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RESEARCH PAPER

Ascorbate oxidation activates systemic defence against root-knot nematode *Meloidogyne graminicola* in rice

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Abstract

Ascorbic acid (AA) is the major antioxidant buffer produced in the shoot tissue of plants. Previous studies on root-knot nematode (RKN; *Meloidogyne graminicola*)-infected rice (*Oryza sativa*) plants showed differential expression of AA-recycling genes, although their functional role was unknown. Our results confirmed increased dehydroascorbate (DHA) levels in nematode-induced root galls, while AA mutants were significantly more susceptible to nematode infection. External applications of ascorbate oxidase (AO), DHA, or reduced AA, revealed systemic effects of ascorbate oxidation on rice defence versus RKN, associated with a primed accumulation of H_2O_2 upon nematode infection. To confirm and further investigate these systemic effects, a transcriptome analysis was done on roots of foliar AO-treated plants, revealing activation of the ethylene (ET) response and jasmonic acid (JA) biosynthesis pathways in roots, which was confirmed by hormone measurements. Activation of these pathways by methyl-JA, or ethephon treatment can complement the susceptibility phenotype of the rice Vitamin C (*vtc1*) mutant. Experiments on the jasmonate signalling (*jar1*) mutant or using chemical JA/ET inhibitors confirm that the effects of ascorbate oxidation are dependent on both the JA and ET pathways. Collectively, our data reveal a novel pathway in which ascorbate oxidation induces systemic defence against RKNs.

Keywords: Ascorbate oxidase, ascorbic acid, dehydroascorbic acid, hormones, reactive oxygen species, rice *Meloidogyne graminicola*, systemic defence.

Introduction

Rice has been cultivated as a staple crop for many years, and sustains approximately half of the world's population (Seck *et al.*, 2012). Rice is also a model plant for molecular studies due to its fully sequenced genome, availability of functional genomic tools, and relatively easy production of transgenic plants (Shimamoto and Kyozuka, 2002; Han *et al.*, 2007; Lyu,

2018). Yield losses in rice because of parasitic nematodes have been estimated to range between 10% and 25% worldwide (Bridge *et al.*, 2005; Kyndt *et al.*, 2014). *Meloidogyne graminicola* or the rice root-knot nematode (RKN) is one of the most prevalent nematodes in various rice-growing agrosystems (Mantelin *et al.*, 2017). The infective second-stage juveniles (J2s) of *M. graminicola* enter the root in the elongation zone and move intercellularly until they reach the vascular cylinder where they induce the formation of feeding sites containing 5–8 giant cells, leading to root galls, preferentially at the root tips (Kyndt *et al.*, 2014; Mantelin *et al.*, 2017; EPPO, 2018). Once the giant cells are established, the nematodes become sedentary, start feeding, and moult three times to reach the adult stage (Triantaphyllou, 1969; Mantelin *et al.*, 2017).

Upon pathogen attack, the plant innate immune system responds with a series of local and systemic defence mechanisms (Jones and Dangl, 2006; Dhankher *et al.*, 2018). The plant immune system can be activated externally, for example by applying hormone analogues [systemic acquired resistance, dependent on salicylate (SA)] or by beneficial microorganisms [induced systemic resistance, usually mediated by jasmonic acid (JA) and ethylene (ET)]. In previous work, it was shown that root application of β-aminobutyric acid (BABA) (Ji *et al.*, 2015; Jisha and Puthur, 2016), thiamine (Huang *et al.*, 2016), or silicon (Zhan *et al.*, 2018) enhances the rice plant defence system, leading to improved tolerance or resistance against root parasitic nematodes. Systemic defence activation has also been observed: foliar application of methyl-JA (MeJA), ethephon, the SA analogue BTH, or COS–OGA [Fytosave®, a mixture of chitosan oligomers (COS) and pectin fragments (oligogalacturonides, OGAs)] activates root defence pathways with varying efficacy (Nahar *et al.*, 2011; Singh *et al.*, 2019).

One of the earliest hallmarks of induced plant defence is the oxidative burst (Foyer and Noctor, 2005). Reactive oxygen species (ROS) can be directly toxic to pathogens and can function as secondary messengers of signal transduction pathways controlling pathogen defence responses (Apel and Hirt, 2004; Miller *et al.*, 2008; Mittler *et al.*, 2011; Gilroy *et al.*, 2016). However, overproduction of ROS can cause oxidative damage to cells (Foyer and Noctor, 2005; Miller *et al.*, 2010; Mukherjee *et al.*, 2010). To keep the levels of ROS under control, plants have an antioxidant-scavenging network consisting of enzymes, such as superoxide dismutase, ascorbate peroxidase (APX), and catalase, together with non-enzymatic antioxidants such as ascorbic acid (AA), glutathione, pyridine, and NAD^+ (Pastori and Foyer, 2002; Gechev *et al.*, 2006; Foyer and Noctor, 2011; Mittler *et al.*, 2011; Pétriacq *et al.*, 2013; Foyer, 2017; Gakière *et al.*, 2018).

Water-soluble AA is the most abundant and ubiquitous cellular antioxidant in plants (Davey *et al.*, 2000; Smirnoff and Wheeler, 2000; Foyer and Noctor, 2005; Foyer, 2017; Smirnoff, 2018). AA biosynthesis in plants is controlled by light and predominantly occurs via the D-Man/L-Gal pathway with D-mannose and L-galactose as intermediates (Ivanov Kavkova *et al.*, 2019), while other pathways are still under debate (Smirnoff and Wheeler, 2000; Wheeler *et al.*, 2015). AA biosynthesis through GDP-L-galactose involves multiple enzymatic steps including phosphomannose isomerase (PMI), GDP-mannose-3,5-epimerase (aka Vitamin C1, VTC1), and GDP-l-galactose phosphorylase (GGP) (Höller *et al.*, 2015). In the symplast, the enzyme APX plays a role in ROS metabolism by catalysing the conversion of hydrogen peroxide (H_2O_2) to H_2O and O_2, using two molecules of AA (Asada *et al.*, 1999). In rice, eight isoforms of APX have been characterized and mostly the thylakoid-bound APX, namely APX8, modulates H_2O_2 content (Jardim-Messeder *et al.*, 2018). Ascorbate has been described to be a cofactor for some enzymes involved in phytohormone biosynthesis (Anjum *et al.*, 2014), such as gibberellins, ET, and abscisic acid (ABA) (Pastori *et al.*, 2003; Barth *et al.*, 2006; Ye *et al.*, 2012; Zhang *et al.*, 2012; Chen *et al.*, 2014). For example, ET, cyanide, and dehydroascorbate (DHA) are formed from 1-aminocyclopropane-1-carboxylate (ACC) and AA through a chemical reaction which is catalysed by ACC oxidase.

The redox status of the apoplastic AA pool, which is regulated by ascorbate oxidase (AO) (Pignocchi and Foyer, 2003; Foyer and Noctor, 2005; Fotopoulos and Kanellis, 2013), influences plant growth and responses to hormones, antioxidant enzyme activities, mitogen-activated protein kinase (MAPK) activity, and transcripts associated with calcium channels (Pignocchi and Foyer, 2003). AO catalyses oxidation of AA to DHA via the unstable radical monodehydroascorbate (MDHA). DHA is transported from the apoplast to the symplast and reduced back to AA through the symplastic AA–glutathione (AA–GSH) cycle (Horemans *et al.*, 2000*a*) including among others dehydroascorbate reductases (DHARs) (Foyer, 2017; Smirnoff, 2018). AO gene expression is induced by light (Pignocchi and Foyer, 2003; Nanasato *et al.*, 2005), auxin (Esaka *et al.*, 1992; Pignocchi and Foyer, 2003), and JA (Sanmartin *et al.*, 2007). Studies show that the apoplastic AO influences both symplastic and apoplastic AA/DHA ratios (Yamamoto *et al.*, 2005), and that apoplastic oxidation has major effects on cell expansion (Esaka *et al.*, 1992; Li *et al.*, 2017). Although both AO and APX are responsible for the oxidation of the ascorbate pool, the electron acceptors are different, namely O_2 in the former and H_2O_2 in the latter reaction.

Several studies show a negative correlation between the amount of AA and resistance to aboveground pathogens. Pavet *et al.* (2005) showed that Arabidopsis *vtc1* mutants exhibit enhanced basal resistance against *Pseudomonas syringae*, accompanied by increased cell death activation. Confirming this view, these mutants were shown to contain elevated levels of SA and H_2O_2, and the authors proposed that AA may hinder H_2O_2-facilitated defence (Mukherjee *et al.*, 2010). However, there are also reports on the role of AA in defence against pathogens. *Brassica rapa* cultivars with naturally high amounts of AA showed enhanced resistance to *Turnip mosaic virus*, while *vtc1* plants are more susceptible than wild-type lines (Fujiwara *et al.*, 2016).

In our previously published transcriptome analyses on galls and giant cells induced by the RKN *M. graminicola* in rice roots, a significant differential expression of several genes encoding proteins involved in AA recycling (e.g. DHAR and APX) was detected in nematode-induced galls and in isolated giant cells (Kyndt *et al.*, 2012; Ji *et al.*, 2013). In a follow-up analysis on aboveground tissue of RKN-infected plants, we revealed that

the rice shoot is under oxidative stress, with significant differential expression of redox signalling genes, associated with accumulation of H_2O_2 and lipid peroxidation (Kyndt *et al.*, 2017). It is well known that upon pathogen infection in shoots, accumulation of ROS directs the AA pool towards a more oxidative state (Foyer and Noctor, 2005). Similarly, although AA levels are generally extremely low in plant roots (Mozafar and Oertli, 1993; Matamoros *et al.*, 2006), oxidized AA—DHA—has been found to accumulate in nodules induced by legumes (Puppo *et al.*, 2005; Matamoros *et al.*, 2006) and feeding sites induced by parasitic cyst nematodes in Arabidopsis (Siddique *et al.*, 2014). When considering RKNs, Arrigoni *et al.* (1979) have shown a significant accumulation of AA in roots of nematode-resistant tomato cultivars upon nematode infection, but not in susceptible cultivars. In the compatible *M. incognita*–tomato interaction, treating tomato with AA suppressed the number of galls, females, and egg masses (Melillo *et al.*, 1983; Al-Sayed and Thomason, 1988), and the activity and gene expression of APX were shown to be induced in galls (Veronico *et al.*, 2017).

In this study, we sought to evaluate the role of AA and its redox state in controlling the susceptibility of rice to RKNs. We demonstrate that oxidation of AA is enhancing systemic defence against *M. graminicola* through activation of JA and ET biosynthesis and is correlated with primed H_2O_2 accumulation upon nematode infection in roots.

Materials and methods

Plant material and growth conditions

Seeds of wild-type rice *Oryza sativa* cv. 'Nipponbare' were obtained from USDA (GSOR-100). Different TOS17 insertional mutants in Nipponbare were obtained from the Rice Genome Resource Center of the National Institute of Agrobiological Sciences (Hirochika *et al.*, 1996; Hirochika, 2001), a mutant in jasmonic acid amido-synthetase (*Osjar1*, NC2728 (Miyao *et al.*, 2003; Riemann *et al.*, 2008), a mutant in GDP-mannose-3,5-epimerase (*gme=vtc1*, ND6172; LOC_Os10g28200), known to contain 20–30% less ascorbate (Frei *et al.*, 2012), a mutant in mannose-6-phosphate isomerase (=phosphomannose isomerase, *pmi*, NF5987; LOC_Os01g03710), and a mutant in thylakoid-bound ascorbate peroxidase APX8 (*apx*, ND0462; LOC_Os02g34810) (Supplementary Table S1 at *JXB* online). Mutant lines were genotyped by triple primer PCR using gene-specific primers and the TOS17-tail6 primer AGGTTGCAAGTTAGTTAAGA to produce homozygous T_2 seeds. The expression level of the mutated gene was checked by quantitative reverse transcription–PCR (qRT–PCR) as described in Nahar *et al.* (2011) using primers listed in Supplementary Table S1. For each mutated gene, the Cq was >39 in the corresponding T-DNA insertion line, while the Cq in wild-type plants varied between 25 and 30, confirming gene inactivation. Seeds were germinated on wet filter paper for 4–5 d at 30 °C and seedlings were transferred to SAP-substrate (Sand mixed with Absorbent Polymer AquaPerla®, DCM, Belgium) and further grown at 28 °C, under a light regime of (day/night) 12 h/12 h. Hoagland solution was given as a source of nutrients three times a week.

Nematode extraction, inoculation, and sample collection

Meloidogyne graminicola originally isolated in the Philippines (Batangas) was cultured on susceptible rice plants. Second-stage juveniles (J2s) were extracted using a sieve/tray extraction method and 250–300 J2s were inoculated per plant as described in Nahar *et al.* (2011). For susceptibility assays, at least two independent experiments were carried out, each time including eight plants per treatment. Twelve days after inoculation (dai), roots were stained using acid fuchsin (Nahar *et al.*, 2011) to count galls and developmental stages of nematodes. In some experiments, length and width of galls were measured using the Fiji software, an image processing package based on ImageJ (Schindelin *et al.*, 2012).

Chemical applications

Reduced AA (20 mM), DHA (20 mM), AO (20 U ml^{-1}), MeJA (100 μM), aminooxyacetic acid (AoA, 12.5 mM), diethyldithiocarbamic acid (DIECA, 100 μM), and ethephon (100 μM) were purchased from Sigma. For foliar application, all chemicals were dissolved in water, and 0.02% (v/v) of Tween-20 as a surfactant was added prior to spraying on aerial portions of the rice plants. Control plants were mock-sprayed with distilled water containing Tween-20, a surfactant with no effect on the enzymatic activity of AO (data not shown). Eight 2-week-old plants per treatment were sprayed until run-off with 6.5 ml of solution (at 24 h prior to nematode inoculation, in cases where infected plants were used). The substrate was covered to avoid direct contact with the roots and/or nematodes. For soil application, 6.5 ml of each solution was drenched on the SAP-substrate, 24 h prior to nematode inoculation.

mRNA sequencing

Four different treatment groups were made: mock-sprayed control plants, AO-sprayed plants, nematode-inoculated plants, and AO-sprayed+nematode-inoculated plants. Nematode inoculation was done 24 h after foliar AO treatment (20 U ml^{-1}). To evaluate systemic effects, root systems were collected 3 d after nematode inoculation, which corresponds to 4 d after treatment with AO. For each treatment, three independent biological replicates, containing a pool of 4–5 individual plants, were taken. RNA was extracted using the QIAGEN RNeasy Plant Mini kit, with additional sonication after adding extraction buffer RLT. RNA integrity was checked using the Agilent BioAnalyzer 2100 (Agilent). Approximately 1 μg of RNA was used for 3' mRNA-Seq library construction using the QuantSeq 3' mRNA-Seq Library Prep Kit FWD for Illumina. To minimize lane effects, the samples were multiplexed, using the multiplexing sequencing adaptors provided in the Multiplexing Sample Preparation Oligo Kit (Illumina). Size selection was performed on a 2% agarose gel (Low Range Ultra Agarose, Biorad 161-3107). The denatured library was diluted to a final concentration of 6 pM and loaded on a flow cell (Illumina). After cluster generation, the multiplexed library was sequenced on an Illumina NextSeq500 (75 cycles, single end, high output).

Reads were trimmed with Trimmomatic (0.36) and mapped to the *O. sativa* subsp. *japonica* reference genome (build MSU7.0) using STAR (2.5.2a). Only uniquely mapped reads were kept for further analysis. BAM files of multiplexed samples were merged using samtools (version 1.3). Count tables were generated by the summarize overlaps function in the genomic alignments R package (version 1.16.0). Differential gene expression analysis was performed using the DESeq2 package (version 2.1.20) with the annotations from the Rice Annotation Project Database (version 38). Genes with a false discovery rate (FDR) <0.05 were considered differentially expressed compared with the control group.

Gene Ontology (GO) analysis and GO enrichment were performed using agriGO (Du *et al.*, 2010). Parametric analysis of gene set enrichment (PAGE) was carried out (Kim and Volsky, 2005), based on differential gene expression levels (log2FC). Benjamin and Hochberg FDR analysis was performed to adjust the PAGE *P*-values. In addition, MapMan (Thimm *et al.*, 2004) was used to visualize the expression of genes onto metabolic pathways, and the WSR-test (with Benjamin and Hochberg correction) was used to test the statistical significance of differential expression of these pathways. The data were submitted to the Gene Expression Omnibus under accession number GSE125427.

Hormone measurements

Fourteen-day-old plants were either mock-sprayed or sprayed with chemicals. One day later, root material was collected. Whole root material was sampled from five biological replicates, each consisting of a pool of at least four individual plants.

Following a cold solvent (modified Bieleski) extraction, filtration, and clean-up, hormone levels were measured on a UHPLC Q-Exactive™ high-resolution Orbitrap mass spectrometer (Thermo Fisher Scientific), according to the protocol described in Haeck *et al.* (2018). For ET measurements, four root systems were pooled per biological replicate (n=6). Roots were cut into ~0.5 cm pieces and placed in separate 9.5 ml glass vials, which were subsequently sealed with a rubber syringe cap to allow ET accumulation in the headspace. After 3 h of incubation at room temperature, 1 ml of headspace was analysed by GC (Thermo Finnigan TRACE GC Ultra; CP-PoraBOND CP7354, 25 m, 0.53 mm, 10 µm, 35 °C) with a flame ionization detector. Standards of 0.4 ppm and 1 ppm ET were used for calibration.

HPLC-based ascorbic acid measurement

For quantification of AA, the HPLC method introduced by Gökmen *et al.* (2000) was optimized for plant tissues. Samples (100–200 mg) were ground in a small mortar by using 400 µl of 1 M $HClO_4$ per 100 mg FW. A total of five biological replicates per treatment were used. For measurement of reduced AA, the samples (extract) were centrifuged at 13 000 rpm for 2 min. A 300 µl aliquot of the clear supernatant was transferred to a new tube and neutralized to pH 4–5 by the addition of ~26 µl of 5 M K_2CO_3. The precipitated $KClO_4$ was removed by centrifugation for 1 min. A 20 µl aliquot of the supernatant was analysed by HPLC-UV within a few minutes after preparation of the extract to avoid oxidation by oxygen.

For total AA, 100 µl of the neutralized extract was incubated with 20 µl of a citric acid (0.3 M)/Na_2HPO_4 (0.5 M) buffer with a pH of 4.6, and with 7 µl of 20 mM Tris (2-carboxyethyl) phosphine (TCEP). Samples were incubated for at least 1 h at room temperature to allow full reduction of oxidized AA to reduced AA. Then 20 µl of this mixture was analysed directly by HPLC-UV. The amount of oxidized AA was calculated as the difference between total and reduced AA. Analysis was done on a Dionex UHPLC with a photodiode array detector. A HILIC column (125×4 mm, Nucleodur Machery-Nagel, Düren, Germany) was used for separation. At a flow rate of 0.6 ml min^{-1}, buffer A was 100 mM ammonium acetate and buffer B was acetonitrile (super gradient grade). Isocratic separation was performed with 80% buffer B. AA was detected at 262 nm. Quantification was done using authentic standards.

Biochemical analyses

Plant roots and the whole shoot region were collected from a pool of 5–6 plants per treatment and crushed in liquid nitrogen. In each assay, 100 mg of crushed material was used per sample, and at least four biological replicates were analysed per treatment. Glutathione was extracted using 1.5 ml of 4% sulfosalicylic acid and 5% insoluble polyvinylpolypyrrolidone (PVPP), and concentrations were determined using a microplate-adjusted spectrometric assay (Matthus *et al.*, 2015). H_2O_2 levels were determined according to Velikova *et al.* (2000), and malondialdehyde (MDA) levels were measured using the protocol described in Hodges *et al.* (1999). Reduced and oxidized AA were measured according to the method described in Ueda *et al.* (2013). Absorbance was monitored using a microplate reader (TECAN—Infinite200pro).

Results

Ascorbate oxidation has a role in plant immunity against RKNs

Previous transcriptome analyses on rice infected by *M. graminicola* revealed that genes involved in recycling of AA are differentially expressed in locally infected tissue (Kyndt *et al.*, 2012; Ji *et al.*, 2013; Supplementary Table S1) as well as in aboveground tissue (Kyndt *et al.*, 2017). Our previously published biochemical measurements showed that shoots of root-infected rice plants are under oxidative stress (Kyndt *et al.*, 2017) and suggested accumulation of DHA in galls and aboveground tissue early upon nematode infection (Foyer *et al.*, 2020). To independently confirm the biochemical analyses, we used a HPLC-UV measurement to quantify the total AA (TAA) levels, the reduced form of AA (redAA), and the oxidized form of AA (DHA) in nematode-induced galls [*M. graminicola* (Mg+)] in comparison with uninfected root tips (no *M. graminicola* (Mg–)] at two different time points (3 and 7 dai). Significant increases in TAA levels, and particularly levels of DHA, were observed in galls at both time points (Fig. 1A). The accumulation of DHA suggests that nematode infection causes oxidative stress in host plants, leading to ascorbate oxidation. As a marker for oxidative stress, H_2O_2 was measured in nematode-induced galls at 3 and 7 dai and compared with uninfected root tips. A significantly higher H_2O_2 level was observed in galls at 3 dai compared with galls at 7 dai (Fig. 1B), showing that oxidative stress signals are triggered as an early response upon nematode infection. Further endorsing oxidative stress, levels of MDA, a by-product of the oxidation of polyunsaturated fatty acids, also increased in galls, most strongly at 3 dai (Fig. 1C). These data suggest that AA oxidation might play a role in the early interaction between RKN and rice.

To further study this potential role, loss-of-function rice mutants for three genes involved in AA biosynthesis or oxidation, namely *pmi*, *vtc1*, and *apx* (collectively called AA mutants hereafter), were selected. Previous work with these or similar mutants has focused on aboveground tissues of the *pmi* and *vtc1* mutants grown under hydroponic conditions (e.g. Frei *et al.*, 2012; Höller *et al.*, 2014) or has investigated shoot tissue of *OsAPX8* RNAi lines (Caverzan *et al.*, 2014). Knowing that *M. graminicola* preferentially infects root tips (Mantelin *et al.*, 2017) and that the AA redox state can vary depending on the growth conditions, the AA levels and redox state were first evaluated in the root tips of the selected mutants, under the growth conditions used in our nematode infection protocol, where plants are grown under aerobic conditions in the SAP-substrate (Reversat *et al.*, 1999). It is noteworthy that under these conditions rice roots are provided with abundant levels of Fe and oxygen, known to promote AA oxidation (Wu *et al.*, 2017). Our data show that wild-type rice root tips grown in this experimental set-up contained considerable amounts of DHA (>50% of the TAA) (Supplementary Table S2). Significantly lower amounts of TAA and more specifically DHA (Supplementary Table S2) were detected in roots of all three mutants in comparison with the wild type, which correlates with increased amounts of oxidative stress markers H_2O_2 and MDA in their roots (Supplementary Fig. S1).

Subsequently, susceptibility of these mutants to nematode infection was investigated. A significant increase in number of galls (Fig. 2A), total number of nematodes (Supplementary Fig. S2A), and gall size (Supplementary Fig. S2B–D) was observed at 12 dai in all AA mutants when compared with the wild type. Considering the fact that glutathione is known to be involved in plant defence activation (Han *et al.*, 2013), and that glutathione is oxidized during ascorbate recycling through the 'Foyer–Halliwell–Asada' pathway, we questioned whether glutathione and/or its oxidation levels might be changed in the

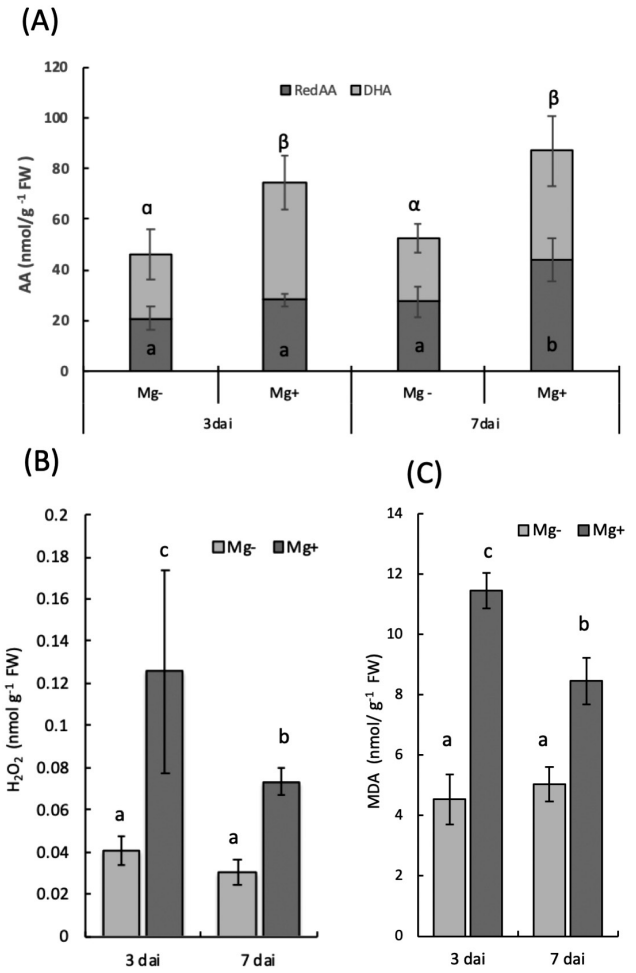

Fig. 1. Oxidative stress markers are accumulating in nematode-infected root tissue. (A) Levels of reduced ascorbic acid (RedAA) and oxidized ascorbic acid (DHA) in galls (induced by *Meloidogyne graminicola*, Mg+) of rice roots at 3 and 7 dai in comparison with uninfected control root tips (Mg–). The ascorbate levels are expressed as nmol mg^{-1} of fresh tissue. Bars show the average and SE of five independent measurements, and letters represent significant differences (Duncan's multiple range test with α=0.05) Each of the five biological replicates consists of a pool of 5–6 plants. (B) Hydrogen peroxide (H_2O_2) content in galls (Mg+) versus uninfected root tips (Mg–) at 3 and 7 dai. Bars represent the average and the SE from four biological replicates, each containing a pool of 4–5 plants. Different letters represent statistically significant differences (Duncan's multiple range test with α=0.05). (C) Malondialdehyde (MDA) content in galls (Mg+) versus uninfected root tips (Mg–) at 3 and 7 dai. Bars represent the average and the SE from four replicates, each containing a pool of 4–5 plants. Different letters represent statistically significant differences (Duncan's multiple range test with α=0.05).

AA mutants. Contradicting this hypothesis, no significant differences in glutathione levels/oxidation were observed in roots and shoots (Supplementary Table S3A, B). These data indicate that AA and/or its oxidation state is involved in plant defence against RKN.

AA oxidation activates systemic root defence against nematodes

Knowing that ascorbate is mainly produced in photosynthetically active aboveground tissues (Foyer, 2017), and based on the observation that the whole rice plant is under oxidative stress upon nematode infection (Fig. 1; Kyndt *et al.*, 2017), we questioned whether AA oxidation could be involved in defence signalling. To investigate this, rice plants were foliar sprayed, or soil drenched with redAA, DHA, or AO (20 U ml^{-1}), 24 h prior to nematode inoculation. It is noteworthy that the effects of soil drenching can be related to direct nematicidal activity and/or induced defence in the rice roots, which cannot be distinguished with this set-up. As previously used for other treatments—with confirmed systemic effects (Nahar *et al.*, 2011)—we added a surfactant to all foliar sprays to promote adherence and uptake of the products.

We first evaluated the effect of foliar application with these chemicals (AA, AO, and DHA) on endogenous redAA and DHA levels, 24 h after their application. The amount of redAA was significantly increased in shoots of redAA-sprayed plants (Supplementary Fig. S3C), while no changes in roots were observed (Supplementary Fig. S3D). Upon foliar AO application, TAA levels were slightly decreased in shoots, while three times higher amounts of TAA were detected in roots of AO-sprayed plants versus control plants, suggesting potential transport and/or systemic signalling towards the roots (Supplementary Fig. S3D). DHA-sprayed shoots contained significantly higher amounts of DHA than control plants, while a minor TAA increase was observed in roots (Supplementary Fig. S3D). These data reveal that foliar AO application is an effective treatment to enhance DHA levels in roots.

Next, we investigated the effect of these treatments on the number of galls and number of nematodes at 12 dai, using either foliar spray or soil drenching. For foliar spraying, there were no significant differences in number of galls and total number of nematodes between the control and redAA-sprayed plants (Fig. 2B), but a significantly lower number of galls (Fig. 2B) and a lower number of nematodes (Supplementary Fig. S3A) was observed in rice plants sprayed with AO or with DHA.

Our results with soil drenching showed no significant difference in number of galls and total number of nematodes between control and AO soil-drenched plants, which is probably related to low enzyme stability in substrate and/or the low presence of AA in root tissues. However, a significantly lower number of galls (Fig. 2C) and lower number of nematodes (Supplementary Fig. S3B) was observed in rice plants soil drenched with AA or with DHA.

Collectively, our data indicate that the level of root DHA plays a role in plant defence against nematodes. The results obtained with foliar applications indicate that endogenous ascorbate oxidation systemically reduces rice susceptibility to RKNs.

Ascorbate oxidation activates systemic rice defence against RKNs through induction of H_2O_2, and JA and ET pathway in the roots

To determine how oxidation of ascorbate leads to enhanced defence in rice roots, we investigated the defence response and the transcriptome of roots upon foliar AO treatment. This analysis was done both with and without nematode inoculation (3 dai), so that we could also evaluate the typical priming phenomenon, where defence metabolites and genes are not

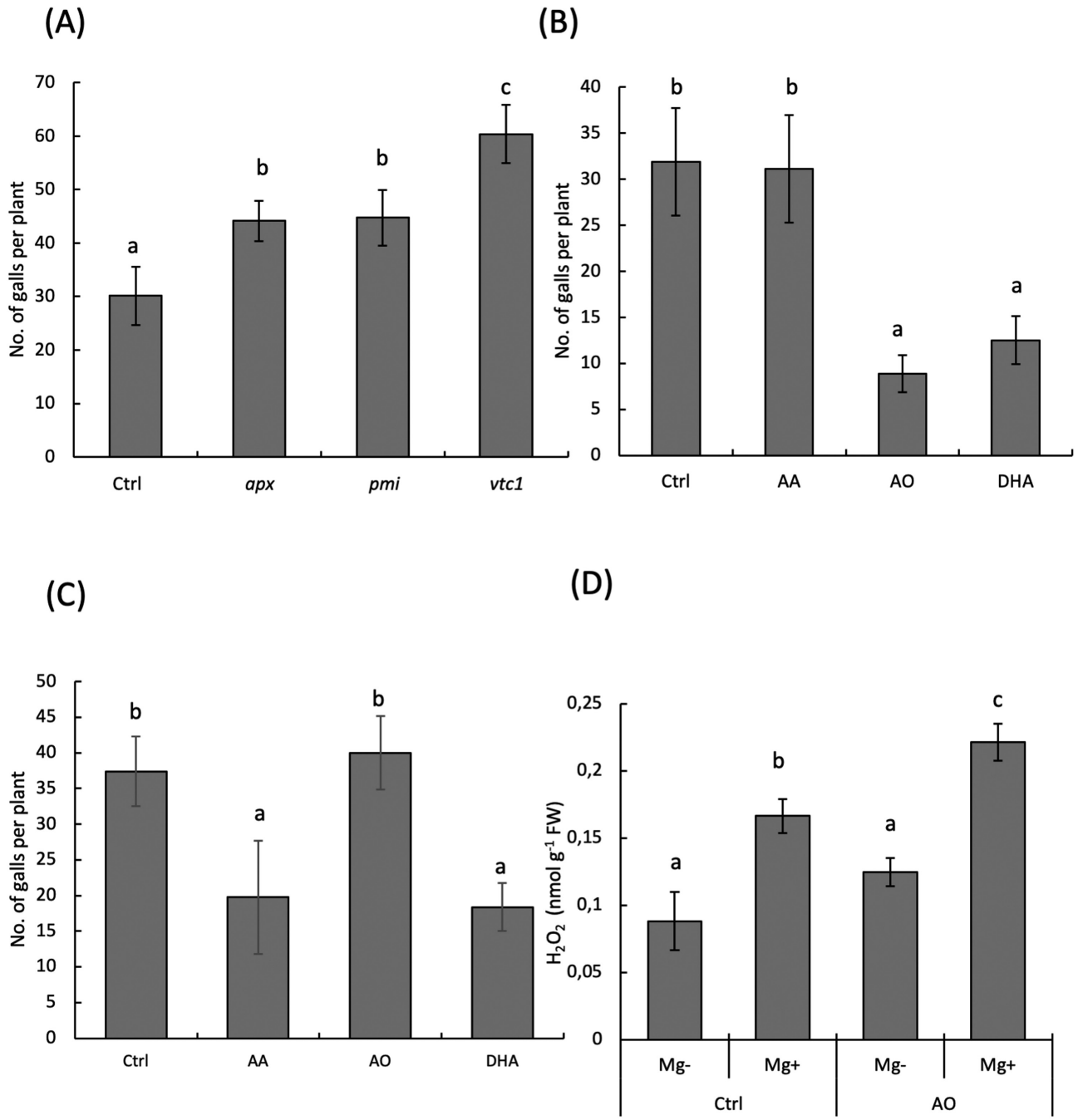

Fig. 2. The role of ascorbate oxidation in rice root defence against the root-knot nematode *M. graminicola*. (A) Nematode susceptibility test in AA mutant lines. Number of galls in AA mutants versus wild-type Nipponbare. Fifteen-day-old plants were inoculated with 250–300 nematodes. At 24 h before the inoculation, plants were sprayed with respective chemicals. The number of galls per plant was counted at 12 dai. Different letters indicate statistically significant differences (Duncan's multiple range test with α=0.05). Bars show the average and SE of eight plants. Data represent one of three independent experiments with similar results. GDP-mannose-3'5'-epimerase (GME/*vtc1*), phosphomannose isomerase (*pmi*), and ascorbate peroxidase (*apx*). (B) Susceptibility assay on rice plants sprayed with AA (20 mM), AO (20 U ml^{-1}), DHA (20 mM), or water as a negative control. Fifteen-day-old plants were inoculated with 250–300 nematodes. At 24 h before the inoculation, plants were sprayed with respective chemicals. The number of galls per plant was counted at 12 dai. Bars represent means and SE of eight plants. Different letters indicate statistically significant differences (Duncan's multiple range test with α=0.05). Data represent one of three independent experiments with similar results. (C) Susceptibility assay on rice plants soil drenched with AA (20 mM), AO (20 U ml^{-1}), DHA (20 mM), or water as a negative control. Fifteen-day-old plants were inoculated with 250–300 nematodes. At 24 h before the inoculation, plants were soil drenched with the respective chemicals. The number of galls per plant was counted at 12 dai. Bars represent means and SE of eight plants. Different letters indicate statistically significant differences (Duncan's multiple range test with α=0.05). Data represent one of three independent experiments with similar results. (D) Hydrogen peroxide (H_2O_2) content in the galls of AO-sprayed plants (Mg+) and in the uninfected root tips of AO sprayed plants (Mg–) versus galls of the mock-sprayed plants (Mg+) and uninfected root tips (Mg–). Fifteen-day-old plants were inoculated with nematodes or mock inoculated. One day before inoculation the plants were sprayed. Samples were collected at 3 dai. H_2O_2 levels are expressed as nmol g^{-1} of fresh tissue. Bars represent the average and SE of four biological replicates, each containing a pool of 4–5 plants. Different letters represent statistically significant differences (Duncan's multiple range test with α=0.05).

strongly induced by a defence-activating molecule but rather vigorously induced upon subsequent pathogen invasion (Conrath *et al.*, 2015). H_2O_2 accumulation is an early hallmark for activated immune responses. Measurements of this compound (Fig. 2D) showed a significant increase in H_2O_2 levels in the galls formed on AO-sprayed plants when compared with galls from non-infected AO-sprayed plants or mock-sprayed plants. These data show that ascorbate oxidation is correlated with primed H_2O_2 generation upon nematode infection in the root system.

Next, mRNA-seq was done on root material of AO-sprayed plants, nematode-infected plants, and AO-sprayed+nematode-infected plants (Supplementary Table S4). When comparing AO-sprayed plants with control plants, minor changes were found in genes related to nitrogen metabolism, proteins, RNA, protein phosphorylation, sugars (glucans), cell wall biosynthesis, chromatin assembly, and oxidative stress. Considering nematode-infected plants, the data confirmed previous observations (Kyndt *et al.*, 2012) showing a disturbance of genes involved in, for example, metabolism, redox, amino acids, starch, regulation, signal transduction, and phenylpropanoids (Supplementary Table S5). When focusing on AO-sprayed+nematode-infected plants, the transcriptional response seemed most similar to the response upon nematode infection alone (Supplementary Fig. S4A; Supplementary Table S5). Parametric analysis of gene enrichment of the mRNA-seq data showed that AO did not lead to statically significant changes in any GO group ($q<0.05$; Supplementary Table S5), revealing a very modest transcriptional response in the plants (Supplementary Fig. S4A). However, the nematodes, which are well known to manipulate cellular metabolism (Kyndt *et al.*, 2012; Ji *et al.*, 2013), are no longer or are less able to do so when plants were pre-sprayed with AO (Supplementary Table S5), confirming the decreased plant susceptibility (Fig. 2B). This phenomenon was observed for several GO groups related to cellular/organelle metabolism and development. The typical priming response, namely activation of plant defence only or more strongly in sprayed+infected plants, was observed for genes related to cell wall biosynthesis such as hemicellulose and xylan metabolic processes, as well as nucleotidyltransferase activity (Supplementary Table S5). The mRNA-seq data confirmed differential expression of several genes related to ascorbate/glutathione metabolism in galls at 3 dai (Supplementary Fig. S4B). At 4 d after AO treatment, no significant induction/repression patterns were observed in genes related to AA biosynthesis or recycling, while induced expression was observed for redox-related genes, more specifically those encoding haem proteins, glutaredoxins, thioredoxins, peroxiredoxins, and dismutases/catalases (Supplementary Fig. S4B). These data confirm that the redox status of the roots is significantly affected upon foliar AO treatment. When specifically focusing on hormone pathways, a higher expression of JA biosynthesis genes was detected in roots of AO-sprayed plants, where some genes were directly induced while others showed a primed response (e.g. *LOX10, AOC, OPR2,* and *JMT1* are only activated in AO-sprayed+nematode-infected plants) (Table 1; Supplementary Fig. S4). Also, ET response genes were activated in roots upon foliar AO treatment (Table 1; Supplementary Fig. S4).

To verify the transcriptome data, the effect of foliar AO application on root levels of plant hormones JA, ET, SA, ABA, and indole acetic acid (IAA) were evaluated. Significantly higher amounts of JA were observed in rice roots 24 h after spraying the plants with AO, while SA was slightly higher and other hormone levels were not affected (Fig. 3A). Considering ET, evaluation was initially done 24 h after AO treatment but, since there were no significant changes at this time point and this volatile hormone tends to accumulate very rapidly, ET levels were also evaluated at earlier time points. Root ET levels were significantly higher 12 h after spraying with AO (Fig. 3B).

To confirm the link between ascorbate oxidation and hormone levels, root tips of 15-day-old AA mutant plants were collected, and hormones of interest, namely JA, ET, and SA, were measured. All three AA mutants, which are hypersusceptible (Fig. 2A), showed significantly lower levels of JA in the roots (Fig. 4A). No significant change in the amount of SA was observed in *pmi* and *apx* mutants in comparison with wild-type plants, but a significantly lower level of SA was observed in the *vtc1* mutant (Fig. 4B). Significantly lower levels of ET were observed in *pmi* and *vtc1* mutants compared with control root tips, and marginally lower levels of ET were found in the *apx* mutant (Fig. 4C).

Based on the knowledge that (i) JA is the key hormone in plant defence against RKNs in rice, and that this hormone is modulated by ET (Nahar *et al.*, 2011); and (ii) that foliar AO treatment activates systemic ET and JA accumulation (Fig. 3A, B), we hypothesized that lower endogenous JA/ET levels in the *vtc1* mutant (Fig. 4) may be a major reason for its hypersusceptibility to RKNs. To test this hypothesis, wild-type Nipponbare and the *vtc1* mutant were sprayed with MeJA or the ET-generator ethephon, 24 h before nematode inoculation. Our results with the wild type confirm published information (Nahar *et al.*, 2011) that aboveground MeJA and ethephon application leads to systemic enhanced defence against RKNs in rice. While the number of galls increased significantly in the *vtc1* mutant as compared with the wild type, this increase was not observed when the *vtc1* mutant was sprayed with MeJA or ET prior to inoculation (Fig. 5A). This shows that application of MeJA or ethephon can rescue the hypersusceptibility phenotype of the *vtc1* mutant.

To further confirm that ascorbate oxidation leads to systemic induced defence against RKNs through activation of ET and JA, an infection experiment was carried out using foliar application of AO or DHA on the jasmonate signalling *Osjar1* mutant, and on plants sprayed with AO or DHA in combination with the JA biosynthesis inhibitor DIECA or the ET biosynthesis inhibitor AoA (Fig. 5B). A significantly increased number of galls was observed in the *Osjar1* mutant and DIECA-sprayed plants when compared with wild-type Nipponbare, confirming that JA is a major player in defence against RKNs in rice (Nahar *et al.*, 2011). Again, a significant reduction in number of galls (reduction of 50–70%) was observed in plants sprayed with AO or DHA. However, only a 25–30% reduction was observed in *Osjar1* mutants sprayed with AO or DHA, and similarly, only a 20% reduction was observed in plants sprayed with a combination of DIECA and AO or DHA. Plants sprayed with the ET biosynthesis inhibitor AoA had a significantly higher number

Table 1. Overview of root expression pattern of JA biosynthesis genes, based on mRNA-seq data of (i) nematode-infected galls (3 dai); (ii) roots of AO-treated plants (4 d after foliar treatment); and (iii) AO-treated+infected plants

Rap-DB locus number	Protein encoded by this locus	FC (infected versus control roots)	FC (AO-treated versus control roots)	FC (infected+AO-treated versus control roots)
Jasmonate biosynthesis genes				
Os03g0699700	LOX3	0.61	**1.71**	**1.69**
Os11g0575600	LOX10	0.97	0.79	**1.59**
Os08g0509100	LOX8	**1.53**	**1.74**	**1.70**
Os08g0508800	LOX2	0.46	0.70	0.62
Os04g0447100	LOX6	0.65	0.71	0.46
Os03g0700400	LOX3	**2.73**	**1.81**	**1.79**
Os03g0700700	LOX4	1.25	0.99	1.16
Os02g0218800	AOS4	0.68	0.92	0.76
Os03g0225900	AOS2	1.43	0.76	1.00
Os03g0767000	AOS1	0.60	0.82	0.53
Os02g0218700	AOS3	0.85	**1.80**	1.46
Os03g0438100	AOC	1.09	0.63	**3.12**
Os08g0459600	OPR7	0.84	0.82	0.78
Os06g0216200	OPR2	1.48	**1.81**	**3.27**
Os01g0370000	OPR9	0.79	1.37	1.06
Os06g0215500	OPR6	**2.22**	**1.68**	**2.36**
Os05g0102000	JMT1	**3.63**	**2.92**	**4.15**
Ethylene response genes				
Os12g0603300	ERF112	1.48	ND	6.59
Os06g0181700	ERF2	**1.64**	0.90	**2.14**
Os03g0183200	ER69	1.01	**2.90**	**2.11**
Os05g0437100	ERF105	**2.77**	**3.31**	5.12
Os08g0545500	ER28	ND	6.93	**1.79**
Os08g0521600	ERF115	0.51	**4.18**	0.31
Os04g0429050	ERF100	0.15	**1.63**	0.36
Os04g0610400	ERF77	0.78	**1.78**	0.48
Os02g0546600	ERF98	ND	**1.81**	ND
Os03g0860100	ERF83	**1.52**	**4.76**	**1.95**
Os05g0497200	ERF130	1.03	**2.16**	1.14
Os09g0572000	ERRF87	0.42	**1.71**	0.93
Os09g0522100	ERF133	0.47	**2.16**	0.11

Data show the fold change (FC) of the number of transcripts detected in each tissue in comparison with control root tissue of untreated and uninfected plants of the same age. Values indicated in bold represent significant induction (FDR <0.05). Rap-DB: locus number based on the Rice Annotation Project Database Genome Annotation. The GEO number for the AO data is GSE125427. LOX, lipoxygenase; AOS, allene oxide synthase; ACO, allene oxide cyclase; OPR, 12-oxo-phytodienoic acid reductase; JMT, jasmonate methyl transferase; ERF, ethylene response factor.

of galls when compared with control plants. However, when AoA was combined with AO or DHA, again only a 20% reduction was seen (Fig. 5B). Furthermore, AoA was applied on the *Osjar1* mutant, and susceptibility of these plants to RKNs was compared with single AoA treatment or only *Osjar1* deficiency, revealing that ET and JA have a synergistic effect on plant defence against nematodes (Fig. 5C). Interestingly, AO-induced defence was negated when the *Osjar1* mutant was treated with AoA, showing that AO-induced defence is dependent on activation of both JA and ET (Fig. 5C).

Discussion

In this research, we show that ascorbate oxidation induces systemic rice defence against RKNs by activating the ET and JA pathway in roots and by priming for increased H_2O_2 generation upon nematode infection.

Genetic AA deficiency leads to significantly higher susceptibility to RKNs. Under basal conditions, roots of the used AA-deficient mutant plants showed lower TAA and DHA, and increased H_2O_2 and MDA levels, confirming that ascorbate is important to scavenge ROS production in rice roots, as previously shown by Frei *et al.* (2012). The fact that all three investigated rice AA mutants contain a higher amount of ROS but are not able to generate defence responses against RKN reveals that ROS signalling in rice root defence is ascorbate dependent. In contrast, Mukherjee *et al.* (2010) showed that the Arabidopsis *vtc1* mutant is more resistant to *P. syringae*, and they proposed that AA may hinder H_2O_2-facilitated defence. Whether this contradiction could be related to the difference in infection strategy between those pathogens or to plant/tissue-specific defence responses remains to be elucidated. It is noteworthy that AA and AO effects have previously been reported to be different when comparing shoot and root tissue. For example, Pignocchi *et al.* (2006) observed an AO-induced auxin insensitivity in tobacco shoots, but not in roots, as stimulation of growth by exogenous auxin was only lost in the shoots of the AO overexpression line.

Arrigoni *et al.* (1979) and Zacheo *et al.* (1981) previously reported AA accumulation in the roots of resistant pea and

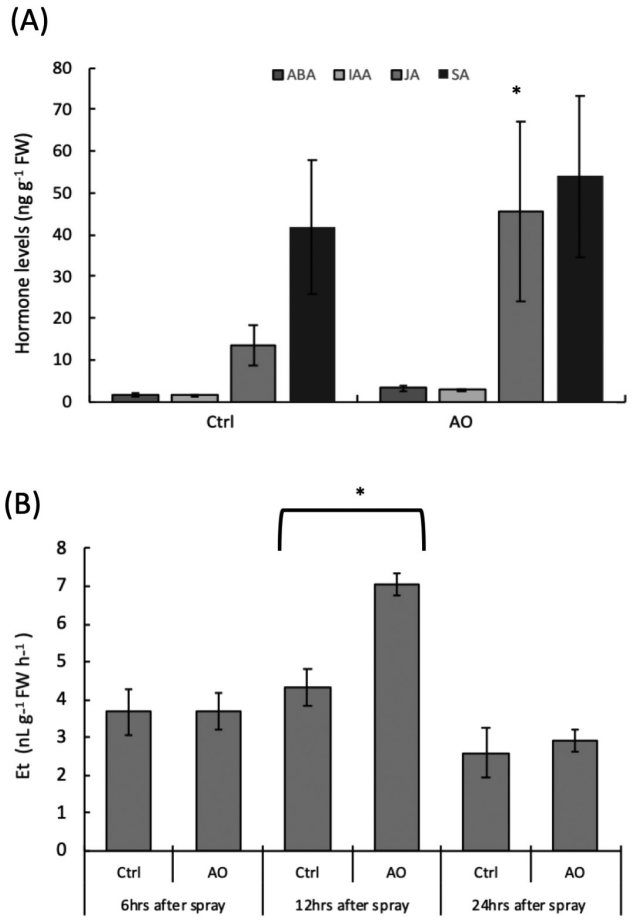

Fig. 3. Ascorbate oxidation triggers ET and JA accumulation in rice roots. (A) JA, SA, IAA, and ABA content in the roots of plants sprayed with ascorbate oxidase (AO) (20 U ml^{-1}), or water as a control. Root samples were collected 24 h after spraying. Values presented are average ±SE of five biological replicates each consisting of at least four individual plants. Asterisks indicate statistically significant differences (Duncan's multiple range test with α=0.05). ABA, abscisic acid; IAA, indole-3-acetic acid; JA, jasmonic acid; SA, salicylic acid. (B) ET levels of roots sampled at 6, 12, and 24 h after spraying with water (ctrl) or AO (20 U ml^{-1}). The plants were 15 days old. Values presented are average ±SE of six biological replicates each consisting of at least four individual plants. Asterisks indicate statistically significant differences between control and AO-sprayed plants (Duncan's multiple range test with α=0.05). ET, ethylene.

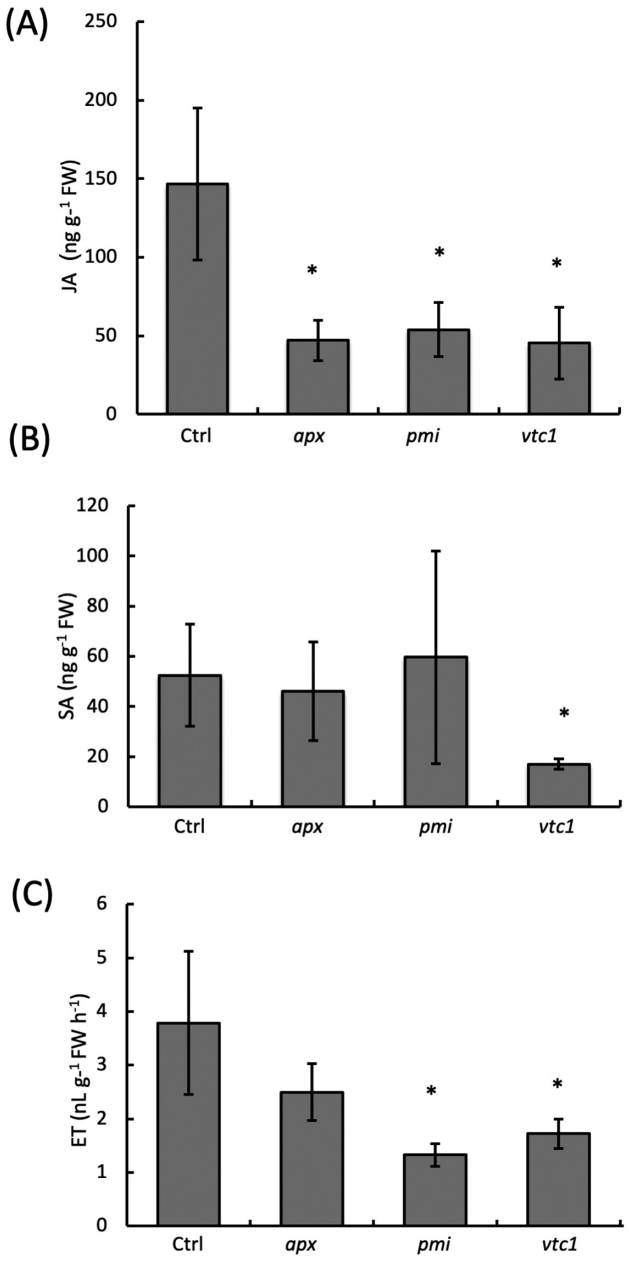

Fig. 4. Hormone measurements in the AA mutants show reduced levels of ET and JA. (A) JA, (B) SA, and (C) ET contents in the roots of 15-day-old AA mutant and wild-type plants. Values presented are average ±SE of five biological replicates, each consisting of at least four individual plants. Asterisks indicate statistically significant differences between the control and mutant plants (Duncan's multiple range test with α=0.05). JA, jasmonic acid; SA, salicylic acid; ET, ethylene; GDP-mannose-3'5'-epimerase (GME/*vtc*), phosphomannose isomerase (*pmi*), and ascorbate peroxidase (*apx*).

tomato upon nematode invasion. Here, we demonstrate that the nematode-induced galls mainly contain DHA, and that this DHA plays a role in plant defence.

Similar to our observations, Arrigoni *et al.* (1977) showed that soil application of redAA inhibits invasion of tomato roots by *M. incognita*. Since direct nematicidal effects of AA or DHA cannot be ruled out in the case of soil applications, we decided to continue with foliar application to stimulate endogenous ascorbate oxidation. Mainly foliar AO application was a potent activator of DHA accumulation in the roots (Supplementary Fig. S3). Foliar application of redAA did not affect nematode infection in roots (Fig. 2B), which is correlated with only a localized accumulation of redAA in shoots, but no effects on roots (Supplementary Fig. S3). It has been previously described that ascorbate preferentially crosses cellular membranes in the oxidized form (Horemans *et al.*, 1997, 2000*b*).

Our data showed that oxidation of the endogenous AA pool—by foliar AO application—results in strongly increased DHA levels in roots (Supplementary Fig. S3), which are negatively correlated with root susceptibility to nematodes. Most probably, a systemic signalling cascade based on redox signals and/or DHA is activated upon foliar AO treatment. Wang *et al.* (2019) demonstrated that a shoot–root signalling pathway, integrating ROS, electric signals, and JA, is required to activate defence against RKNs in tomato. Further investigations, for example by labelling of candidate compounds or grafting,

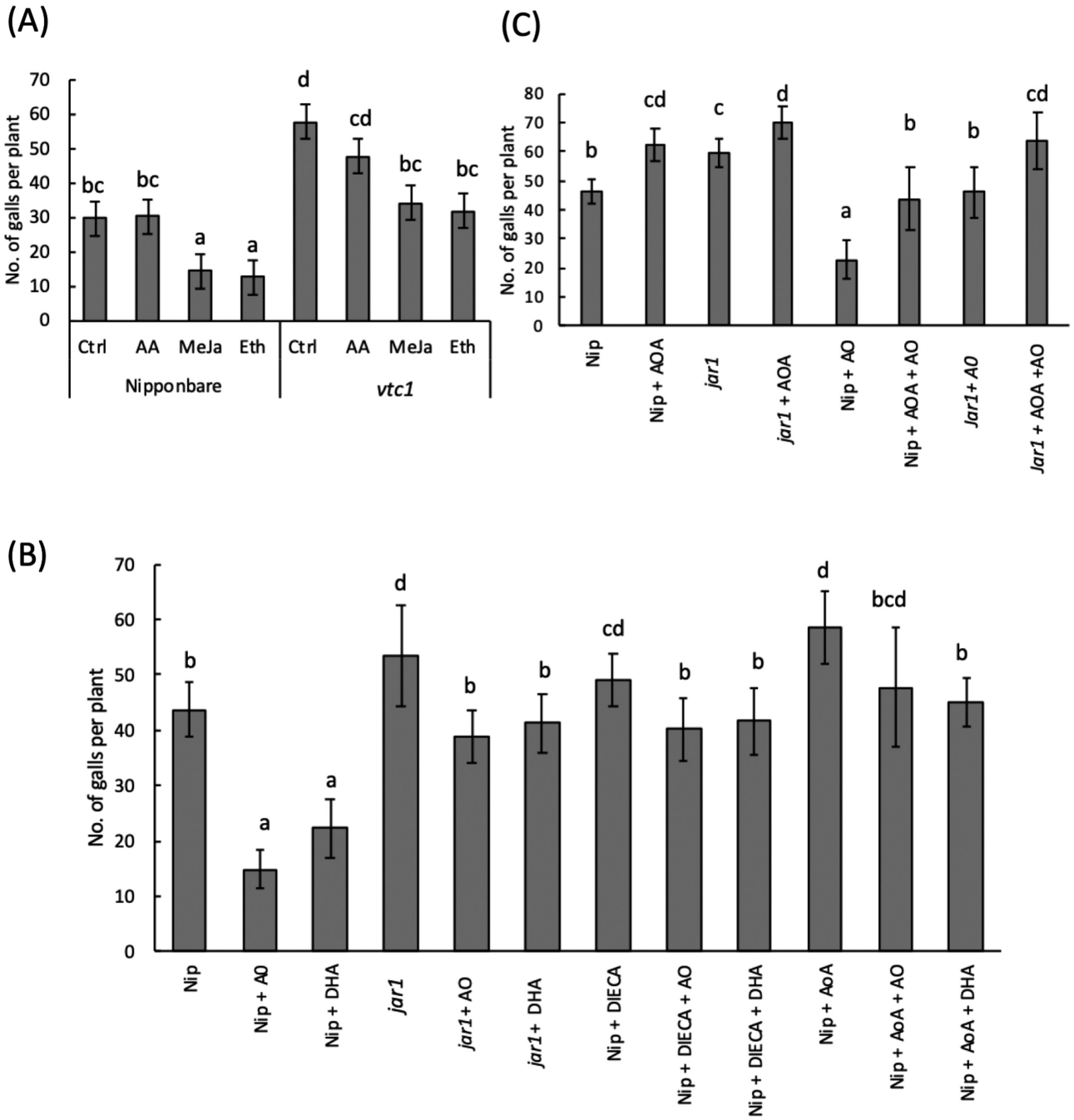

Fig. 5. Ascorbate oxidation-induced defence against root-knot nematodes is dependent on the JA and ET pathways. (A) Wild-type and *vtc1* mutant plants were sprayed with water as negative control, AA (20 mM), MeJA (100 µM), or ethephon (100 µM). (B) Wild-type Nipponbare (Nip) and the JA signaling mutant *jar1* were sprayed with water, AO (20 U ml^{-1}), DHA (20 mM), DIECA (JA biosynthesis inhibitor, 100 µM), or AoA (12.5 mM) either alone or in combination with AO (20 U ml^{-1}) or DHA (20 mM). (C) The wild-type Nip ponbare (Nip) and the JA signalling mutant *jar1* were sprayed with AO (20 U ml^{-1}) or AoA (12.5 mM) either alone or in combination. In all experiments (A, B, C), plant roots were inoculated with 300 *M. graminicola* juveniles at 24 h after spraying with the respective chemicals. Bars represent means ±SE of the number of galls of eight plants at 12 dai. Different letters indicate statistically significant differences (Duncan's multiple range test with α=0.05). Data represent one of three independent experiments with similar results. MeJA, methyl jasmonate; AO, ascorbate oxidase; DHA, dehydroascorbate; AoA, aminooxyacetic acid; DIECA, diethyldithiocarbamic acid.

could shed light on the nature of the transported signal upon AO treatment.

Overexpression of AO in *Nicotiana tabacum* resulted in H_2O_2 and ABA accumulation, and increased susceptibility to *Botrytis cinerea* and to agents provoking oxidative stress (Fotopoulos *et al.*, 2008). Enhanced H_2O_2 production was also observed here (Fig. 2D), but, in contrast to these observations, we found rather that foliar AO application activates systemic defence against RKNs in rice and increases plant stress tolerance. In contrast to external applications, ectopic overexpression of AO might put the plant's immune system in non-responsive modus, leading to cell death and lack of long-term stress tolerance. Hormone measurements and transcriptome data confirmed enhanced activation of root defence upon foliar applications with AO, pointing to an indirect and systemic signalling effect of ascorbate oxidation on plant immunity against nematodes. It

is noteworthy that the AO treatment is inactive when applied on soil (Supplementary Fig. S3B), most probably because the substrate of this enzyme is not present in root tissue or because of enzyme instability in the SAP-substrate.

The role of AA in production of plant defence metabolites has been described for anthocyanins, flavonoids and glucosinolates (Turnbull *et al.*, 2004), zeaxanthin (Müller-Moulé *et al.*, 2004; Giacomelli *et al.*, 2006), and the hormones ABA, gibberellic acid, and ET (Arrigoni and De Tullio, 2002; Mirica and Klinman, 2008). Foyer and Noctor (2011) proposed two possible reasons for the effect of AA oxidation on defence pathways: (i) the availability of AA may regulate the synthesis and abundance of hormones; and (ii) there is a compensation for a decrease in AA by an increase in the abundance of glutathione, potentially enhancing redox signalling. The second option can be ruled out here, as we did not observe shifts in glutathione levels or oxidation in the investigated AA mutants here (Supplementary Table S3). Rather, transcriptome analysis revealed that the JA biosynthesis pathway and ET-responsive genes were activated in the rice roots upon foliar AO application (Table 1; Supplementary Fig. S4). Confirming a link between DHA and JA/ET in rice roots, AA mutants contain lower root levels of JA and ET as well as lower DHA levels, while foliar AO-treated rice contains enhanced levels of DHA and both hormones in roots (Figs 3, 4). The JA pathway, modulated by ET, is a key player in protecting rice from *M. graminicola* (Nahar *et al.*, 2011) and also protects other plants from RKNs (Soriano *et al.*, 2004; Cooper *et al.*, 2005; Gleason *et al.*, 2016; Martínez-Medina *et al.*, 2017) and *Heterodera schachtii* (Kammerhofer *et al.*, 2015). AA is a well-known cofactor during the formation of ET through ACC oxidase, and DHA is formed as a co-product. By applying AO onto plants, this reaction seems to have been positively influenced in rice roots.

Work with genetic and chemical inhibition of JA/ET confirms that systemic defence activated by AO or DHA is dependent on activation of the JA/ET pathways (Fig. 5). AO-induced defence involves an early accumulation of ET and subsequent JA accumulation in the rice roots (Fig. 3). This is a novel link establishing that ascorbate oxidation leads to a ET/JA-based defence response in roots. While in our research we reveal that AA oxidation activates JA, the inverse has also been shown where treatment of *N. tabacum* and *Arabidopsis thaliana* suspension cells with MeJA stimulated *de novo* biosynthesis of AA (Wolucka *et al.*, 2005). Interestingly, a combined application of JA and AA enhances the accumulation of sakuranetin, a phytoalexin identified from blast-resistant rice cultivars, compared with JA alone (Tamogami *et al.*, 1997). Hence, we hypothesize that ascorbate oxidation and JA together form a positive feedback loop to prime the plant for enhanced activation of immunity upon pathogen attack. Some reports have previously suggested that the quantity and redox state of AA may influence JA signaling. For example, in tomato, silencing of the terminal enzyme in the Man/Gal pathway resulted in DHA accumulation and increased expression of JA-responsive transcripts such as proteinase inhibitors and arginine decarboxylase (Suza *et al.*, 2010). Although JA is known to activate AA synthesis and recycling in Arabidopsis, Brassica, and tobacco (Sasaki-Sekimoto *et al.*, 2005; Wolucka *et al.*, 2005; Suza *et al.*, 2010; Fujiwara *et al.*, 2016), no evidence of AA acting as a cofactor or trigger for JA biosynthesis has been reported before in the scientific literature.

The primed accumulation of H_2O_2 in AO-treated plants (Fig. 2D) indicates that oxidation of the endogenous AA pool hampers H_2O_2 detoxification, permitting H_2O_2 to accumulate strongly and exert its role in root defence against RKNs in rice. Additionally, it is possible that DHA formed upon ascorbate oxidation is hydrolysed to diketogulonic acid and eventually to oxalic acid and H_2O_2 (Green and Fry, 2005; Dewhirst and Fry, 2018). AA accumulation upon pathogen infection was found to be correlated with increased oxalic acid biosynthesis and increased H_2O_2 in cacao, as a defence response upon fungal infection (Dias *et al.*, 2011). This reflects the concept of defence 'priming' as coined by Conrath *et al.* (2006, 2015), where defence responses are not activated directly, but are 'memorized' and expressed in an accelerated manner once an actual stress signal is being perceived (Conrath *et al.*, 2015). Although for ET and JA direct accumulation was observed (Fig. 3), our data do reveal a primed increase in H_2O_2 levels in AO-pre-sprayed nematode-inoculated plants (Fig. 2D).

Based on the results presented here, we conclude that ascorbate oxidation has a positive role in rice defence against RKNs by systemically activating the ET/JA pathway and increased H_2O_2 accumulation upon nematode infection. Application of AO or DHA could be used as a novel strategy to protect plants from these damaging pathogens.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Oxidative stress markers in AA mutants.

Fig. S2. Details of nematode susceptibility assays on AA mutants.

Fig. S3. Details of nematode susceptibility upon foliar and soil applications with AA, AO, or DHA, and evaluation of redAA/DHA in the shoots and roots upon foliar applications.

Fig. S4. Visualization of mRNA-seq results.

Table S1. Overview of results from previously published transcriptome analyses of galls and giant cells induced by the RKN *M. graminicola* in rice (*Oryza sativa*) roots.

Table S2. Ascorbate levels in roots and shoots of AA mutants.

Table S3. Glutathione levels in roots and shoots of AA mutants.

Table S4. Number of reads and mapping percentage of the transcriptome data.

Table S5. Gene set enrichment analyses on transcriptome data.

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Author contributions

TK, RS, GG, MF, and SS planned and designed the research. RS and AMT performed all infection experiments. SP and MF measured glutathione levels. BV and TK performed the mRNA sequencing analysis. AH, KD, RT, and RS executed hormone measurements. RS, SS, and TK wrote the manuscript, with input from all authors.

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