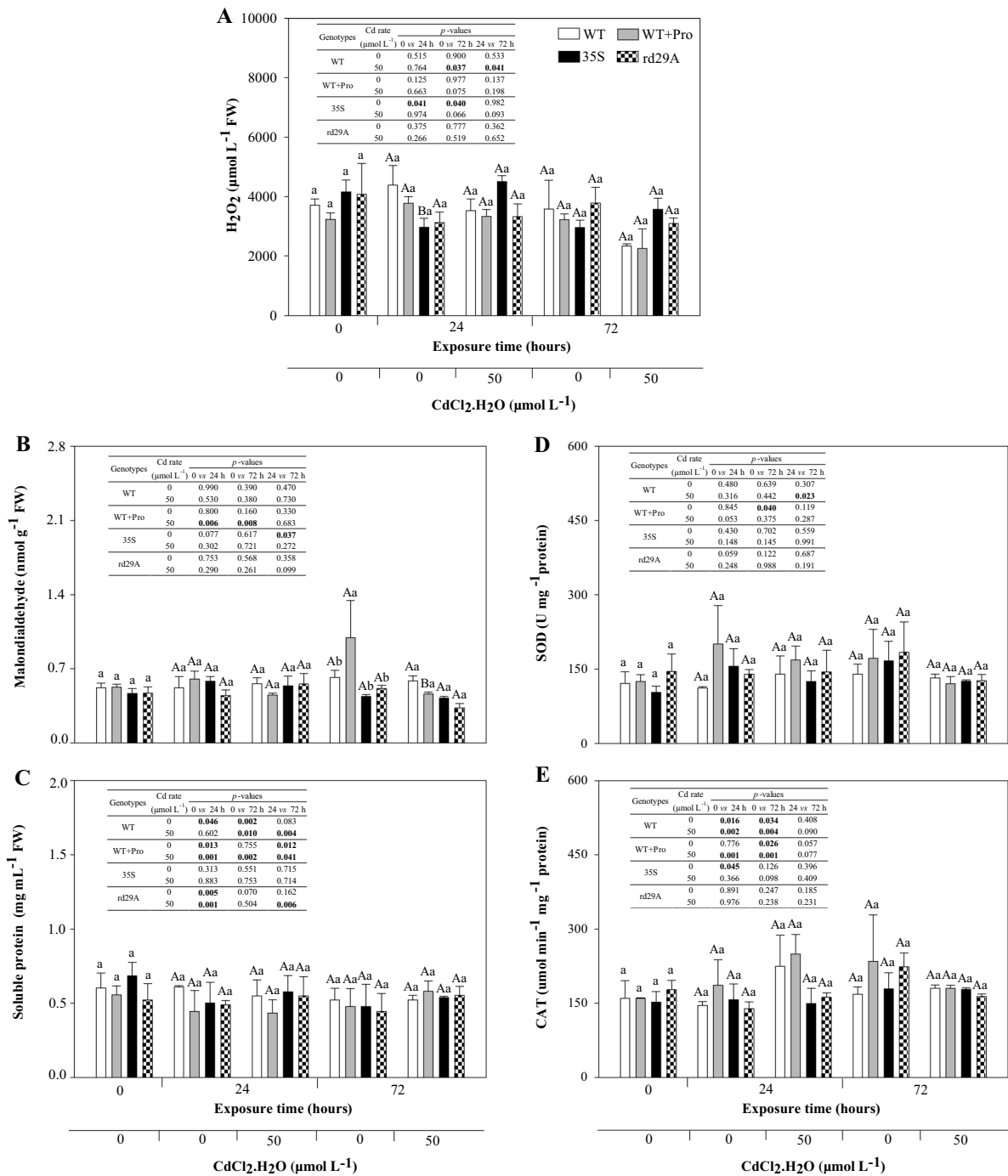
A — H_2O_2 (μmol L^{-1} FW)

Genotypes	Cd rate (μmol L^{-1})	0 vs 24 h	0 vs 72 h	24 vs 72 h
WT	0	0.515	0.900	0.533
	50	0.764	**0.037**	**0.041**
WT+Pro	0	0.125	0.977	0.137
	50	0.663	0.075	0.198
35S	0	**0.041**	**0.040**	0.982
	50	0.974	0.066	0.093
rd29A	0	0.375	0.777	0.362
	50	0.266	0.519	0.652

B — Malondialdehyde (nmol g^{-1} FW)

Genotypes	Cd rate (μmol L^{-1})	0 vs 24 h	0 vs 72 h	24 vs 72 h
WT	0	0.990	0.390	0.470
	50	0.530	0.380	0.730
WT+Pro	0	0.800	0.160	0.330
	50	**0.006**	**0.008**	0.683
35S	0	0.077	0.617	**0.037**
	50	0.302	0.721	0.272
rd29A	0	0.753	0.568	0.358
	50	0.290	0.261	0.099

D — SOD (U mg^{-1} protein)

Genotypes	Cd rate (μmol L^{-1})	0 vs 24 h	0 vs 72 h	24 vs 72 h
WT	0	0.480	0.639	0.307
	50	0.316	0.442	**0.023**
WT+Pro	0	0.845	**0.040**	0.119
	50	0.053	0.375	0.287
35S	0	0.430	0.702	0.559
	50	0.148	0.145	0.991
rd29A	0	0.059	0.122	0.687
	50	0.248	0.988	0.191

C — Soluble protein (mg mL^{-1} FW)

Genotypes	Cd rate (μmol L^{-1})	0 vs 24 h	0 vs 72 h	24 vs 72 h
WT	0	**0.046**	**0.002**	0.083
	50	0.602	**0.010**	**0.004**
WT+Pro	0	**0.013**	0.755	**0.012**
	50	**0.001**	**0.002**	**0.041**
35S	0	0.313	0.551	0.715
	50	0.883	0.753	0.714
rd29A	0	**0.005**	0.070	0.162
	50	**0.001**	0.504	**0.006**

E — CAT (μmol min^{-1} mg^{-1} protein)

Genotypes	Cd rate (μmol L^{-1})	0 vs 24 h	0 vs 72 h	24 vs 72 h
WT	0	**0.016**	**0.034**	0.408
	50	**0.002**	**0.004**	0.090
WT+Pro	0	0.776	**0.026**	0.057
	50	**0.001**	**0.001**	0.077
35S	0	**0.045**	0.126	0.396
	50	0.366	0.098	0.409
rd29A	0	0.891	0.247	0.185
	50	0.976	0.238	0.231

Legend: □ WT ▨ WT+Pro ■ 35S ▧ rd29A

Exposure time (hours): 0, 24, 72
CdCl$_2$·H$_2$O (μmol L^{-1})

Fig. 3 Concentrations of hydrogen peroxide (H_2O_2, **A**), malondialdehyde (**B**) and soluble protein (**C**) and activities of superoxide dismutase (SOD, **D**) and catalase (CAT, **E**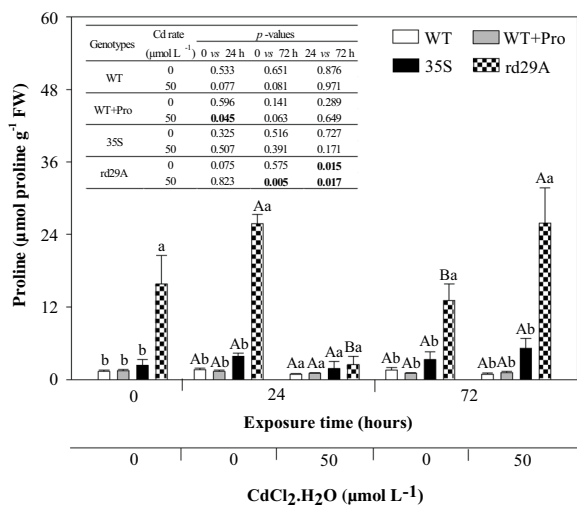) in the leaves of wild type (WT), WT supplied with 1 mmol L^{-1} of exogenous proline 24 h before Cd exposure (WT+Pro), and transgenic tobacco (*Nicotiana tabacum* L.) plants containing the mutated *P5CSF129A* gene under control of the cauliflower mosaic virus CaMV35S (35S) or stress inducible rd29A (rd29A) promoters, exposed to Cd (0 and 50 µmol L^{-1} CdCl$_2$·H$_2$O) for 0 (control), 24 and 72 h. Distinct upper case letters on the bars indicate difference between Cd rate (0 vs 50 µmol L^{-1} CdCl$_2$·H$_2$O) within each genotype (WT, WT+Pro, 35S, and rd29A) over the time, and distinct lower case letters indicate difference between genotypes within each Cd rate for each exposure time (Tukey test, $n = 3$, $p \leq 0.05$). *p*-values in bold in the tables inside each figure indicate difference between exposure times within each genotype and Cd rate ($p \leq 0.05$)

occurred with plants supplied with exogenous proline or overproducing proline after 72 h of Cd exposure (Fig. 3C).

The activities of the enzymes SOD and CAT of plants supplied with exogenous proline or overproducing proline did not differ from WT, regardless of Cd rate (Fig. 3D, E). There was also no effect of Cd exposure on SOD and CAT activities, regardless of genotypes. Over the time, we can observe that tobacco grown without exogenous proline supply or proline overproduction tended to present lower SOD activity due to Cd exposure for 72 h (Fig. 3D). Meanwhile, WT supplied or not with exogenous proline presented an increase on CAT activities in their leaves after 72 h compared to WT of the control, regardless of Cd rate (0 vs 72 h; Fig. 3E).

Plants Overproducing Proline Under the Control of the rd29A Promoter Presented Higher Proline Accumulation, Which Correlated with Genes Controlling the Metabolism of Proline

In the absence of Cd, rd29A presented higher proline concentrations compared to other plants at the times 0 and 24 h

Fig. 4 Proline concentration in the leaves of wild type (WT), WT supplied with 1 mmol L^{-1} of exogenous proline 24 h before Cd exposure (WT+Pro), and transgenic tobacco (*Nicotiana tabacum* L.) plants containing the mutated *P5CSF129A* gene under control of the cauliflower mosaic virus CaMV35S (35S) or stress inducible rd29A (rd29A) promoters, exposed to Cd (0 and 50 μmol L^{-1} $CdCl_2 \cdot H_2O$) for 0 (control), 24 and 72 h. Distinct upper case letters on the bars indicate difference between Cd rate (0 vs 50 μmol L^{-1} $CdCl_2 \cdot H_2O$) within each genotype (WT, WT+Pro, 35S, and rd29A) over the time, and distinct lower case letters indicate difference between genotypes within each Cd rate for each exposure time (Tukey test, $n=3$, $p \leq 0.05$). *p*-values in bold in the tables inside each figure indicate difference between exposure times within each genotype and Cd rate ($p \leq 0.05$)

(Fig. 4). Differently from the results observed in Cd absence, both transgenic tobacco lines presented similar proline concentrations to WT after 24 h of Cd exposure, regardless of exogenous proline supply. 72 h after, rd29A presented higher proline concentration in relation to other plants, regardless of Cd rate. Proline concentration was not affected by Cd exposure in the leaves of WT and 35S. On the other hand, proline concentrations in the leaves of rd29A exposed to Cd was 90% lower compared to plants unexposed to Cd after 24 h, but after 72 h, proline concentrations in the leaves of rd29A exposed to Cd was 97% higher compared to rd29A unexposed to Cd. The analysis of the data over the time showed that proline concentrations in the leaves of rd29A exposed to Cd after 72 h were 63% higher in relation to control (Fig. 4).

The higher proline concentration observed in rd29A collected at the times 0 and 24 h, in the absence of Cd (Fig. 4), probably occurred due to a higher *P5CSF129A* expression (Fig. 5A), since there was no significant difference in the expression of *P5CS-1*, *P5CS-2*, and *P5CR* (Fig. 5B–D) as compared to the other plants. Although unexposed rd29A presented the highest expression of *PDH1* compared to the other plants (Fig. 5E), proline was highly accumulated in these conditions (Fig. 4). There was no variation between genotypes unexposed to Cd concerning the expression of *P5CDH* (Fig. 5F). On the other hand, 24 h after Cd exposure plants overproducing proline presented similar proline concentrations to WT, regardless of exogenous proline supply (Fig. 4), even the transgenic plants presenting lower *P5CDH* expression compared to WT (Fig. 5F). This result probably is related to the low expression of *P5CSF129A* and *P5CR* under Cd exposure after 24 h (Fig. 5A and D).

The Synthesis of GSH and the Capacity to Keep Glutathione in Its Reduced Form Was Limited in Tobacco Plants Overproducing Proline Under Control of 35S Promoter

The concentrations of GSH and GSH + GSSG, and the redox state of GSH in tobacco supplied with exogenous proline or overproducing proline did not differ from that of WT collected immediately before Cd supply, differently from the concentration of GSSG that was lower in 35S (Fig. 6A–D). 24 and 72 h after the beginning of the study, there was no difference in the concentrations of GSH, GSSG, and GSH + GSSG (Fig. 6A–C), and in the redox state of GSH (Fig. 6D) between the genotypes unexposed to Cd. On the other hand, when the tobacco lines were exposed to Cd for 72 h, 35S presented lower concentrations of GSH and GSH + GSSG and a lower redox state of GSH (Fig. 6A and C, D). Cadmium exposure induced higher GSH accumulation in WT (regardless of exogenous proline supply) and rd29A compared to the same plants
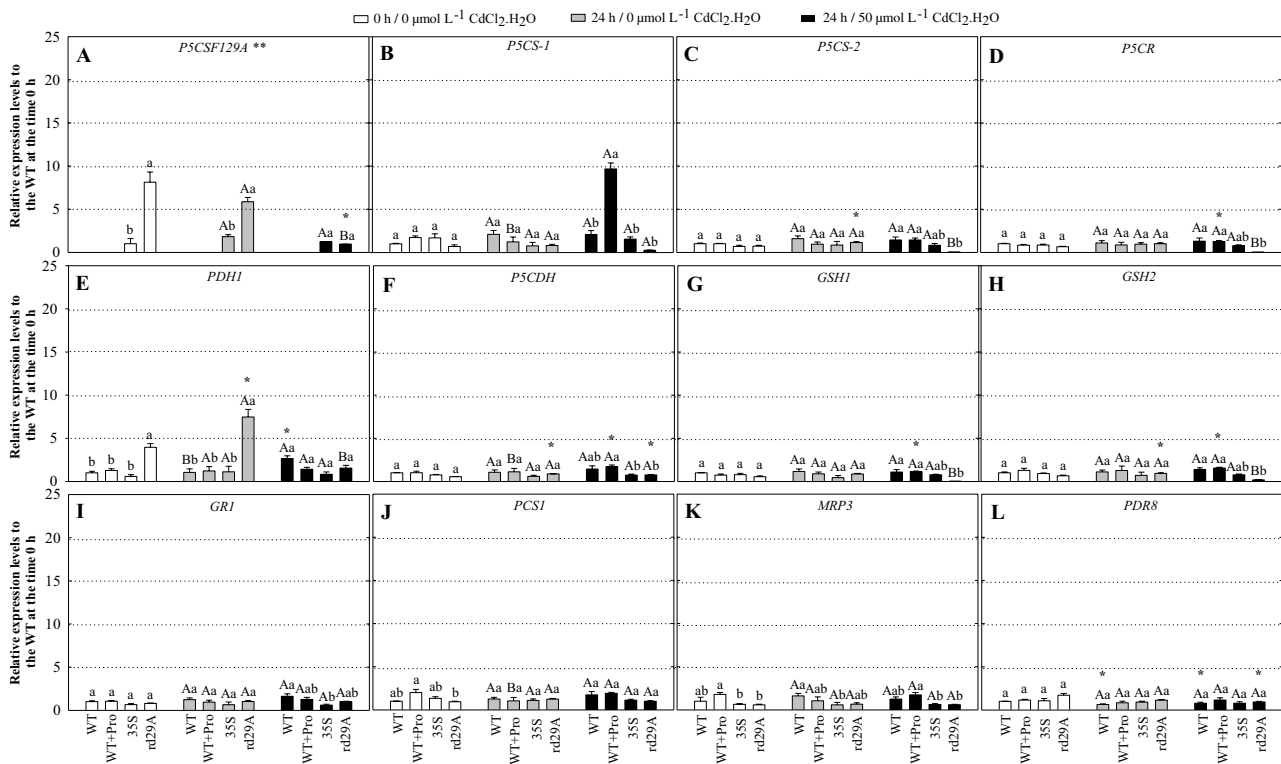
Fig. 5 Relative expression (fold change) of the genes *P5CSF129A* (**A**), *P5CS-1* (**B**), *P5CS-2* (**C**), *P5CR* (**D**), *PDH1* (**E**), *P5CDH* (**F**), *GSH1* (**G**), *GSH2* (**H**), *GR1* (**I**), *PCS1* (**J**), *MRP3* (**K**), and *PDR8* (**L**) in the leaves of wild type (WT), WT supplied with 1 mmol L^{-1} of exogenous proline 24 h before Cd exposure (WT+Pro), and transgenic tobacco (*Nicotiana tabacum* L.) plants containing the mutated *P5CSF129A* gene under control of the cauliflower mosaic virus CaMV35S (35S) or stress inducible rd29A (rd29A) promoters, exposed to Cd (0 and 50 μmol L^{-1} CdCl$_2$·H$_2$O) for 0 (control), 24 and 72 h. Distinct upper case letters on the bars indicate difference between Cd rate (0 vs 50 μmol L^{-1} CdCl$_2$·H$_2$O) within each genotype (WT, WT+Pro, 35S, and rd29A) after 24 h, and distinct lower case letters indicate difference between genotypes within each Cd rate for each exposure time (Tukey test, $n = 3$, $p \leq 0.05$). Asterisks (*) over the letters on the bars inside each figure indicate difference between exposure times within each genotype and Cd rate ($p \leq 0.05$). **Relative expression levels to transgenic tobacco overexpressing proline under control of constitutive promoter CaMV35S (35S) unexposed to Cd at the time 0 h (control)

unexposed to Cd after 72 h (Fig. 6A). The concentration of GSH+GSSG was higher in WT exposed to Cd compared to WT unexposed to Cd after 72 h, regardless of exogenous proline supply (Fig. 6C). The redox state of GSH in WT and rd29A exposed to Cd was higher than in the same plants unexposed to Cd after 72 h (Fig. 6D). Analyzing these results over the time, 35S have low capacity to keep glutathione in its reduced form (GSH) under Cd exposure (Fig. 6A–D), even when presenting similar GR activities as in other plants after 24 and 72 h of Cd exposure (Fig. 6E).

There was no difference in the expression of the genes *GSH1* and *GSH2* in the leaves of unexposed plants (Fig. 5G, H), but after 24 h of Cd exposure the expression of *GSH1* and *GSH2* in rd29A was lower than WT. The low expression of *GSH1* and *GSH2* did not limit the synthesis of GSH in rd29A in relation to other plants exposed to Cd for 24 h (Fig. 6A). Although the expression of *GR1* (encoding for GR) was lower in the leaves of 35S compared to other plants

after 24 h of Cd exposure (Fig. 5I), this genotype presented similar GR activity as the other plants (Fig. 6E).

There was no difference in *PCS1* expression between the plants after 24 h, regardless of Cd rate. Taking together the results of *PCS1* expression (Fig. 5J) and GSH concentration (Fig. 6A) we can conclude that PCs concentrations in the genotypes assayed should be similar to each other after 24 h. Nevertheless, the higher expression of the gene *MRP3* that encodes for the ABCC3 transporters (vacuolar membrane-localized protein involved in the vacuolar transport of PC–Cd complexes) (Brunetti et al. 2015) in the leaves of WT supplied with exogenous proline compared to 35S and rd29A after 24 h of Cd exposure (Fig. 5K) suggests that PCs synthesis was possibly higher when there was exogenous proline supply. There was no effect of genotypes nor Cd rate on the expression of *PDR8* (pleiotropic drug resistance) that is involved in Cd cellular efflux (Kim et al. 2007). However, *PDR8* expression
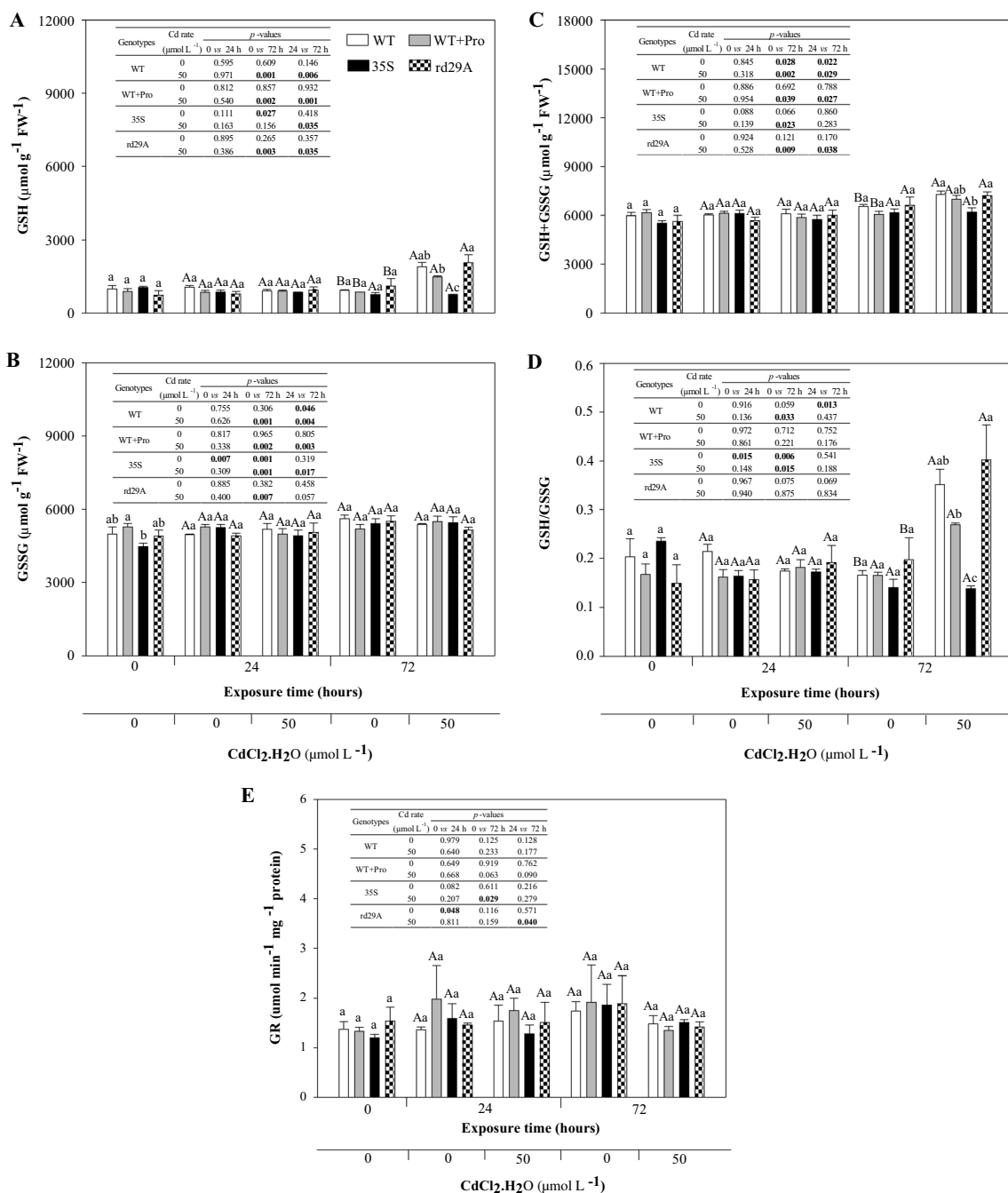
Fig. 6 Concentrations of reduced glutathione (GSH, **A**), oxidized glutathione (GSSG, **B**) and total glutathione (GSH+GSSG, **C**), redox state of glutathione (GSH/GSSG, **D**) and activity of glutathione reductase (GR, **E**) in the leaves of wild type (WT), WT supplied with 1 mmol L^{-1} of exogenous proline 24 h before Cd exposure (WT+Pro), and transgenic tobacco (*Nicotiana tabacum* L.) plants containing the mutated *P5CSF129A* gene under control of the cauliflower mosaic virus CaMV35S (35S) or stress inducible rd29A (rd29A) promoters, exposed to Cd (0 and 50 μmol L^{-1} CdCl$_2$·H$_2$O) for 0 (control), 24 and 72 h. Distinct upper case letters on the bars indicate difference between Cd rate (0 vs 50 μmol L^{-1} CdCl$_2$·H$_2$O) within each genotype (WT, WT+Pro, 35S, and rd29A) over the time, and distinct lower case letters indicate difference between genotypes within each Cd rate for each exposure time (Tukey test, $n=3$, $p \leq 0.05$). *p*-values in bold in the tables inside each figure indicate difference between exposure times within each genotype and Cd rate ($p \leq 0.05$)

in the leaves of WT and rd29A exposed to Cd after 24 h decreased over the time (Fig. 5L).

Discussion

Although it is believed that proline accumulation plays an adaptive role in plant tolerance against Cd-induced toxicity (Islam et al. 2009; Zouari et al. 2016; Repkina et al. 2019), this process is not completely known. In this study we assayed the effect of exogenous proline supply and proline overproduction on tolerance mechanisms of tobacco plants exposed to Cd in order to better understand the role of proline in mitigating Cd-induced toxicity. It is known that Cd exposure can change a series of physiological, biochemical, and molecular events in plants that leads to inhibition of biomass production (Clemens 2006; Gallego et al. 2012), as occurred with all tobacco plants after 72 h of Cd exposure (Fig. 1A, B). The biomass production of plants supplied with exogenous proline or overproducing proline under control of 35S and rd29A promoters did not differ from that of the WT, under Cd exposure (Fig. 1A, B). Differently from our results, Zouari et al. (2016) described that olive plants exposed to Cd produced more biomass when it received exogenous proline supply, and attributed this result to a differentiated proline-induced distribution of Cd between roots and leaves. In our study, only WT plants grown with exogenous proline supply exhibited Cd concentration in their leaves below 100 mg kg^{-1} DW after 72 h of Cd exposure (Fig. 1C), which can be attributed to a higher Cd accumulation in the roots (Fig. 1D) and a lower root-to-shoot Cd translocation (Fig. 1E) in this treatment. It is known that Cd accumulation in the roots is strongly related to PCs synthesis in this tissue (Clemens 2006). Therefore, it is possible that exogenously applied proline allowed high proline accumulation in the roots of tobacco exposed to Cd helping to reduce oxidative damage and contributing to a more reducing cellular environment, which may then have increased GSH concentration, allowing higher PCs synthesis (Siripornadulsil et al. 2002).

Some studies have also pointed out beneficial effects of proline on maintaining the nutritional plant status (Islam et al. 2009; Zouari et al. 2016), which is desirable since the modulation of nutritional status is involved in plant tolerance against Cd-induced stress (Carvalho et al. 2020). Indeed, in general tobacco lines overproducing proline were less susceptible to Cd-induced nutritional changes compared to WT (Tables 2 and 3). However, Cd exposure increased P concentrations in the leaves of rd29A and decreased Mn and Zn concentrations in the leaves of WT plants, regardless of exogenous proline supply, 24 h after the beginning of the study (Tables 2 and 3). Interestingly, when P concentration in the leaves of rd29A increased due

Cd to exposure (Table 2), there was a decrease in proline concentration (Fig. 4). Aleksza et al. (2017) mentioned that phosphate starvation led to gradual increase in proline concentration in *A. thaliana* as well as in transcriptional activation of *P5CS-1*. In fact, the expression of *P5CS-1* in our study was higher in tobacco plants which presented lower P concentrations after 24 h of Cd exposure (Fig. 5A; Table 2). However, there is no relationship between P concentration and *P5CSF129A* expression (Table 2; Fig. 5A). Bertoli et al. (2012) evaluated the effect of Cd on uptake and translocation of nutrients in tomato (*Solanum lycopersicum* L.) and also found lower Mn and Zn concentrations in the leaves of plants exposed to Cd, which was attributed to the antagonistic competition between Cd and Mn/Zn. In our study, Cd exposure also decreased S concentration in the leaves of 35S plants after 72 h (Table 2), which possibly contributed to the lower concentrations of GSH and GSH + GSSG in 35S compared to other plants after 72 h (Fig. 6A and C), since S is a component of cysteine that is used for GSH synthesis (Yadav 2010).

Tobacco 35S also presented lower Fe concentrations in their roots after 24 h of Cd exposure, which was different from WT plants supplied with exogenous proline that presented higher Fe concentration after 72 h of Cd exposure (Table 3). Sharmila et al. (2017) suggested that proline accumulation in leaves of Indian mustard exposed to Cd was coupled to Fe depletion. If we consider that the same could occurs in the roots, our results make sense since 35S plants should synthesize more proline and WT plants do not need to synthesize proline due to exogenous supply. Furthermore, both transgenic plants overproducing proline presented decreased Mn concentrations in their roots after 72 h of Cd exposure (Table 3). The lower concentrations of cationic micronutrients, such as Mn, in the roots of plants have been often attributed to competition of Cd^{2+} for the same nutrient transporters (Bertoli et al. 2012), but lower Mn concentrations in the roots can indicate protective mechanisms to counteract the entrance of positive charges originating from Cd accumulation in roots (Carvalho et al. 2020). Changes on nutrient concentrations are often coupled to negative outcomes on plant development under Cd exposure, but the degree of plant tolerance to short Cd exposure is related to its capacity to adjust the nutritional status in order to improve its performance under Cd-induced stress (Carvalho et al. 2020). Manganese deficiency can increase root endodermal suberization (Chen et al. 2019), which is an important mechanism to immobilize Cd in cell walls (Gallego et al. 2012). Dicotyledonous species such as tobacco present several proline- or hydroxyproline-rich glycoprotein constituents in the cell wall (Stiefel et al. 1988). Thereby, it is probable that there is some link between proline, Mn status, and root suberization, but this assumption needs to be carefully investigated. Cd-induced changes on nutrient

homeostasis can also affect the metabolic network of plants that must be reconfigured under stress conditions to allow the maintenance of metabolic homeostasis and the production of compounds that ameliorate the stress (Obata and Fernie 2012). Thus, the ameliorated capacity of tobacco plants overproducing proline to cope with nutritional alterations (Tables 2 and 3) could facilitate metabolic adjustments under Cd-induced stress (Fig. 2).

Only 3 metabolites were significantly different in WT plants grown with exogenous proline supply (Fig. 2A and D), but 18 and 34 responsive metabolites were identified in plants overproducing proline under control of 35S and rd29A promoters, respectively (Fig. 2E, F), indicating us that proline is involved in metabolic network adjustments, especially under stress conditions. In 35S plants, Cd exposure induced the accumulation of sugars (lactulose and similar to glycerolaldopyranosid) and organic acids (glyceric and galactaric acids) (Fig. 2E). Karalija and Selović (2018) related proline seed priming with an increased sugar content in maize (*Zea mays* L.), indicating an interaction of increased proline and soluble sugars on antioxidant plant protection. Sugars are involved in direct ROS quenching in different organelles and act in an integrated cellular redox network (for a comprehensive review see Keunen et al. 2013). Like sugars, organic acids are important metabolites to decrease the Cd-induced stress in plants (Sun et al. 2010). Sun et al. (2006) pointed out that organic acids are related to Cd hyperaccumulation in the leaves of *Solanum nigrum* L. due to their roles in Cd complexation, transportation and storage, mainly in the vacuoles. Similarly to what was observed in 35S plants, proline overproduction under control of rd29A promoter allowed accumulation of sugars (sucrose, galactose-6-phosphate, and mannitol), organic acids (erythronic acid), and amino acids (cysteine and glutamic acid) after 24 h of Cd exposure (Fig. 2F). The interaction between proline and sugars (Karalija and Selović 2018), such as sucrose that acts on hydroxyl (°OH) scavenging (Van den Ende and Valluru 2009), is important to reduce oxidative damage and contribute to a more reducing cellular environment (Siripornadulsil et al. 2002). Thus, as speculated by Siripornadulsil et al. (2002), a more reducing cellular environment probably contributed for the increased cysteine concentration in rd29A plants after Cd exposure (Fig. 2F), which is essential for both GSH and PCs synthesis (Yadav 2010). The synthesis of antioxidants like sugars and GSH is indispensable to avoid the Cd-induced oxidative damages.

Cd-induced oxidative damage leads to a vast number of responses in plants depending on both, the Cd concentration and the exposure time (Gallego et al. 2012). These triggered responses are particularly important to be understood in leaves, since ROS production can impair photosynthesis that in turn is one of the main causes of Cd-induced growth inhibition (Zouari et al. 2016). Whereas oxidative stress has

often been discussed as a primary effect of Cd^{2+} exposure (Clemens 2006), only the plants overproducing proline under the control of the 35S promoter presented higher H_2O_2 concentrations in their leaves after 24 h of Cd exposure compared to unexposed plants (Fig. 3A). However, Cd exposure did not induce lipid peroxidation (Fig. 3B) in all tobacco genotypes assayed, regardless of exposure time. Our results are similar to those described by Repkina et al. (2019) who also did not observe any symptom of Cd-induced oxidative stress in wheat leaves in the first 48 h of Cd exposure and attributed this result to an efficient action of non-enzymatic antioxidants and Cd-chelators (e.g., sugars, GSH, and PCs). In our study, the activities of SOD and CAT also did not increase due to Cd exposure, regardless of exogenous proline supply or proline overproduction (Fig. 3D, E). Plants not supplied with exogenous proline or overproducing proline tended to present lower SOD activity over time after Cd exposure (Fig. 3D). Perhaps SOD and CAT are not the main line of defense against Cd-induced oxidative stress in tobacco plants, as previously speculated in other studies. Martins et al. (2014) pointed out that SOD was not heavily involved in antioxidative responses of tobacco exposed to Cd. Iannone et al. (2015) stated that CAT did not play a crucial role in tobacco protection against Cd toxicity, since this species is able to activate alternative defense mechanisms such as ameliorated synthesis of proline and GSH.

Proline accumulation is a common physiological response in many plant species under biotic and abiotic stresses (Hong et al. 2000; Sharma and Dietz 2006), such as Cd exposure (Islam et al. 2009; Sun et al. 2010; Iannone et al. 2015; Zouari et al. 2016; Repkina et al. 2019), since this amino acid has protective functions such as osmotic adjustment, stabilization of cellular structures, and ROS scavenging (Verbruggen and Hermans 2008; Borgo et al. 2015). However, in our study Cd exposure only led to proline accumulation in tobacco plants overproducing proline under the control of the rd29A promoter, after 72 h (Fig. 4). This result is probably related to the expression of the genes linked to proline synthesis, since the results measured in the first 24 h of study indicate that the expression of *P5CSF129A* in transgenic tobacco was higher in rd29A than 35S plants (Fig. 5A). The first glutamate reduction in tobacco overproducing proline is regulated by *P5CSF129A*. Kumar et al. (2010) reported that proline accumulation in salt-stressed rice (*Oryza sativa* L.) was closely correlated with the expression of the *P5CSF129A* gene. Moreover, the 35S promoter is presumed to be a constitutive promoter, but it contains several domains and subdomains that can confer different developmental and tissue-specific expression patterns in different species (Borgo et al. 2015). These same authors reported that tobacco overproducing proline under control of 35S promoter presented higher proline concentrations in the roots than in leaves. Despite the fact that tobacco overproducing

proline under control of the 35S promoter, they did not present higher proline concentrations in their leaves after Cd exposure (Fig. 4). Nevertheless, the concentration of this amino acid was two-fold higher compared to WT plants after 24 and 72 h of Cd exposure, which is in agreement with the results described by Hong et al. (2000) for this genotype.

In non-transgenic plants, the levels of both *P5CS* and *P5CR* transcripts were correlated with proline concentration in *A. thaliana* leaves (Verbruggen and Hermans 2008). In the case of WT plants supplied with exogenous proline that presented Cd-induced expression of *P5CS-1* (Fig. 5B) and low proline concentration (Fig. 4) after 24 h, it is possible that post-transcriptional changes took place, which limited proline synthesis. The low proline concentration in the leaves of WT that received exogenous proline supply (Fig. 4) can also be associated to low proline exportation via xylem from roots to leaves due to the local proline storage in root cells, in the absence and presence of Cd. This assumption makes sense considering that *PDH1* expression is strongly induced by exogenous proline addition (Verbruggen and Hermans 2008), but *PDH1* expression in the leaves of WT plants grown with or without exogenous proline addition was similar to each other (Fig. 5E). Gagneul et al. (2007) reported that excessive proline was sequestered into the vacuoles in the roots of non-stressed *Limonium latifolium* L., whereas high proline concentrations were detected in the cytosol of plants under salt-stress. Therefore, proline coming from exogenous supply could be stored in the roots and used when necessary in plants under Cd-induced stress. Zouari et al. (2016) determined proline concentrations in the roots and leaves of olive plants exposed to Cd in response to exogenous proline supply and observed higher proline concentrations in the roots (low exportation to leaves), which was the tissue more damaged by the Cd-induced oxidative stress. Although proline can also accumulate due to a low degradation rate in reactions catalyzed by proline dehydrogenase (PDH, EC 1.5.99.8) and pyrroline-5-carboxylate dehydrogenase (P5CDH, EC 1.2.1.88) (Verbruggen and Hermans 2008), there was no clear correlation between proline concentration and the expression of the *PDH1* and *P5CDH* genes in rd29A plants (Figs. 4 and 5E, F). With the exception of rd29A that presented high proline concentration, there was no clear action of proline on the absence of Cd-induced oxidative stress in the leaves of the other plants, which can be associated with changes in Cd translocation from roots to shoots and with the synthesis of non-enzymatic antioxidants, such as sugars, organic acids, and GSH.

Glutathione is the most important non-enzymatic antioxidant in plants exposed to Cd (Gratão et al. 2005; Yadav 2010; Hendrix et al. 2020a). However, *GSH1* and *GSH2* genes that encode for enzymes controlling GSH synthesis (γ-glutamylcysteine synthetase—GSH1, EC 6.3.2.2; and glutathione synthetase—GSH2, EC 6.3.2.3) were not induced upon Cd exposure in the first 24 h (Fig. 5G, H), as well as the GSH concentration in the leaves remained the same in all genotypes (Fig. 6A). Vögeli-Lange and Wagner (1996) proposed that leaf cells of tobacco have a responsive 'sensing system' to keep GSH concentration at a fixed level even under stress conditions. On the other hand, some studies have shown that GSH induction by Cd in the leaves of plants occurs more often under prolonged Cd exposure and depends on signaling and substrates for GSH synthesis, PCs synthesis from GSH, action of other antioxidants over ROS, and Cd concentrations in this tissue (Clemens 2006; Mendoza-Cózatl and Moreno-Sánchez 2006). Thus, it is probable that the higher Cd concentration detected in the leaves of the plants after 72 h of Cd exposure compared to 24 h of Cd exposure (Fig. 1C) contributed to the higher GSH concentration observed in the leaves of WT, regardless of exogenous proline supply, and rd29A plants at the end of the study (Fig. 6A). Hendrix et al. (2020a) reported that GSH concentrations in the leaves of *A. thaliana* exposed to Cd also did not differ from the control in the first 24 h, but its concentration increased in relation to control plants after 72 h of Cd exposure. Mendoza-Cózatl and Moreno-Sánchez (2006) suggested that plants exposed to Cd rates up to 50 μmol L^{-1} can present an increase in GSH concentration, but high Cd concentrations often lead to GSH depletion due its use as a substrate for PCs synthesis. In our study, the higher Cd-induced *PCS1* expression (Fig. 5J) and the higher *MRP3* expression in the leaves of WT that received exogenous proline supply compared to the other tobacco plants after 24 h of Cd exposure (Fig. 5K) suggest a possible higher PCs synthesis in this treatment. *PCS1* encodes for phytochelatin synthase (PCS, EC 2.3.2.15) (Wojas et al. 2008). So, the lower concentrations of GSH and GSH + GSSG observed in the leaves of 35S plant in comparison to the other plants after 72 h of Cd exposure (Fig. 6A and C) possibly is related to lower Cd-induced S concentrations (Table 2) or some Cd-induced limitation on GSH synthesis or both. Wojas et al. (2008) reported that Cd-induced γ-glutamylcysteine (γ-EC) accumulation in tobacco leaves that resulted in lower GSH concentrations in this tissue. As *GSH2* expression (GSH2 ligates a glycine residue with γ-EC to form GSH) in 35S did not decrease after 24 h of Cd exposure (Fig. 5H), it is more likely that γ-EC has been used to bind Cd, as related by Wojas et al. (2008) in tobacco overexpressing PCS under control of a 35S promoter.

The transgenic tobacco 35S also presented a lower redox state of GSH after 72 h of Cd exposure (Fig. 6D), and a lower capacity to keep glutathione in its reduced form (GSH) over time under Cd exposure (Fig. 6A–D), even when presenting similar GR activities as the other plants (Fig. 6E). These results could indicate a relationship between proline and GSH, but in our study there was no significative correlation between proline and GSH concentrations for all genotypes

assessed (data not shown). Thus, besides the possible limitation in GSH synthesis due to the use of γ-EC to bind Cd, the lower redox state of GSH in 35S plants after 72 h of Cd exposure can also be associated to the presence of glutathione in its oxidized form due to an inefficient action of CAT on H_2O_2 scavenging in tobacco (Iannone et al. 2015). Queval et al. (2011) observed that under limited CAT activity in the leaves of *A. thaliana* plants in response to increased H_2O_2 availability, glutathione metabolism was changed and there was predominance of GSSG. This fact helps to explain the higher GSSG concentration in relation to GSH in all plants, even those unexposed to Cd (Fig. 6A, B). Noctor et al. (2012) pointed out that there is a close relationship between H_2O_2 concentration and GSH status, and at moderate rates of endogenous H_2O_2 production a decrease in the leaf GSH/GSSG ratio is common. Although the low redox state of GSH can limit the action of ascorbate–glutathione cycle on ROS scavenging (Gratão et al. 2005), Jozefczak et al. (2015) described that in GSH-deficient mutants of *A. thaliana* exposed to Cd, the more oxidized environment contributed to the activation of alternative pathways using both $O_2^{\cdot-}$ and H_2O_2 scavengers. Thus, it is possible that the Cd-induced increase in sugars such as lactulose and organic acids such as glyceric and galactaric acids in the leaves of 35S plants after 24 h of Cd exposure (Fig. 2E) may also have occurred after 72 h of Cd exposure, contributing for ROS scavenging. As mentioned before, both sugars and organic acids play important roles on Cd-tolerance (Sharma and Dietz 2006; Sun et al. 2006, 2010; Keunen et al. 2013; Soares et al. 2019).

Conclusion

Exogenous proline supply induced different responses compared to endogenous proline overproduction in tobacco exposed to Cd. Plants supplied with exogenous proline presented lower Cd translocation from roots to leaves compared to plants overproducing proline, which certainly contributed to attenuate oxidative damages in the leaves of plants. On the other hand, tobacco overproducing proline was less susceptible to Cd-induced nutritional changes as compared to wild type and showed better metabolic adjustment under stress conditions than plants grown with exogenous proline supply. In this sense, the absence of Cd-induced oxidative stress in the leaves of plants overproducing proline is associated with an enhanced proline-induced synthesis of metabolites such as sugars and organic acids, since both SOD and CAT were not the main line of defense against Cd-induced oxidative stress in tobacco. Moreover, the absence of Cd-induced oxidative stress in tobacco overproducing proline under control of the stress inducible rd29A promoter can be attributed to a higher proline and GSH concentrations. Transgenic tobacco

under the control of the stress inducible rd29A promoter exhibited higher proline concentrations than plants overproducing proline under the control of the constitutive 35S promoter. With exception of rd29A plants that presented high proline and GSH concentrations, the others presented an inverse correlation between proline and GSH synthesis after 72 h of Cd exposure, suggesting a signaling network between proline and GSH. However, new studies are necessary to elucidate this assumption and to assay the action of exogenous proline supply and proline overproduction under prolonged Cd exposure, in roots and leaves.

Declarations

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