Copper amine oxidase 8 regulates arginine-dependent nitric oxide production in Arabidopsis thaliana

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Abstract

Nitric oxide (NO) is a key signaling molecule in plants, regulating a wide range of physiological processes. However, its origin in plants remains unclear. It can be generated from nitrite through a reductive pathway, notably via the action of the nitrate reductase (NR), and evidence suggests an additional oxidative pathway, involving arginine. From an initial screen of potential Arabidopsis thaliana mutants impaired in NO production, we identified copper amine oxidase 8 (CuAO8). Two cuao8 mutant lines displayed a decreased NO production in seedlings after elicitor treatment and salt stress. The NR-dependent pathway was not responsible for the impaired NO production as no change in NR activity was found in the mutants. However, total arginase activity was strongly increased in cuao8 knockout mutants after salt stress. Moreover, NO production could be restored in the mutants by arginase inhibition or arginine addition. Furthermore, arginine supplementation reversed the root growth phenotype observed in the mutants. These results demonstrate that CuAO8 participates in NO production by influencing arginine availability through the modulation of arginase activity. The influence of CuAO8 on arginine-dependent NO synthesis suggests a new regulatory pathway for NO production in plants.

Key words: Arginase, arginine, copper amine oxidase, nitric oxide, nitric oxide production, nitrate reductase, plant.

Introduction

Nitric oxide (NO) is a ubiquitous radical gas that possesses a wide range of functions in plants. Indeed, this signaling molecule is involved in developmental processes, such as germination or flowering, as well as in the adaptive response to biotic or abiotic stresses (for reviews, see Scheler et al., 2013; Yang et al., 2014; Hichri et al., 2015; Sanz et al., 2015; Simontacchi et al., 2015). Over the last years, the complex mechanisms underlying these effects have been studied in depth, including the NO-dependent post-translational modifications of proteins or the NO crosstalk with phytohormones (see, for example, Astier and Lindermayr, 2012; Freschi, 2013; Mur et al., 2013). However, the origins of NO in plants are not fully understood. In animals, NO is mainly produced via nitric oxide synthase.
synthases (NOSs), which catalyze a two-step oxidation of l-arginine into l-citrulline and NO, using reduced NADPH as the electron donor, oxygen as co-substrate, and (6R)-5,6,7,8-tetrahydrobiopterin (BH2), FAD, FMN, and calmodulin (CaM) as cofactors (Forstermann and Sessa, 2012).

In plants, the picture is more complex and the subject of debate (Moreau et al., 2010; Frohlich and Durner, 2011). An extensive in silico study demonstrated that NOS homologs could not be found in the transcriptome of >1000 different photosynthetic organisms, with the notable exception of ~12 algae, including the recently characterized NOS from Osterococcus tauri (Foresi et al., 2010; Jeandroz et al., 2016). Importantly, no homologs were found in the transcriptomes of embryophytes (Jeandroz et al., 2016).

In fact, two pathways for NO production have been described in plants. The reductive pathway converts nitrite to NO notably through the nitrite reductase activity (Ni-NR activity) of nitrate reductase (NR). The principal activity of NR concerns the reduction of nitrate to nitrite. However, it can also reduce nitrite into NO (Ni-NR activity) in an NADH-dependent reaction. This Ni-NR activity represents only ~1% of the total NR activity in normal conditions but can be promoted by several conditions such as acidic pH, anoxia, or a substantial increase in nitrite content (Rockel et al., 2002; Meyer et al., 2005). Although NR is the best characterized NO source in plants, other routes for nitrite reduction have also been described. Nitrite can be reduced to NO via the mitochondrial electron transport system (Gupta and Igamberdiev, 2016), or non-enzymatically in the case of high nitrite concentrations, low pH, or highly reducing conditions (Bethke et al., 2004). Some evidence suggests that molybdoenzymes could also reduce nitrite in some conditions (Maia and Moura, 2015).

The second main pathway of NO production in plants is an oxidative one. Even if the enzymes involved in this pathway are unknown, several works have correlated consumption of l-arginine with a production of NO in plants, similar to the NOS activity present in animals (Gas et al., 2009; Del Rio, 2011; Corpas and Barroso, 2014). This NOS-like activity is further supported by the expression of recombinant rat NOS in Arabidopsis thaliana and Nicotiana tabacum, which results in increased NO production and higher resistance to biotic and abiotic stresses (Chun et al., 2012; Shi et al., 2012). In addition, several other experimental data support the existence of an oxidative route for NO production in plants, such as external addition of hydroxylamine or polyamines (PAs), which both produce NO in plant cells (Tun et al., 2006; Rümer et al., 2009; Yang et al., 2014; Jeandroz et al., 2016).

Besides these works directly linking the NO produced in plants to a putative NOS activity, several indirect reports also favor the existence of arginine-dependent NO production in plants. For example, an increased arginine activity, an enzyme directly modulating the arginine and PA bioavailability in the plant, resulted in an impaired NO production in A. thaliana correlated with higher salt stress resistance (Flores et al., 2008; Shi et al., 2013; Meng et al., 2015). Moreover, copper amine oxidase 1 (CuAO1) mutants displayed decreased NO production in A. thaliana after ascorbic acid (ABA) or PA treatment (Wimalasekera et al., 2011). CuAOs are homodimeric enzymes that control PA metabolism by catalyzing the oxidation of the primary amine groups of PAs, with a higher affinity for putrescine and cadaverine (Cona et al., 2006). The catabolic products from these oxidation reactions are also involved in signaling and regulation of primary metabolism. As an example, the 4-aminobutanal produced by CuAO from putrescine is a precursor of γ-aminobutyric acid (GABA), a well-known signaling compound involved in plant stress responses (Moschou et al., 2012). To date, 10 different putative CuAOs have been annotated in A. thaliana, four of them (AtAO1, AtCuAO1, AtCuAO2, and AtCuAO3) being recently characterized (Wimalasekera et al., 2011; Planas-Portell et al., 2013; Ghuge et al., 2015).

In this study, CuAO8 was identified by a targeted screen to be involved in the regulation of NO production. We were able to show that CuAO8 participates in NO production after 2,6-dichloroisonicotinic acid (INA) and salt stress, but also impacts primary root growth. We demonstrated that CuAO8, a true copper amine oxidase, modulates NO production by regulating arginase activity, thereby affecting the bioavailability of arginine.

### Materials and methods

#### Plant growth

All A. thaliana T-DNA insertion lines were in the Columbia (Col-0) background and grown vertically in square Petri dishes on half-strength Murashige and Skoog (1/2 MS) medium [1% sucrose, 1.2% phytoagar (Duchefa)] in short-day conditions (10 h light day−1) unless indicated otherwise. Seeds were surface sterilized for 3 h with chlorine gas and vernalized for 2 d at 4 °C in the dark.

#### T-DNA insertional knockout lines

T-DNA insertion lines used were tested for homozygosity by PCR on genomic DNA using gene-specific primers in combination with the LbB1.3 T-DNA insertion primer. The primer sequences were obtained from the SALK Institute with the iSect Primers tool: http://signal.salk.edu/tdnaprimer2.html, last accessed 24 February 2017 (not shown). For knockout verification of cuao8-1 and cuao8-2, total RNA was isolated (Qiagen), cDNA was synthesized (Qiagen), and CDs (coding sequence) transcript absence checked by RT-PCR using CuAO8-specific primers (see Supplementary Table S1 at JXB online).

#### Relative quantitative PCR

RNA (Qiagen) was isolated from 5-day-old seedlings harvested before or after NaCl (200 mM/6 h) treatment. Afterwards cDNA was synthesized (QuantiTect Reverse transcriptase kit, Qiagen) according to the manufacturer's instructions. Primers (Supplementary Table S1) were designed using QuantPrime software discriminating splice variants and span exon–exon borders (http://quantprime.mpimp-golm.mpg.de/, last accessed 24 February 2017) (Arvidsson et al., 2008). The qPCR was performed with Applied Biosystems 7500 (Fast) and the Sequence Detection Software 1.3.1 from Applied Biosystems. Raw data analysis and calculation of the efficiency (E) and cycle threshold (CT) values were carried out with the PCR-Miner software http://rewindup.info/miner/, last accessed 24 February 2017 (Zhao and Fernald, 2005). The relative transcript abundance was calculated as (1+E)−ΔCT for every well and normalized against the geometric mean of the reference genes ubiquitin 5, tubulin 9, and ribosomal protein S16 (Scheler et al., 2015; Supplementary Table S1).
Total protein extraction for CuAO8 knockout analysis

Total protein of 4-week-old leaves was extracted using the Plant Total Protein Extraction Kit (Sigma-Aldrich) according to the manufacturer’s instructions.

Quantification of NO and H$_2$O$_2$ production

Five-day-old seedlings were stained with diverse dyes reflecting the NO/H$_2$O$_2$ production during stress treatments. Different dyes were used in different concentrations and for different incubation times: DAF-FM DA (4-amino-5-methylamino-2'7'-difluorofurorescein diacetate; 15 µM/15 min; Sigma-Aldrich), DAR-4AM, AM (diaminohydroxylamine; 5 µM/60 min; Sigma-Aldrich); Cu$_6$(FL2E) (5 µM/60 min; STREM Chemicals); Amplex Red (100 µM/20 min; Invitrogen); and DCF-DA (2',7'-dichlorofluorescein diacetate; 20 µM/15 min; Sigma-Aldrich). All stainings and treatments were performed in STM buffer (50 mM MES-KOH pH 5.7, 0.25 mM KC1, 1 mM CaCl$_2$). After staining, the seedlings were washed three times in STM buffer and treated with 2 mM INA (Sigma-Aldrich) for 45 min or 200/150 mM NaCl for 6/5.5 h, respectively. INA is an analog of salicylic acid, resulting in a strong NO production when perceived in plants (Floryszak-Wieczorek et al., 2012). ePTTIO [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; 200 µM; Sigma-Aldrich], l-arginine (1 mM; Sigma-Aldrich), or nor-NOHA (No-hydroxy-nor-arginine; 0.1 mM; Sigma-Aldrich) were applied during both the staining and the stress application phase. The roots were observed under an epifluorescence microscope (Olympus BX61, ×4 objective) with enhanced green fluorescent protein (eGFP; excitation filter, 474/23; emission filter, 525/45) or red (excitation filter, 585/20; emission filter, 647/57) filter settings. The microscope software (cellP/cellSens, Olympus Soft Imaging) was set on optimized histogram and flexible exposure time for single optimized images. The microscope image each time included both the tested sample and a stress-treated Col-0 sample as internal control. The fluorescence intensity of the sample was further quantified relative to this internal control using ImageJ software. Afterwards, the values of the relative quantification were normalized to the genotype-specific non-treated control.

Primary root length measurements

Plants were grown on vertical 1/2 MS plates (1% sucrose, 1.2% phytagar) supplemented with either l-arginine (1 mM), GABA (1 mM), GSNO (S-nitrosoglutathione; 50 µM), or SNAP (S-nitroso-N-acetylpenicillamine; 50 µM) (Sigma-Aldrich). After vernalization, they were subjected to long-day conditions (14 h light/dark; cycles). CuAO8 activity measurement

In vitro CuAO8 activity measurement

The H$_2$O$_2$ production by CuAO8 was measured with the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Planas-Portell et al., 2013) (ThermoFisher Scientific, No. A22118) according to the manufacturer’s instructions. Briefly, 100 ng of CuAO8 was incubated with 1 mM of substrate (Sigma-Aldrich) in a total reaction volume of 100 µl. H$_2$O$_2$ production was detected using resorufin (detecting in a 1:1 ratio) at the specific excitation and emission wavelengths (excitation, 571 nm; emission, 585 nm) with the TECAN Reader Infinite M1000pro spectrofluorometer using a black 96-well plate. The amine oxidase inhibitor aminoaguandine (0.1 mM, Sigma-Aldrich) was used in combination with the provided substrates. In parallel, NO-producing activity was assayed in the same conditions replacing the Amplex Red probe with DAR-4AM DA (excitation, 543 nm; emission, 575 nm).

Determination of polyamines

The levels of free putrescine, spermidine, and spermine in 5-day-old seedlings were quantified with HPLC after pre-column derivatization with FMOC-Cl (Fellenberg et al., 2012). A 100 µg aliquot of plant material was ground in liquid nitrogen and extracted with 1 ml of 5% perchloric acid for 1 h at room temperature in the dark with frequent vortexing. After centrifugation (18 000 g, 10 min), 15 µl of the supernatant was neutralized with 360 µl of 0.1 M NaHCO$_3$, supplemented with 1,7-diaminohexane as internal standard. After the addition of 100 µl of aceton and 200 µl of 6 mM FMOC, the samples were incubated for 5 min at room temperature and for 10 min at 50 °C. The reaction was stopped at –20 °C for 5 min and 300 µl of methanol were added. The derivatized PAs were separated by reverse phase chromatography on a Luna C18 column [5 µm 100 Å C18(2) 250 × 4.6 mm column, Phenomenex] connected to a Beckman System Gold HPLC equipped with a Shimadzu RF 10AXL fluorescence detector (excitation, 260 nm; emission, 313 nm). The flow rate and the column temperature were set at 1 ml min$^{-1}$ and 40 °C. Elution was performed with water as eluent A and methanol as eluent B. Elution conditions were (min/B%): 0/80; 30/100, 60/100, 20/40, and 45/80. Quantification was based on the external standard method using calibration curves fitted by linear regression analysis in combination with the internal standard method (standards: putrescine dihydrochloride, 1,7-diaminohexane, spermidine, and spermine), where the response factor of each derivative was corrected with respect to that of the internal standard.

Measurement of nitrate reductase activity

The NR activity was measured as the rate of nitrite production determined with a spectrophotometric assay (Frugillo et al., 2014).
All samples were protected from light. Total protein from seedlings was extracted in 50 mM HEPES-KOH pH 7.5, 0.5 mM EDTA, 100 μM FAD, 5 mM Na₂MoO₄, 6 mM MgCl₂, protease inhibitor cocktail (Roche), and the homogenate was incubated for 10–15 min on ice with periodic vortexing. After centrifugation, the supernatant was rebuffered using 10K Amicon Ultra 0.5 centrifugal filter units (Millipore, No. UFC501008) and the protein concentration was measured with Bradford reagent. The protein activity assay was performed in a clear 96-well plate (Greiner) in a total reaction volume of 200 μl. The reaction mixture consisted of 1 mM KNO₃, 1 mM NADH, and either 2 mM EDTA or 6 mM MgCl₂, and was incubated for 55 min at room temperature with gentle shaking. Then, a 1:1 mixture of 1% (w/v) sulfuramidine in 1.5 M HCl and 0.02% (w/v) N-(1-naphthyl)-ethylenediamine dihydrochloride in 1.5 M HCl was added. After 15 min incubation at room temperature, the A₅₄₀ was measured with the TECAN Reader Infinite M1000pro spectrophotometer. The activity in the presence of EDTA represents the total NR activity and the activity, measured in the presence of MgCl₂ represents the actual NR activity.

Measurement of nitrite and nitrate content

The nitrite and nitrate contents in 5-day-old seedlings were determined with the Nitric Oxide Analyzer Sievers 280i from GE Healthcare. A 500 μl aliquot of 1× PBS was added to 200 mg of seedlings ground in liquid nitrogen and incubated on ice for 10 min with periodic vortexing. After centrifugation (12 000 rpm, 10 min, 4 °C), the supernatant was used for analysis. For nitrite detection, NO₃⁻ was reduced to NO by I⁻ (triiodide, 30 °C). For nitrite detection, nitrite and nitrate were reduced using VCl⁻ (vanadium chloride, 90 °C) and the measured amount of nitrite was subtracted. The nitrite/nitrate content was normalized to the protein content in the supernatant measured with Bradford reagent.

Measurement of arginase activity

To measure arginase activity in total protein extracts of 5-day-old seedlings, the Arginase Activity Assay Kit (Sigma-Aldrich, No. MAK112) was used according to the manufacturer’s instructions. Total protein extracts were homogenized in 50 mM Tris–HCl pH 9 and the supernatant was rebuffered using a 10K Amicon Ultra 0.5 centrifugal filter (Millipore). The protein concentrations were measured with Bradford reagent (BioRad).

Amino acid analysis

Proteinogenic amino acids were measured according to Thiele et al. (2008) with modifications. The extraction was conducted from 100 mg of 5-day-old seedlings homogenized with an aqueous HCl–ethanol mixture containing an internal standard (d₅-Phc). After incubation on ice and centrifugations, the supernatant was analyzed by liquid chromatography electrospray ionization–tandem mass spectrometry technique (LC-ESI-MS-MS) in the positive electrospray ionization mode. Analyses were conducted with a Waters ACQUITY UPLC system (binary pump, autosampler) coupled to a Waters Xevo TQ-S triple–quadrupole mass spectrometer (Waters Technologies Corp., MA, USA). A 10 μl extract was injected into the UPLC system. Amino acids were separated on a Nucleosil 100-5 SA column (150 × 2 mm, 5 μm particle size) equipped with a pre-column filter from Macherey-Nagel (Düren, Germany). The mobile phase consisted of 5% acetic acid (A) and 30 mM ammonium acetate (pH 6, B). Samples were separated at 40 °C and a flow rate of 500 μl min⁻¹ using two consecutive isocratic steps: isocratic at 87.5% A for 15 min, linear gradient to 0% A over 0.5 min, isocratic at 0% A for 4.5 min, linear gradient to 87.5% A over 0.5 min, and equilibration at 87.5% A for 9.5 min. The capillary voltage was set to 2.5 kV. The cone voltage was 20 V. The dissolution temperature was 500 °C and source temperature 150 °C. The dissolution gas flow was set to 800 l h⁻¹, the cone gas flow was set at 150 l h⁻¹ using nitrogen in both cases. MRM was used for specific quantification of the amino acids and the internal standard, applying a dwell time of 0.018 s. Nitrogen was used as the collision gas at a flow rate of 0.25 ml min⁻¹. MS-MS parameters of the amino acids were determined by flow injection analysis of amino acid standard solutions using the inbuilt syringe pump (Supplementary Table S3).

Transient expression and localization of GFP–CuAO8 in N. benthamiana

GFP–CuAO8 was obtained using the Gateway recombination system (Invitrogen) with the pENTR/D-TOPO entry vector and the pK7WG2F expression vector (adding an N-terminal cGFP). The CuAO8 gene sequence was amplified with specific primers (Supplementary Table S1) applied on cDNA synthesized from 4-week-old A. thaliana leaves. Agrobacterium tumefaciens GV3101 pmP90 was transformed either with pK7WG2F:CuAO8 or with p19 RNA silencing suppressor vector. The agrobacteria were grown in selective LB medium at 28 °C until an OD₆₀₀ of 1.7–2.2. After harvesting by centrifugation (4500 rpm, 10 min), the pellet was washed twice with buffer (10 mM MES-KOH pH 5.7, 10 mM MgCl₂), resuspended to an OD₆₀₀ of 1.3, and incubated for 3 h at room temperature. Then suspensions were mixed in a 1:1 ratio and infiltrated with a syringe in the abaxial side of 5-week-old N. benthamiana leaves. A 5 dpi, the GFP expression was monitored with a confocal microscope (Zeiss 510 META, C-Apochromat ×40/1.2 water objective). Leaf discs were vacuum infiltrated three times in FM4–64 (Biotium) solution (20 μM) followed by a 15 min incubation in the dark to visualize the plasma membrane (excitation/emission filter: GFP, 488 nm/BP 505–530; FM4–64, 514 and 543/BP 565–615 IR).

Results

The mutant cuao8–1 displays an impaired NO production after elicitor treatment in seedling root tips

We performed an initial screening experiment to monitor the NO production in several CuAO mutant lines of A. thaliana (Planas-Portell et al., 2013; Supplementary Table S2). The noa1 (nitric oxide associated 1) mutant, which is impaired in NO production (Guo et al., 2003), and an overexpression line of the rat neuronal nitric oxide synthase 35S::nNOS-2 (Shi et al., 2012) were included as negative and positive controls, respectively (Fig. 1A). NO production was triggered with INA treatment in root tips of 5-day-old seedlings and estimated using the fluorescent probe DAF–FM DA, which reacts with intracellular NO and emits fluorescence (Kojima et al., 1998).

Of the 13 lines tested, the lines noa1, ataa1 (aldehyde oxidase 1), cuao1, cuao6, cuao8, and cuao9 displayed a reduced NO production, even if the difference was not significant. The cuao1 and noa1 lines presented a reduced signal, in agreement with the previously published data (Guo et al., 2003; Wimalasekera et al., 2011). Interestingly, only the line impaired in the expression of the putative CuAO8 showed a significantly decreased fluorescence after INA treatment (Fig. 1A). In contrast, the 35S::nNOS-2 overexpressor showed a drastically increased NO production, in accordance with expectations.

To confirm these preliminary results, two independent cuao8 knockout lines (Supplementary Table S2) were confirmed at the transcriptomic (Fig. 1B) and the proteomic level (Fig. 1C). We further checked the NO production
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capacities of these lines after INA treatment, using DAF-FM DA (Fig. 2A) and the more selective NO probe Cu$_2$(FL2E) (McQuade and Lippard, 2010; Fig. 2B). Both mutant lines displayed a strong reduction of NO production after INA stress, confirming the results obtained in the initial screen. As a control, a Col-0 induced sample was co-treated with the NO scavenger cPTIO, and showed non-significantly reduced NO production. These results demonstrate the existence of a CuAO8-dependent NO production in seedling root tips after elicitor treatment.

cuao8-1 and cuao8-2 are impaired in NO production after salt stress

To determine the role of CuAO8 in NO production, we subjected cuao8-1 and cuao8-2 seedlings to salt stress. Both cuao8 mutants displayed an impaired NO production in root tips after salt stress, verified with the two different fluorescent probes DAF-FM DA (Fig. 3A) and DAR-4M AM (Fig. 3B). Remarkably, no induction of NO production was measured after salt stress in the mutants, as compared with the wild type.

These results suggest the existence of a general CuAO8-dependent NO production in seedlings root tips of A. thaliana.

CuAO8 impacts primary root length

We further analyzed the phenotype of the cuao8 mutants looking for primary root length of seedlings grown of 1/2 MS medium (Fig. 4). Mutants displayed 15% and 30% shorter primary root length, respectively, as compared with Col-0, to a significant extent for the cuao8-2 line. Interestingly, the inclusion of NO donors (GSNO and SNAP) in the medium could restore the primary root length to the Col-0 level (Fig. 4). These results demonstrate that the CuAO8-dependent NO production is involved in controlling the primary root growth of seedlings.

CuAO8 possesses a typical copper amine oxidase activity

To characterize further the function of CuAO8, we transiently expressed the recombinant protein in N. benthamiana leaves. Recombinant CuAO8-His$_6$ was obtained after an
Ni-NTA column purification as shown by SDS–PAGE and verified by western blot analysis applying anti-His and anti-CuAO8 antibody (Fig. 5A). The band appeared at 95 kDa, which is higher than predicted, but glycosylation events, as already shown in apples and human amine oxidases, are likely to increase the size of the protein (Airenne et al., 2005; Zarei et al., 2015).

The recombinant CuAO8 displayed no NO production activity (data not shown). The enzymatic activity of CuAO8 was further examined in vitro by measuring the amount of H$_2$O$_2$ released from the oxidative deamination of PAs. H$_2$O$_2$ was detected using the Amplex Red reagent as previously described (Planas-Portell et al., 2013). The results showed that CuAO8 possesses a typical CuAO activity, with the highest activity for putrescine, spermidine, spermine, and agmatine (Fig. 5B). This activity was almost completely abolished with co-application of the irreversible inhibitor of amine oxidases aminoguanidine (AG), implying specific CuAO activity of CuAO8. These results demonstrate that CuAO8 is a typical copper amine oxidase.

$H_2O_2$ production and polyamine accumulation in cuao8 mutants

Since CuAO8 enzymatic activity results in the production of H$_2$O$_2$, the in vivo generation of this compound was followed in root tips of seedlings from Col-0 and mutant lines after salt stress exposure using Amplex Red reagent and the fluorescent dye DCF-DA. Only a minor and non-significant reduction of H$_2$O$_2$ production was measured in cuao8 mutants (Fig. 6).

Although H$_2$O$_2$ is able to induce NO production in seedlings of A. thaliana (Wang et al., 2010), this suggests that the minor reduction of H$_2$O$_2$ production cannot explain the reduction in NO production observed in cuao8-1 and cuao8-2.

We then determined the different PA contents of Col-0 and mutant lines in control and salt-treated seedlings by HPLC (Table 1). Without stress, no significant differences were
observed between Col-0, cuao8-1, and cuao8-2 for any of the PA contents. Interestingly, after salt stress, a higher amount of putrescine was detected in mutant lines as compared with Col-0, significantly for cuao8-2. This demonstrates that CuAO8 deaminates putrescine in response to salt stress in A. thaliana.

These results support the activity tests done in vitro and argue in favor of a typical CuAO activity for CuAO8 in vivo being involved in the salt stress response in seedling root tips.

Nitrate reductase activity is not impaired in cuao8 mutant lines

As we revealed a typical CuAO activity for CuAO8, we investigated the possible reason for the impairment of NO production observed after elicitor and salt treatment in cuao8-1 and cuao8-2.

To investigate the role of NR after salt stress in the cuao8 mutants, we followed by qPCR the expression of the two genes encoding nitrate reductases in A. thaliana, namely NIA1 and NIA2 (Fig. 7A). The expression of these genes followed a similar pattern in the different lines tested, NIA1 expression being stable and NIA2 being induced after salt stress compared with the control (Fig. 7A). However, NIA1 and NIA2 gene expression was reduced in cuao8-1 and cuao8-2, both with and without salt stress.

To test whether this down-regulation was related to a change in the enzymatic activity, the NR activity was then determined in the Col-0 and mutant lines, submitted or not to salt stress (Fig. 7B). Since NR activity is tightly regulated at the post-translational level by phosphorylation, subsequent 14-3-3 protein binding, and inhibition in an Mg²⁺-dependent manner (Kaiser and Huber, 2001; Heidari et al., 2011), we assayed NR activity in the presence of EDTA.
Groß et al. (total NR activity) or excess Mg\(^{2+}\) (actual NR activity). The total NR activity was found to be significantly reduced in the two mutant lines, which fits with the qPCR results obtained (Fig. 7B). However, the actual NR activity was similar in Col-0, *cuao8-1*, and *cuao8-2*, which suggests that NR activity is not the reason for the impaired NO production observed in the mutants. This result shows that despite a modification of the expression of NR genes, the NR activity itself is not responsible for the decreased NO production observed in *cuao8* mutant lines after salt stress.

The reductive NO-producing pathway from nitrite depends on the nitrate/nitrite content and is promoted by high nitrite concentrations. To test whether the decreased NO production in the mutant lines was due to decreased nitrite concentrations, we determined the amount of nitrite and nitrate in the seedlings (Table 2). The impaired NO production of CuAO8 mutants did not correlate with a decreased nitrite availability, as no significant differences were found in mutant nitrite contents as compared with Col-0. In contrast, higher but not significant amounts of nitrite were monitored in these lines. A similar trend was observed for nitrate contents. These results exclude any implication of the nitrite/nitrate content in the impairment of NO production observed in the mutant lines.

**Arginine bioavailability drives the cuao8-dependent phenotype**

Several lines of evidences argue for the existence of an arginine-dependent NO production pathway in higher plants similar to that which exists in animals, where arginine is converted into citrulline and NO (Gas et al., 2009; Del Rio, 2011; Corpas and Barroso, 2014). Arginine bioavailability in plants is controlled notably by arginase, which exists under two isoforms in *A. thaliana*, namely ARGH1 and ARGH2, the second counting for ~85% of the total arginase activity (Winter et al., 2015). Interestingly, the manipulation of expression of arginase was previously correlated with modifications of NO production, signaling, and plant stress resistance, possibly via the arginine-dependent NO production pathway (Flores et al., 2008; Shi et al., 2013; Meng et al., 2015). To assay whether this pathway could be involved in the decreased NO production measured in *cuao8* mutants, a series of experiments was performed.

Initially, the expression of *ARGH1* and *ARGH2* was followed in the different lines subjected or not to salt stress (Fig. 8A). Salt stress induced an increase in the expression of both genes in Col-0 seedlings, more pronounced for

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**Table 1. Free polyamine levels (nmol g FW\(^{-1}\)) in 5-day-old seedlings of Col-0, *cuao8-1*, and *cuao8-2* after NaCl (200 mM, 6 h) or control (buffer, 6 h) treatment**

| Polyamine   | Control       | cuao8-1       | cuao8-2       |
|-------------|---------------|---------------|---------------|
| Putrescine  | 41.60 ± 3.55  | 49.41 ± 2.76  | 44.25 ± 8.43  |
|             | 31.01 ± 1.95  | 49.55 ± 9.55  | 51.08 ± 5.65* |
| Spermidine  | 213.37 ± 0.24 | 282.58 ± 12.67| 239.57 ± 4.05 |
|             | 294.45 ± 57.93| 217.58 ± 12.78| 248.50 ± 15.33|
| Spermine    | 23.65 ± 1.11  | 21.76 ± 0.89  | 20.70 ± 1.55  |
|             | 20.33 ± 4.56  | 23.92 ± 1.75  | 22.51 ± 1.29  |

Means ±SE of three individual experiments are shown. *Significant difference P<0.05 based on t-test with respect to the corresponding Col-0.

**Fig. 6.** NaCl-induced H\(_2\)O\(_2\) production in Col-0, *cuao8-1*, and *cuao8-2* root tips. Fluorescence quantification analysis of 5-day-old seedlings from Col-0, *cuao8-1*, and *cuao8-2* stained with Amplex Red (A) or DCF-DA (B) and treated with NaCl (200 mM, 6 h). The fluorescent signal in the root tips was observed with an epiﬂuorescence microscope. Shown is the relative fluorescence normalized to non-treated controls of each genotype. Means ±SE of at least three individual experiments are shown (Amplex Red, n=22–30; DCF-DA, n=21–28). Different letters indicate a statistically signiﬁcant difference based on ANOVA on ranks (Dunn’s test. P<0.05).
CuAO8 regulates arginine-dependent NO production in A. thaliana in accordance with previously published data (Shi et al., 2013). A different pattern was observed in the mutant lines, where no significant change for \textit{ARGH2} expression was measured after salt stress.

To confirm this observation further, the total arginase activity from crude extract of Col-0, \textit{cuao8-1}, and \textit{cuao8-2} lines was determined in seedlings exposed or not to salt stress. The two insertion lines displayed a strongly and significantly increased arginase activity as compared with Col-0 after salt stress. These results imply a link between CuAO8 and arginase activity in \textit{A. thaliana} during salt stress response.

We then verified whether this link could explain the impaired NO production observed in the \textit{cuao8} mutant lines. We performed NO production tests (DAR-4M AM staining) during salt stress in root tips of seedlings co-treated with the specific reversible arginase inhibitor \textit{nor}-NOHA (Custot et al., 1997) or supplemented with arginine (Fig. 8C). Interestingly, \textit{nor}-NOHA, as well as arginine, could, at least partially, significantly restore the NaCl-induced NO production in both mutant lines. Furthermore, none of these compounds impacted significantly the salt-induced NO production in Col-0. These data confirm the implication of arginase and arginine in CuAO8-dependent NO production during salt stress.

Based on these results, we examined the effect of arginine on the \textit{cuao8}-dependent reduction of the primary root length (Fig. 9). For this purpose, seedlings were grown on 1/2 MS medium supplemented with arginine. Given that GABA

\begin{table}
\centering
\begin{tabular}{lccc}
\hline
 & Col-0 & \textit{cuao8-1} & \textit{cuao8-2} \\
\hline
\textbf{Nitrite (pmol mg protein$^{-1}$)} & \\
Control & 146.3 ± 27.8 & 350.8 ± 83.9 & 451.8 ± 96.6 \\
NaCl & 84.4 ± 22.6 & 172.4 ± 20.6 & 168.4 ± 49.1 \\
\textbf{Nitrate (µmol mg protein$^{-1}$)} & \\
Control & 14.9 ± 3.6 & 28.9 ± 5.1 & 29.2 ± 4.1 \\
NaCl & 10.2 ± 2.0 & 25.1 ± 4.7 & 21.0 ± 3.9$^*$ \\
\hline
\end{tabular}
\caption{Nitrate and nitrite quantification in 5-day-old seedlings of Col-0, \textit{cuao8-1}, and \textit{cuao8-2} after NaCl (200 mM, 6 h) or control (buffer, 6 h) treatment}
\end{table}

\textit{ARGH2}, in accordance with previously published data (Shi et al., 2013). A different pattern was observed in the mutant lines, where no significant change for \textit{ARGH2} expression was measured after salt stress.

To confirm this observation further, the total arginase activity from crude extract of Col-0, \textit{cuao8-1}, and \textit{cuao8-2}
synthesis may result from a product generated by the CuAO enzymatic activity (Seiler, 2004), we also included a GABA-negative control to confirm the causality between CuAO activity itself and the phenotype. GABA treatment could not restore the normal primary root growth, in contrast to arginine supplementation. This demonstrates that the mutant phenotype is due to the consumption of the substrate rather than the generation of the product from CuAO activity since the supplementation with arginine significantly restored the primary root length up to Col-0 levels. Taken together, these results show that the mutant phenotype observed results from a change in arginine bioavailability due to an elevated arginase activity caused by the knockout of CuAO.

Discussion

Deciphering the complex mechanisms responsible for the NO production in higher plants is a challenging issue of great interest for a better understanding of the role of NO in plant physiology. NR, putative NOS, electron transport, and putative polyamine oxidases have been suggested as sources of NO in plants (for reviews, see Moreau et al., 2010; Frohlich and Durner, 2011). We were able to demonstrate that the knockout of CuAO led to a decreased NO production due to a higher arginase activity (Fig. 8B). Furthermore, reduced primary root growth in cuao8-1 and cuao8-2 was correlated to lower NO levels and could be rescued by arginine supplementation. These results point to a regulatory effect of CuAO on arginine-dependent NO production during stress responses (Figs 2, 3) but also during primary root growth (Figs 4, 10).

As PAs are able to induce NO production in root seedlings (Scheler et al., 2013) and the cuao1 mutant displayed an impaired NO production in response to ABA or PA treatment (Wimalasekera et al., 2011), we sought to determine the NO production in mutant lines for each CuAO predicted gene of A. thaliana (Planas-Portell et al., 2013) using seedlings submitted to an elicitor treatment (Fig. 1A). The screen of A. thaliana T-DNA insertion lines revealed a reduced NO
production to a similar extent in noa1 and half of the CuAO insertional lines (atao1, cuao1, cuao6, cuao8, and cuao9). This tendency, even if not significant, correlates with the impairment of several CuAO mutant lines in NO synthesis, which reinforces the existing link between PA metabolism and NO production in plants (Tun et al., 2006; Wimalasekera et al., 2011; Scheler et al., 2013; Yang et al., 2014). In our screening system, cuao8-1 displayed a strong and significant reduction of NO production. To confirm and strengthen this initial observation, we obtained two independent cuao8 insertion lines, knocked out at the transcript and protein level (Fig. 1B, C), and validated the impaired NO production after elicitor (INA) treatment (Floryszak-Wieczorek et al., 2012; Fig. 2). Because the use of DAF-FM DA for NO detection has been the subject of debate particularly in plant biology (Rumer et al., 2012), we also assayed the impairment of NO production with the Cu2(FL2E) probe. This recent tool is much more specific for NO than DAF-FM DA and insensitive to other reactive oxygen or reactive nitrogen species present in the medium (McQuade and Lippard, 2010). The data were confirmed with this second probe, validating the use of DAF-FM DA in our experiments. For the screening experiment, a rather high concentration of INA was applied to provoke a fast and strong NO response. The treatment was afterwards substituted by NaCl to analyze NO production in Col-0, cuao8-1, and cuao8-2 seedlings in a more physiological context. NaCl was chosen since PAs and NO are clearly connected in promoting salt stress resistance in A. thaliana (Quinet et al., 2010; Tanou et al., 2014). Almost no NO production was detected in seedling root tips of cuao8-1 and cuao8-2 after salt stress, again using two different NO dyes (Fig. 3). In addition to the NO impairment observed after INA treatment, these results demonstrate an involvement of CuAO8 in the general NO production in seedlings.

NO is a signaling compound involved in a wide range of developmental processes in plants, notably controlling primary root growth (Fernandez-Marcos et al., 2011; Liu et al., 2015). The reduced NO production is involved in the observed reduced primary root growth in cuao8-1 and cuao8-2 since NO donors could at least partially rescue the root growth phenotype (Fig. 4). This supports the connection of CuAO8 and NO production also in a developmental process, independent of an elicitor or stress treatment.

However, the molecular mechanism linking the knockout of CuAO8 to a reduced NO production is ambiguous. The characterization of the CuAO8 enzymatic activity in vitro revealed a typical CuAO activity (Fig. 5B), displaying the highest activity with putrescine as a substrate, as previously described for other CuAOs (Cona et al., 2006; Planas-Portell et al., 2013). Since NO-producing activity was not found in recombinant CuAO8 (data not shown), the effect of CuAO8 on NO formation was more likely to be indirect.

In plants, nitrite reduction is described to be one of the major sources of NO (Meyer et al., 2005; Stöhr et al., 2001; Wimalasekera et al., 2011; Yang et al., 2014). However, the CuAO activity was not able to reduce nitrite to NO in our in vitro assay, which suggests that the effect of CuAO8 on NO formation is indirect and possibly through other pathways.

Fig. 9. Relative root growth of Col-0, cuao8-1, and cuao8-2 after 11 d of growth on vertical 1/2 MS plates or 1/2 MS plates supplemented with GABA (1 mM) or arginine (1 mM). The means ±SE of at least three individual experiments are shown (n=14–22), normalized to Col-0. Different letters indicate a statistically significant difference based on ANOVA on ranks (Dunn’s test. P<0.05).

Fig. 10. Schematic representation of the arginine metabolic pathway in A. thaliana. Arginine is a common substrate for the NOS-like activity, arginase activity, and polyamine synthesis pathway in A. thaliana. ADC, arginine decarboxylases; AIH, agmatine iminohydrolase; NCPH, N-carbamoyl putrescine hydrolase; NOS, nitric oxide synthase; SPDS, spermidine synthases; SPMS, spermine synthase.
In these plants, a decreased NIA1/NIA2 gene expression was found as compared with Col-0, correlated with a decrease in the potential total NR activity. However, no differences in the actual NR activity between the mutants and Col-0 were measured (Kaiser and Huber, 2001; Rockel et al., 2002; Berkowitz et al., 2010; Jeandroz et al., 2016). Interestingly, it has been reported that putrescine is able to reduce the NR activity (Athwal and Huber, 2002; Rosales et al., 2012), and we measured an increased putrescine content in cuao8-1 and cuao8-2 (Table 1).

In these mutants, the decrease in NO production in root tips of seedlings and in the shoots of mature plants, as well as the decrease in NO production in root tips of seedlings submitted to an elicitor treatment or to exposure to salt stress, demonstrates that the influence of CuAO8 on arginase activity is unlikely, since both cuao8-1 and cuao8-2 displayed an impaired NO production in these mutants was not caused by a change in the NR activity. However, a higher arginase activity was measured in cuao8-1 and cuao8-2, and the inhibition of arginases partially recovered the impaired NO production in root tips after salt stress (Fig. 8B, C). Moreover, supplementation with arginine restored the NO production in the mutants to almost the Col-0 level and could also reverse the reduced primary root growth phenotype observed for cuao8-1 and cuao8-2 (Fig. 9).

The higher arginase activity found in cuao8-1 and cuao8-2 seems to result from a post-transcriptional regulation as no changes in gene expression of ARGH1/ARHG2 could be measured in the mutants (Fig. 8A). Moreover, a direct modulation of arginase activity by CuAO8 is unlikely, since both arginases are localized in the mitochondrion (Flores et al., 2008) and a GFP-CuAO8 construct localized in the cytosol and at the plasma membrane (Supplementary Fig. S2). However, nothing is known about a post-translational regulation of A. thaliana arginases so far which could reveal a possible regulatory pathway associated with the function of CuAO8 in PA metabolism. Taken together, our work demonstrates that the influence of CuAO8 on arginase activity affects the arginine bioavailability, which has an impact on the putative NOS-like NO production pathway.

Summarizing, this work characterizes for the first time the copper amine oxidase CuAO8 from A. thaliana. Insertional mutant lines for this enzyme displayed an impaired NO production in root tips of seedlings submitted to an elicitor treatment or to exposure to salt stress. The cuao8 mutants displayed an NO-dependent shorter primary root length phenotype. The recombinant protein presented a typical CuAO activity, metabolizing PA and producing H2O2, with a higher affinity for putrescine. We could demonstrate that the impairment of NO production in these mutants was not caused by a change in the NR activity. However, a higher arginase activity was detected. The resulting modulation of the bioavailability of arginine affected the NO formation probably via a NOS-like NO production route. The impact of CuAO8, and possibly also other CuAOs on NO production constitutes a new regulatory level of NO signaling in plants during stress response and developmental processes.

**Supplementary data**

Supplementary data are available at *JXB* online.

Fig. S1. Metabolic network between polyamines and amino acids, and influence of NaCl treatment on the amino acid levels in Col-0, cuao8-1, and cuao8-2.

Fig. S2. Subcellular localization of transiently expressed GFP–CuAO8 in N. benthamiana.

Table S1. List of PCR primers.

Table S2. List of T-DNA insertion lines.

Table S3. Precursor, product ions, collision energy, and cone voltage of underivatized amino acids and d3-Phe for LC-ESI-MS-MS.

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