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Research article

The nitric oxide suppressed *Arabidopsis* mutants- *Atnoa1* and *Atnia1nia2noa1-2* produce nitric oxide in MS growth medium and on uranium exposure

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A B S T R A C T

The mutants *Atnoa1* and *Atnia1nia2noa1-2* having a defective chloroplast developmental process, showed enhanced chlorophyll levels when they were grown on Murashige and Skoog (MS) medium and on exposure with uranium (U) on Hoagland medium. Thus we hypothesized that these mutants probably produced NO in MS medium and on exposure with U. Wild-type Col-0, *Atnoa1*, *Atnia1nia2noa1-2* plants were cultured on modified Hoagland and 1/10 MS media and NO generation in the roots of these mutants was monitored using NO selective fluorescent dyes, DAF-2DA and Fl2E. Both *Atnoa1* and *Atnia1nia2noa1-2* triple mutants produced NO as observed by increases in DAF-2T and Fl2E fluorescence when these mutants were grown on MS medium but not on Hoagland medium. In presence of NO scavenger, methylene blue (MB, 200 μM), DAF-2T and Fl2E fluorescence was completely abolished. On the other hand treatment of the plants with 25 μM U triggered NO generation. U-treated *Atnoa1* and *Atnia1nia2noa1-2* plants upregulated genes (*POR B, POR D, CHL D*) involved in the chlorophyll biosynthesis. From these results it was concluded that *Atnoa1* and *Atnia1nia2noa1-2* are conditional NO producers and it appears that NO generation in plants substantially depends on growth medium and NIA1, NIA2 or NOA1 does not appear to be really involved in NO generation in MS medium or after U exposure.

1. Introduction

Nitric oxide (NO) is a biologically active, diffusive, water and lipid soluble diatomic gaseous free radical. It has been shown to be a ubiquitous signalling molecule in animal, plant and microbial systems effective even at nanomolar concentration. In plants, NO has been implicated in the regulation of stomatal aperture (Desikan et al., 2002; Garcia-Mata and Lamattina, 2001; Shi et al., 2015), pathogen defence (Durner et al., 1998), developmental transitions including directed pollen tube growth (Prado et al., 2004, 2008), abiotic stress responses (Kumar et al., 2010; Wendehenne et al., 2004), and regulate cellular redox balance (Correa-Aragunde et al., 2015). Recently, we demonstrated that NO is also important in the response of plants to U (Tewari et al., 2015). Despite the potential significance of NO in plant growth and development and environmental responses, the process of NO generation in plant cells is still far of being completely understood (Chamizo-Ampudia et al., 2017; Gupta et al., 2011). In animals, NO synthase (NOS) catalyses the conversion of L-arginine to L-citrulline and NO in the presence of O_2. In plants, although several reports on NOS-like activity are available (Corpas et al., 2009, 2011; Neill et al., 2003); the sequences of two functional NOS proteins catalyzing L-Arginine dependent NO synthesis, have only been revealed in green algae *Ostreococcus* species indicating that this might be lost early in the evolution of plants (Chamizo-Ampudia et al., 2017; Foresi et al., 2010).

Arabidopsis Nitric Oxide Associated 1 (NOA1), also identified as RIF1 (Flores-Perez et al., 2008), was reported initially to encode a protein with NOS activity (Guo et al., 2003). However, subsequent experimental evidences did not support NOA1 dependent catalytic

Abbreviations: DAF-2DA, *4,5*-Diaminofluorescein diacetate; DAF-2T, DAF-2 triazole; MB, Methylene blue; NO, Nitric oxide; NOA1, Nitric oxide associated1; NOS, Nitric oxide synthase; NR, Nitrate reductase; NIA1, nitrate reductase (NADH)1; NIA2, nitrate reductase (NADH)2

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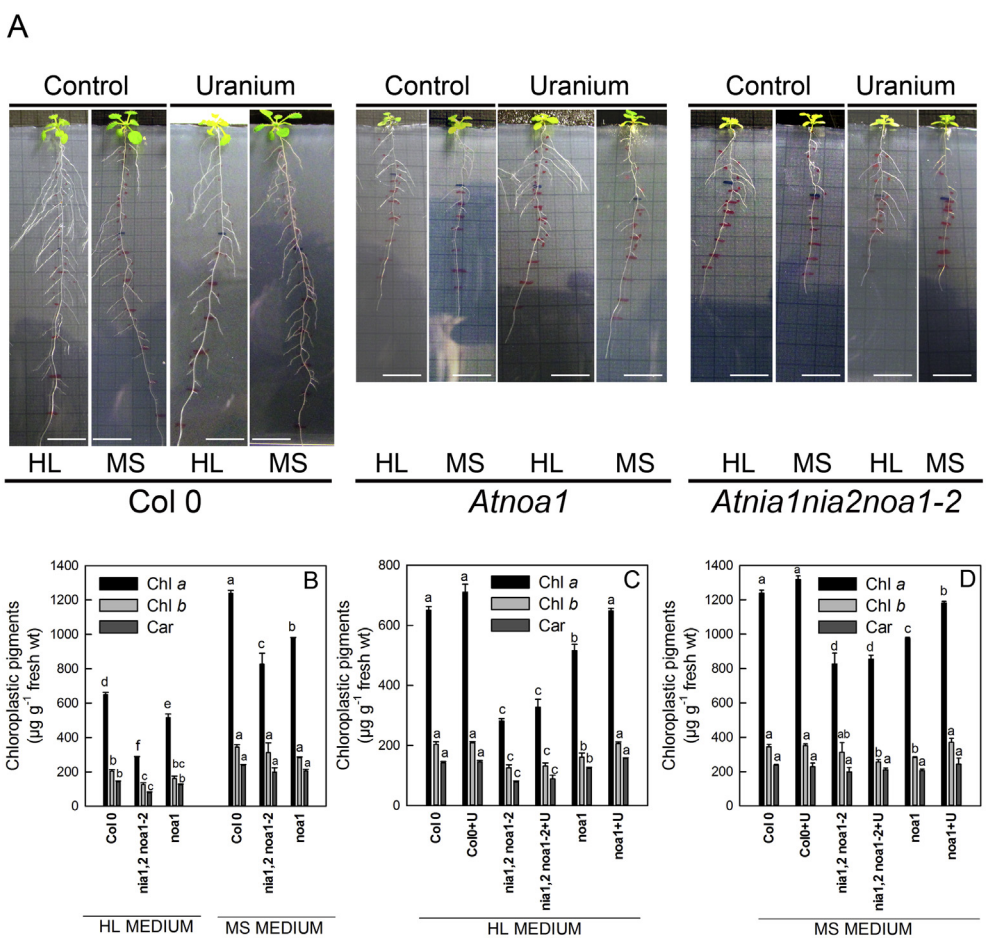

Fig. 1. *Atnoa1* and *Atnia1nia2noa1-2* phenotypes of delayed maturation and pale green leaves are showing increased greenness in the leaves on MS medium (A) in vertical agar plates (VAPs). Photographs of 15-days-old Col-0 and *Atnoa1* and *Atnia1nia2noa1-2* seedlings grown either on Hoaglands (HL) or Murashige and Skoogs' (MS) medium in the presence and absence of 25 µM uranium. Scale bar: 1 cm. Chlorophyll and carotenoids concentrations in Col-0, *Atnia1nia2noa1-2* and *Atnoa1* plants grown on Hoagland or MS medium (C-D) and on exposure with 25 µM U in Hoagland or MS medium (C-D). Data are mean of six replicates (± SE). Bars carrying different letters are significantly different ($p \leq .0.05$) by Fisher-LSD method. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

conversion of L-arginine to L-citrulline and NO (Sanz et al., 2015). Supplementing NO-producing sodium nitroprusside (SNP) in growth medium improves the *Atrif1-1* (Flores-Perez et al., 2008) and *Atnoa1* (Guo et al., 2003) growth phenotypes. Furthermore, NOA1 and a related homolog from *Bacillus subtilis*, YqeH display GTPase activity (Crane et al., 2010) but no detectable NOA1 dependent conversion of L-arginine to L-citrulline and NO. However, YqeH can rescue *Atnoa1* phenotypes (Crane et al., 2010; Flores-Perez et al., 2008). Several reports are available which indicate that *Atnoa1* mutant has relatively reduced levels of NO compared to wild-type, Col-0 plants (Bright et al., 2006; Chen et al., 2010; Guo and Crawford, 2005; Guo et al., 2003; Tewari et al., 2013; Zeidler et al., 2004). These reports suggest an indirect role of NOA1/RIF1 in NO biosynthesis.

Another potential enzymatic NO source in plants is nitrate reductase (NR), the critical enzyme responsible for nitrate assimilation (Neill et al., 2003, 2007). A plasma membrane-bound nitrate reductase (NR) that reduces nitrate to nitrite (Besson-Bard et al., 2008; Gupta et al., 2011). Subsequently, nitrite-NO reductase (Ni-NOR) activity catalyses conversion of nitrite to NO (Besson-Bard et al., 2008; Gupta et al., 2011). NO is produced by one-electron reduction of nitrite by heme or Cu-containing nitrite reductases and then further reduced to N_2O by NO reductase (Gupta et al., 2011; Lozano-Juste and Leon, 2010; Xie et al., 2013). In *Arabidopsis*, NR is encoded by two genes, *NIA1* and *NIA2* (Lozano-Juste and Leon, 2010; Park et al., 2011). The involvement of NR-mediated NO biosynthesis in stomatal closure and cold acclimation has been demonstrated genetically (Bright et al., 2006; Zhao et al., 2009). The triple mutant *nia1nia2noa1-2* (developed by crossing double mutant *nia1nia2* with *noa1-2*) has been shown to have an impaired NIA1, NIA2 and NOA1 dependent NO biosynthesis but these plants exhibit an enhanced abscisic acid-mediated closure of stomata and hence a tolerance to drought stress (Lozano-Juste and Leon, 2010). Finally, nitrite is a non-enzymatic source of NO as it can be directly reduced to NO in the presence of reductant such as ascorbic acid or an acidic pH (Bethke, 2004; Tewari et al., 2009).

The pale phenotype of *Atnoa1/Atrif1* and *Atnia1nia2noa1-2* mutants having defective chloroplast development show reduced carbon fixation ability (Van Ree et al., 2011). The small, pale and slow-growing *Atnoa1* phenotypes have been shown to be rescued by including sucrose in growth medium (Van Ree et al., 2011). NO producer, SNP, has been reported to induce greening in etiolated barley seedlings (Zhang et al., 2006) and Fe-deficient maize plants (Kumar et al., 2010). Uranium (U^{238}), a primordial radionuclide and heavy metal, induces chemical toxicity and inhibits plant growth (Saenen et al., 2013; Vanhoudt et al., 2014) as well as induces NO and H_2O_2 production (Tewari et al., 2015). *Atnoa1* and *Atnia1nia2noa1-2* plants showed greening in the leaves when they were grown on MS growth medium. Therefore, it is hypothesized that these alleged NO-suppressed mutants generate NO in MS medium or after U exposure. To prove this hypothesis, we performed a comparative study of NO generation in roots of *Arabidopsis thaliana* Col-0 (wild-type), *Atnoa1* and *Atnia1nia2noa1-2* mutants grown in Hoagland and MS medium. Moreover, we studied NO generation in *Atnoa1* and *Atnia1nia2noa1-2* plants exposed to 25 µM U as it induced NO generation in *Arabidopsis* plant roots (Tewari et al., 2015). Data presented in this manuscript suggest that NOA1/RIF1 and NIA1and NIA2 are not required for NO production in roots of *Arabidopsis thaliana* plants growing in MS medium nor when plants are exposed to U in Hoagland medium.

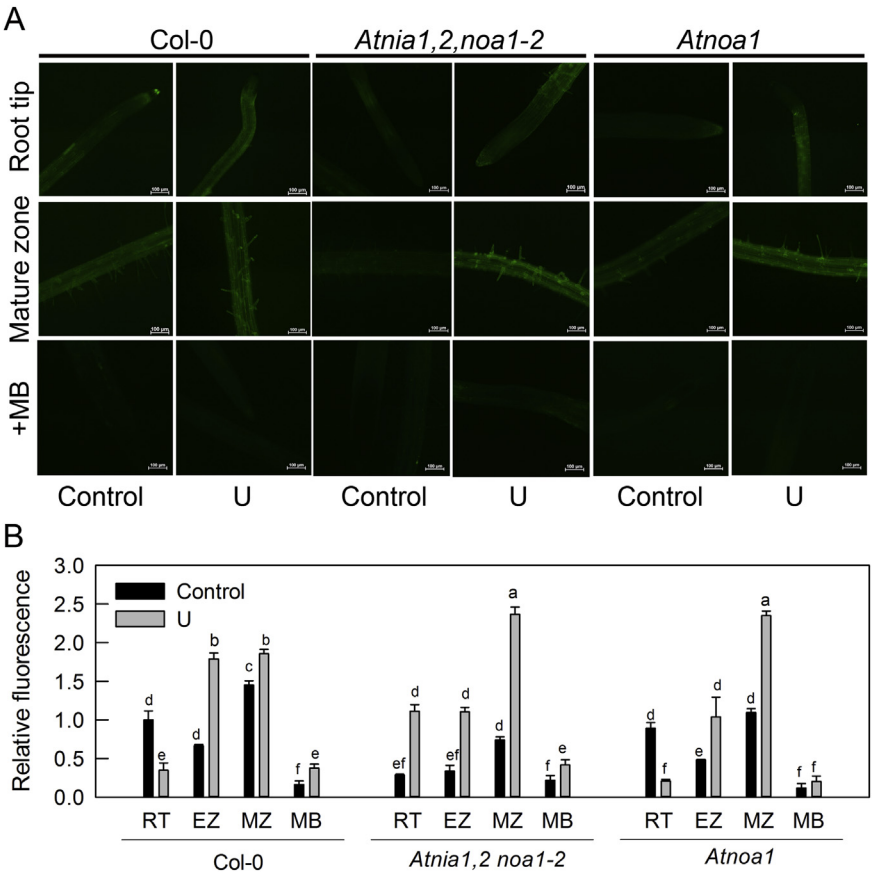

Fig. 2. DAF-2T fluorescence is increased when *Atnia1nia2noa1-2* and *Atnoa1* are grown on Hoaglands medium in the presence of 25 μM U for 7days in VAPs. (A) Fifteen-days-old Col-0 and *Atnia1nia2noa1-2* and *Atnoa1* seedlings grown either with or without 25 μM U on Hoagland medium were pre-incubated for 30 min with (+) or without (−) 200 μM methylene blue (MB), a NO scavenger and roots were stained with the NO-sensitive fluorescent dye, DAF-2DA with (+) or without (−) MB for 1 h. Fluorescence was detected with a FITC filter; excitation, 490 nm; emission, 515 nm. Scale bars: 100 μm. (B) Relative DAF-2T fluorescence in different regions of roots (RT, root tip; EZ, elongation zone; MZ, mature zone) of Col-0, *Atnia1,2noa1-2* and *Atnoa1* plants as described above. Data are mean of five plant roots of three biological replicates (± SE). Bars carrying different letters are significantly different ($p \leq .0.05$) by Fisher-LSD method. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma-Aldrich unless otherwise stated. Depleted U salt, the Uranyl nitrate [$UO_2(NO_3)_2.6H_2O$] was purchased from SPI Chemicals, USA.

2.2. Plant material and growth conditions

Wild-type *Arabidopsis thaliana,* Col-0 was the genetic background of the two mutant plants used in this work. Seeds from the *Atnoa1* mutant were obtained from the Nottingham *Arabidopsis* Stock Centre seed bank (N2356). Seeds form *Atnia1nia2noa1-2* were a kind gift from Dr. José León, (Universidad Politécnica de Valencia). To obtain a sufficient seedstock Col-0 and above mentioned mutants were cultivated in 1/10 Murashige and Skoog (MS) medium on hydroponic culture conditions for one generation.

Two different experimental set-ups were used, a first one in which plants were grown for two weeks on vertically placed agar plates (VAPs) in order to follow root growth and U-induced NO generation and a second hydroponic set up as described by Vanhoudt et al. (2014) for further testing of NO generation. For the agar plates *Arabidopsis thaliana* seeds were stratified and grown on with either modified Hoagland medium (final concentration of salts: 1.0 mM KNO_3, 0.3 mM $Ca(NO_3)_2$, 0.2 mM $MgSO_4$, 0.1 mM $NH_4H_2PO_4$, 1.62 μM $FeSO_4$, 0.78 μM $Na_2EDTA.2H_2O$, 4.6 μM H_3BO_3, 0.9 μM $MnCl_2$, 32 nM $CuSO_4$, 56.6 nM H_2MoO_4, 76.5 nM $ZnSO_4$) or 1/10-strength MS medium (final concentration of salts: 2.061 mM NH_4NO_3, 1.879 mM KNO_3, 0.299 mM $CaCl_2$, 0.15 mM $MgSO_4$, 0.125 mM KH_2PO_4, 9.998 μM $FeSO_4.7H_2O$, 10 μM $Na_2EDTA.2H_2O$, 9.998 μM $MnSO_4.H_2O$, 0.029 μM $ZnSO_4.7H_2O$, 0.01 μM $CuSO_4.7H_2O$, 0.103 μM $Na_2MoO_4.2H_2O$, 10.027 μM H_3BO_3, 0.0105 μM $CoCl_2.6H_2O$, 0.499 μM KI, 2.664 μM Glycine, 0.055 mM myo-Inositol, 0.404 μM Nicotinic acid, 0.243 μM Pyridoxine hydrochloride, 0.033 μM Thiamine hydrochloride) (Murashige and Skoog, 1962) supplemented with 1.0% (w/v) Agar (Agar No 4, Lab M Ltd., UK) at pH 5.5 on agar plates (12 cm × 12 cm × 1 cm). Plates were placed vertically in a growth chamber held at day/night temperature 22/18 °C, light/dark period 14/10 h, humidity 65% and light intensity 150 μmol m^{-2} s^{-1}.

In the second series of experiments *Arabidopsis thaliana* plants were grown in aerated hydroponic culture conditions in modified Hoagland or 1/10 MS media with exactly same nitrate and ammonium concentrations. Hoagland medium was supplemented with 0.38 mM additional KNO_3 and 1.96 mM NH_4NO_3 to maintain similar NH_4^+ and NO_3^- concentrations as described for 1/10 MS medium. At the beginning of the experiment the pH of Hoagland and MS media was adjusted to 5.5 with NaOH, if required. Concentration of other salts in Hoagland and MS media was same as described before. Medium was refreshed twice a week until U contamination. Ambient growth conditions were exactly the same as for the VAPs and as previously described (Tewari et al., 2015). Two different Hoagland and MS growth media were tested here as these two media are commonly used to grow plants for experimental purposes.

2.3. Uranium treatment

Treatment of U induced NO generation in *Arabidopsis* Col-0 seedlings grown on modified Hoagland medium (Tewari et al., 2015). To see whether NO suppressed mutants also produce NO on exposure with U, in first series of experiments, plantlets were initially grown on agar plates containing modified Hoagland medium for 7 days and subsequently these plantlets were transferred on the new plates containing modified Hoagland medium with or without 25 μM filter-sterilized uranyl nitrate (100 mM uranyl nitrate stock dissolved in 0.1 M HCl) for 7 days. The pH of Hoagland medium was adjusted to 5.5 with NaOH, if

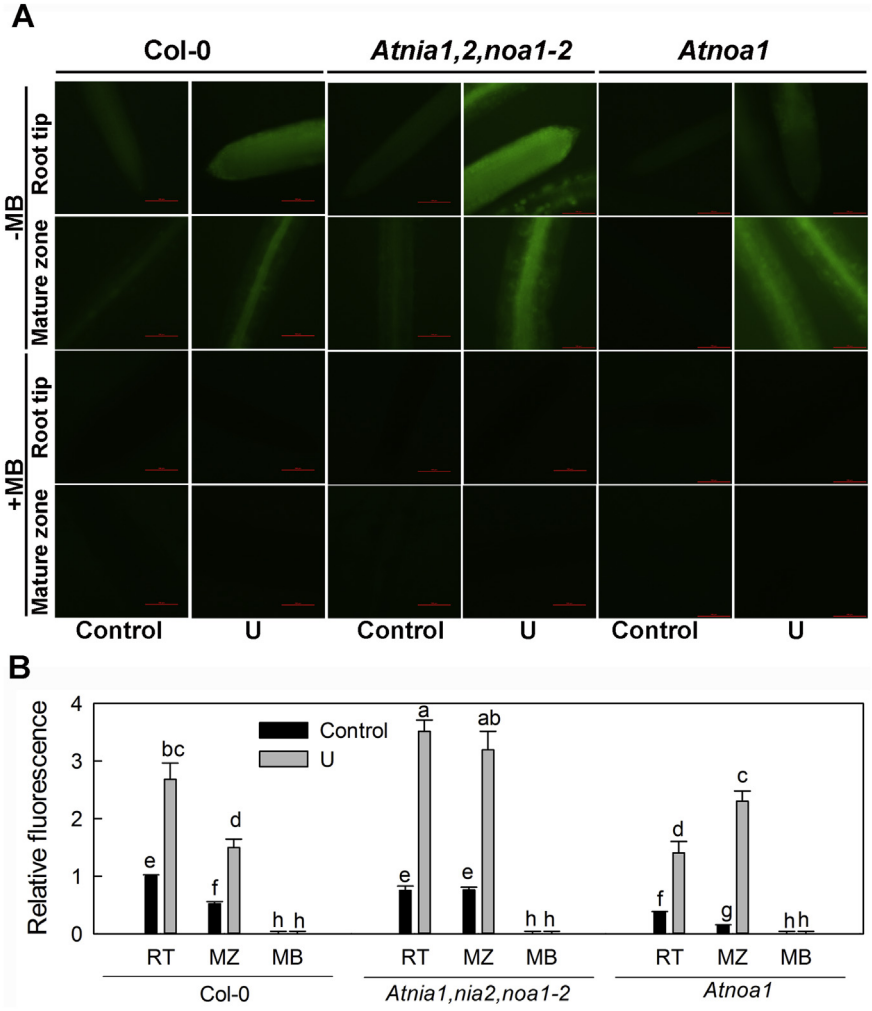

Fig. 3. Fl2E fluorescence is increased when *Atnia1nia2noa1-2* and *Atnoa1* plants are grown on Hoaglands medium and exposed to 25 µM U for 5 days in hydroponic culture. (A) Col-0 (26 days-old) and *Atnia1nia2noa1-2* (33 days-old) and *Atnoa1* (33 days-old) seedlings grown either with or without 25 µM U on Hoagland medium were pre-incubated for 30 min with (+) or without (−) 200 µM methylene blue (MB), a NO scavenger and roots were stained with the NO-sensitive fluorescent dye, Fl2E with (+) or without (−) MB for 1 h. Fluorescence was detected with a FITC filter; excitation, 490 nm; emission, 515 nm. Scale bars: 100 µm. (B) Relative Fl2E fluorescence in different regions of roots (RT, root tip; EZ, elongation zone; MZ, mature zone) of Col-0, *Atnia1,2noa1-2* and *Atnoa1* plants as described above. Data are mean of five plant roots of three biological replicates (± SE). Bars carrying different letters are significantly different ($p \leq .0.05$) by Fisher-LSD method. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

required.

Second series of experiments *Arabidopsis* Col-0 and NO-suppressed mutants (*Atnia1nia2noa1-2* and *Atnoa1*) plantlets were initially grown under hydroponic culture condition for 21 days and 28 days, respectively, in modified Hoagland medium. Due to the very slow growth of the mutants it was chosen to treat them when they reached on average the same number of leaves (i.e. at 28 days) as the wild type plants on 21 days. At 21 or 28 days the plantlets were treated with or without 25 µM filter-sterilized uranyl nitrate (100 mM uranyl nitrate stock dissolved in 0.1 M HCl). The pH of modified Hoagland medium was adjusted to 5.5 with NaOH, if required and plants were exposed for 5 days after which samples were taken for NO localization.

2.4. Chlorophylls and carotenoids

Chlorophylls and carotenoids were extracted from fresh leaves (weighted rosette) by incubation in 1 mL dimethylformamide (DMF) for 24 h at 4 °C under dark conditions. Chlorophyll *a*, chlorophyll *b* and carotenoids concentrations were measured at 664, 647 and 480 nm respectively were calculated according to Wellburn (1994).

2.5. Nitric oxide localization

Seedlings grown on agar plates or under hydroponic culture condition were placed in a loading buffer [5 mM 4-morpholineethanesulfonic acid (MES)-KOH, pH 5.7, 0.25 mM KCl, 1 mM $CaCl_2$] with or without 200 µM methylene blue (MB, a NO scavenger (Vandana et al., 2012)) for 30 min prior to the addition of NO selective dyes, 5 µM diaminofluorescein-2 diacetate (DAF-2DA) or 5 µM Fl2E (Pluth et al., 2011) for 1 h in dark, and then rinsed in loading buffer for 3 times for 5 min each. The fluorescence of distal regions roots was monitored with a fluorescein isothiocyanate (FITC) filter; excitation, 490 nm; emission, 515 nm using Nikon Eclipse Ti fluorescence microscope (Nikon, Japan). Similarly treated unstained roots were used as controls to subtract autofluorescence from all experimental samples. Images were analyzed by selecting region of interest using ImageJ software (http://imagej.nih.gov/ij/) and data were normalized against background.

2.6. Gene expression

After RNA extraction (RNeasy Plant Mini Kit, Qiagen), followed by the determination of the RNA quantity (Nanodrop) and quality (Bioanalyzer, Agilent Technologies), first strand cDNA was prepared using the PrimeScript ™ 1st strand cDNA Synthesis Kit (Takara) and equal amounts of starting material were used (1 µg). Quantitative real time PCR was performed using specific primers of the genes (Supplementary Table 1) with the 7500 Fast Real-Time PCR System (Applied Biosystems) and Sybr Green chemistry. Gene expression data were normalized against multiple housekeeping genes (*YSL8, UBQ10, PPR*) and presented relative value compared to the control plants as described previously (Vanhoudt et al., 2011).

2.7. Statistical analysis

Significant differences between means were determined by ANOVA and subsequent multiple pairwise comparison by 95% Fisher's LSD

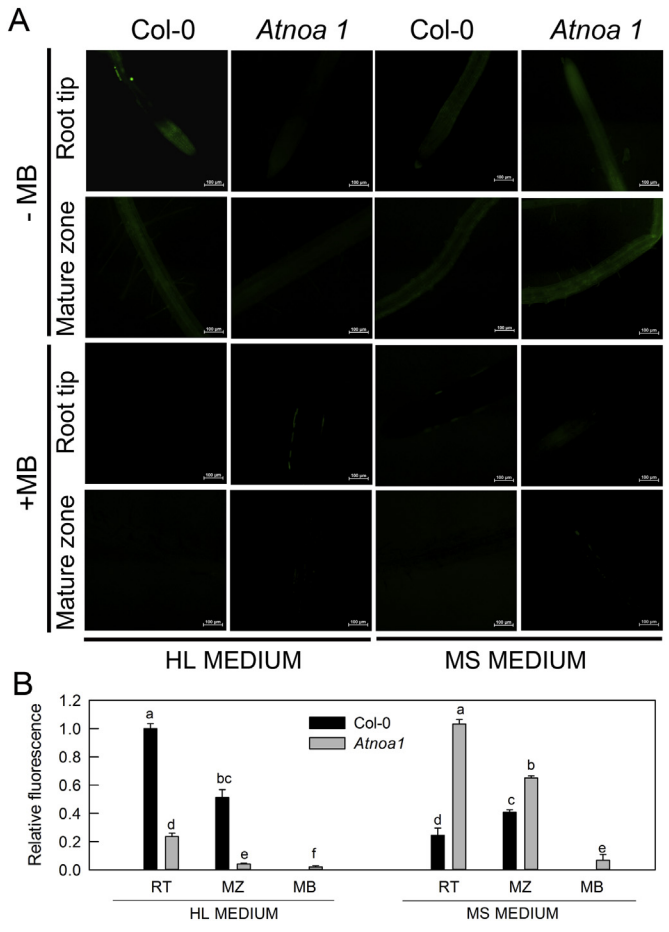

Fig. 4. DAF-2T fluorescence is increased when *Atnoa1* is grown on MS medium in VAPs (A) Fifteen-days-old Col-0 and *Atnoa1* seedlings grown either on Hoagland or MS media were pre-incubated for 30 min with (+) or without (−) 200 μM methylene blue (MB), a NO scavenger and roots were stained with the NO-sensitive fluorescent dye, DAF-2DA with (+) or without (−) MB for 1 h. Fluorescence was detected with a fluorescein isothiocyanate (FITC) filter; excitation, 490 nm; emission, 515 nm. Scale bars: 100 μm. (B) Relative DAF-2T fluorescence in different regions of roots (RT, root tip; MZ, mature zone) of Col-0 and *Atnoa1* plants grown in Hoaglands (HL) or MS medium as described above. Data are mean of five plant roots of three biological replicates (± SE). Bars carrying different letters are significantly different ($p \leq .0.05$) by Fisher-LSD method. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

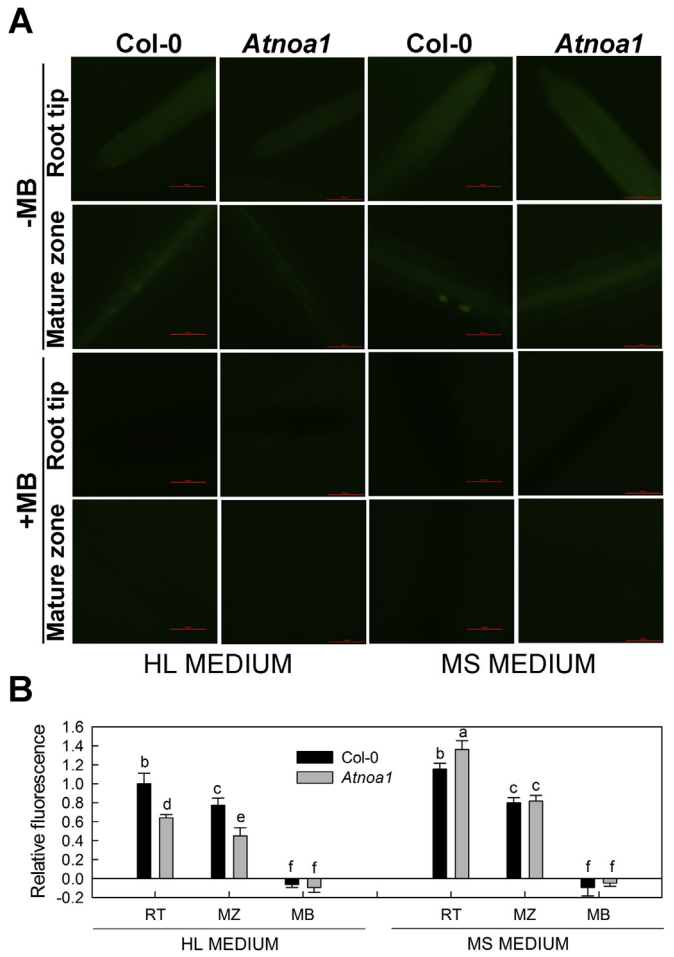

Fig. 5. Fl2E fluorescence is increased when *Atnoa1* is grown on MS medium in hydroponic culture. (A) Col-0 (26 days-old) and *Atnoa1* (33 days-old) seedlings grown either on Hoagland or MS media were pre-incubated for 30 min with (+) or without (−) 200 μM methylene blue (MB), a NO scavenger and roots were stained with the NO-sensitive fluorescent dye, Fl2E with (+) or without (−) MB for 1 h. Fluorescence was detected with a fluorescein isothiocyanate (FITC) filter; excitation, 490 nm; emission, 515 nm. Scale bars: 100 μm. (B) Relative Fl2E fluorescence in different regions of roots (RT, root tip; MZ, mature zone) of Col-0 and *Atnoa1* plants grown in Hoaglands (HL) or MS medium as described above. Data are mean of five plant roots of three biological replicates (± SE). Bars carrying different letters are significantly different ($p \leq .0.05$) by Fisher-LSD method. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

method using Sigma Stat (Systat Software Inc. San Jose, CA).

3. Results

3.1. Plant growth

Root growth, followed on vertical agar plates in seedlings from day 7 till 14 was highest in wild-type Col-0 plants followed by *Atnoa1* and *Atnia1nia2noa1-2* mutants. For all plant types both the main roots and secondary rootlets were more elongated on Hoagland medium than those grown on MS medium (Fig. 1A). Uranium (25 μM) did not show significant inhibition in root growth (Fig. 1A).

3.2. Atnoa1 and Atnia1nia2noa1-2 show greening on MS medium and on exposure with uranium in Hoagland medium

Visible comparison of the plants showed that *Atnoa1* and *Atnia1nia2noa1-2* mutants grown on 1/10 strength MS medium induced greening in leaves compared to those grown in Hoagland medium (Fig. 1B). Moreover, exposing these mutant plants to 25 μM U in modified Hoagland medium also improved greening of leaves (Fig. 1C). Enhanced greening in the leaves of *Atnoa1* and *Atnia1nia2noa1-2* mutants on MS medium (Fig. 1B) and *Atnoa1* on exposure with U was observed by enhanced levels of chlorophyll and carotenoids concentrations (Fig. 1B, D).

3.3. Atnoa1 and Atnia1nia2noa1-2 produced NO generation on exposure of uranium in Hoagland medium

It was shown before that U can induce NO in wild-type *Arabidopsis* plants (Tewari et al., 2015). Here possible NO generation was tested in NO-suppressed mutants (*Atnoa1 and Atnia1nia2noa1-2*) using two NO selective dyes in roots of *Atnoa1* and *Atnia1nia2noa1-2* seedlings grown on VAPs with Hoagland medium and treated with 25 μM U for 7 days. Uranium exposure indeed produced NO in *Atnoa1* and *Atnia1nia2noa1-2* mutants roots to an extent similar to wild-type Col-0 plants grown on VAPs as indicated by enhanced DAF-2T fluorescence (Fig. 2A and B). In

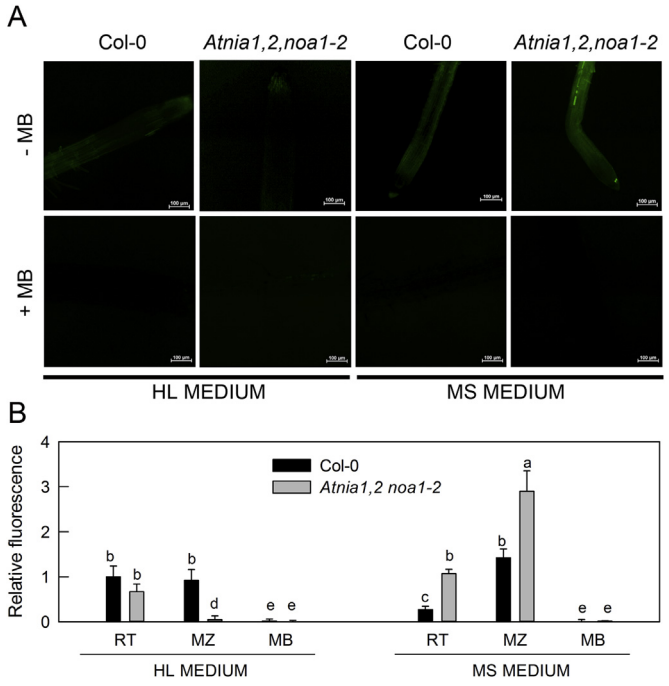

Fig. 6. DAF-2T fluorescence is increased when *Atnia1,2noa1-2* is grown on MS medium in VAPs. (A) Fifteen-days-old Col-0 and *Atnia1,2noa1-2* seedlings grown either on Hoagland or MS media were pre-incubated for 30 min with (+) or without (−) 200 μM methylene blue (MB), a NO scavenger and roots were stained with the NO-sensitive fluorescent dye, DAF-2DA with (+) or without (−) MB for 1 h. Fluorescence was detected with a FITC filter; excitation, 490 nm; emission, 515 nm. Scale bars: 100 μm. (B) Relative DAF-2T fluorescence in different regions of roots (RT, root tip; MZ, mature zone) of Col-0 and *Atnia1,2noa1* plants grown in Hoaglands (HL) or MS medium as described above. Data are mean of five plant roots of three biological replicates (± SE). Bars carrying different letters are significantly different ($p \leq .0.05$) by Fisher-LSD method. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

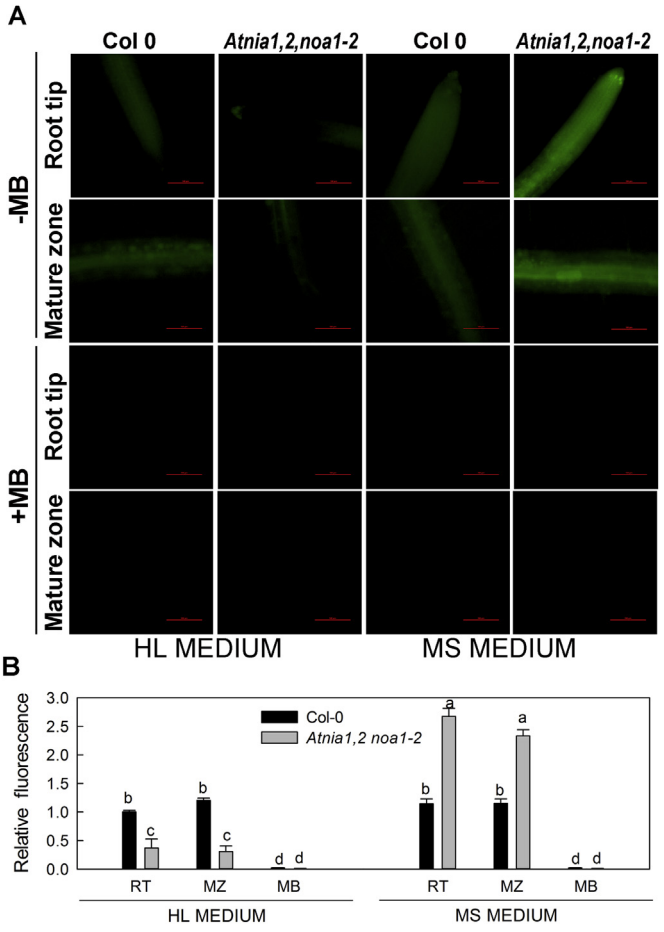

Fig. 7. Fl2E fluorescence is increased when *Atnia1,2noa1-2* is grown on MS medium in hydroponic culture. (A) Col-0 (26 days-old) and *Atnia1,2noa1-2* (33 days-old) seedlings grown either on Hoagland or MS media were pre-incubated for 30 min with (+) or without (−) 200 μM methylene blue (MB), a NO scavenger and roots were stained with the NO-sensitive fluorescent dye, Fl2E with (+) or without (−) MB for 1 h. Fluorescence was detected with a FITC filter; excitation, 490 nm; emission, 515 nm. Scale bars: 100 μm. (B) Relative Fl2E fluorescence in different regions of roots (RT, root tip; MZ, mature zone) of Col-0 and *Atnia1,2noa1* plants grown in Hoaglands (HL) or MS medium as described above. Data are mean of five plant roots of three biological replicates (± SE). Bars carrying different letters are significantly different ($p \leq .0.05$) by Fisher-LSD method. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the presence of 200 μM MB, DAF-2T fluorescence was completely abolished (Fig. 2A and B). Additionally NO generation was detected with an alternative dye, Fl2E, a highly selective dye for NO (Ghosh et al., 2013; McQuade et al., 2010). A similar increase in NO generation in U-treated *Atnoa1 and Atnia1nia2noa1-2* plants (growing under hydroponics culture condition) was observed with Fl2E (Fig. 3A and B).

3.4. NO suppressed mutant (Atnoa1) produces less NO on Hoagland medium but produces more NO on MS medium

DAF-2 DA staining revealed that *Atnoa1* mutants produce a little NO when they were grown on Hoagland medium compared to wild counterpart Col-0 plants (Fig. 4A and B). However, considerable NO-production was found in the *Atnoa1* mutant when it was grown in MS medium as indicated by increased DAF-2T fluorescence (Fig. 4A and B) compared to wild-type Col-0 plants. In the presence of 200 μM MB DAF-2T fluorescence was completely abolished (Fig. 4A and B).

As nitrogen status was slightly different in the two media used in the experimental set up of the agar plates and the role of agar cannot be checked, an alternative experimental set up was used in which plants were grown in hydroponics with an identical nitrogen status both in Hoagland and MS medium. Adjusting the nitrogen status in the hydroponically grown plants did not alter the fact that NO-generation was detected in MS grown mutants and a little in Hoagland grown plants as observed by higher Fl2E fluorescence compared to Col-0 plants (Fig. 5A and B).

3.5. NOA1 and NIA1 and NIA2 do not appear to be involved in NO generation in MS medium

To see whether NR is involved in NO generation in *Atnoa1* in MS medium, experiment with a triple mutant *Atnia1nia2noa1-2*, a mutant with impaired NIA1, NIA2 and NOA1-2 dependent NO biosynthesis, was conducted both on VAPs and in hydroponically grown plants. Similar to *Atnoa1*, this triple mutant, *Atnia1nia2noa1-2*, also produced NO in 1/10 MS medium but not in Hoagland medium as indicated by increase in DAF-2T fluorescence (Fig. 6A and B). DAF-2T fluorescence was suppressed in the presence of 200 μM MB (Fig. 6A and B), Use of Fl2E for NO detection also showed an increase in the level of fluorescence indicating NO generation in triple mutant *Atnia1nia2noa1-2* plants grown under hydroponic culture condition in MS medium with similar nitrogen status as in Hoagland medium (Fig. 7A–B).

3.6. Gene expression

U-treated NO-suppressed mutants, *Atnoa1 and Atnia1nia2noa1*-2,

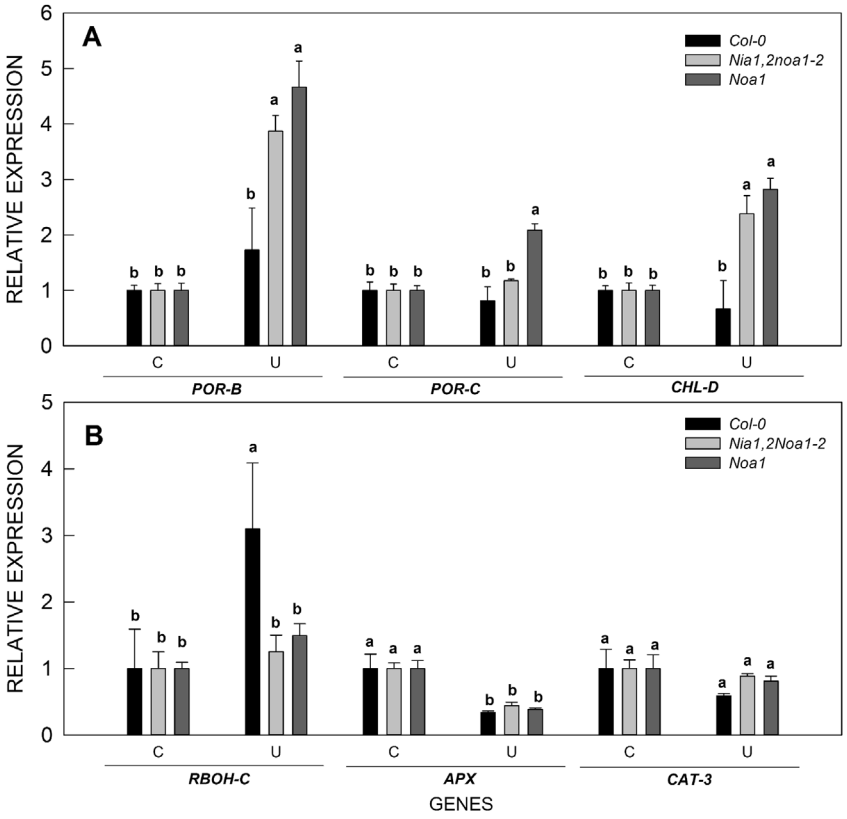

Fig. 8. Relative gene expression levels of the genes involved in chlorophyll biosynthesis (A) and reactive oxygen species (ROS) production and antioxidant defence (B) in Col-0, *Atnia1nia2noa1-2* and *Atnoa1* mutants plants of *Arabidopsis thaliana* after exposure to 25 μM U for 3 days in Hoagland medium under hydroponic culture condition. Data are mean of five plants of three biological replicates (± SE). Bars carrying different letters are significantly different ($p \leq .0.05$) by Fisher-LSD method.

exhibited greening of their pale foliage. To see whether U-induced greening in the leaves of these mutants, is due to activation of chlorophyll biosynthesis related genes, we analyzed few related transcripts. The alleged NO-suppressed mutants indeed activated genes *(POR B, POR C, and CHL D)* related with chlorophyll biosyntheses under U treatment (Fig. 8A). These mutants did not activate or suppress the transcripts involved in oxidative stress (*RBOH C*) and antioxidant defense (*APX* and *CAT3*) under U-treatment. Irrespective of wild type or mutants, U-treated plants suppressed the expression *APX* gene (Fig. 8B).

4. Discussion

The hypothesis was tested here whether NO can be generated in wild type and NO-suppressed mutants depending on the growth medium. Two different media were used for experiments viz. Murashige and Skoog revised medium (Murashige and Skoog, 1962) and Hoagland medium (Kent and Läuchli, 1985). MS medium is a standard nutrient medium utilized for plant, tissue and organ culture and growing plants under axenic culture condition. Hoagland medium (Kent and Läuchli, 1985) is also a frequently used medium for hydroponic culture of plants. In addition U-treated *Arabidopsis* plants also showed NO generation (Tewari et al., 2015). MS medium has been utilized as a plant growth medium to study function of NO or its generations in *Arabidopsis* mutants suppressed in NO biosynthesis (Van Ree et al., 2011; Xie et al., 2013; Van Ree et al., 2011). It has been reported that *Atnoa1* (Van Ree et al., 2011; Xie et al., 2013) and *Atnia1nia2noa1-2* (Lozano-Juste and Leon, 2010, 2011; Xie et al., 2013) plants produced relatively lower levels of NO on MS medium compared to wild-type, Col-0 plants. These studies suggest that NOA1 and/or NIA1 and NIA2 are indirectly and/or directly involved in NO generation. *Atnoa1* and *Atnia1nia2noa1-2* generally exhibit a pale phenotype (Lozano-Juste and Leon, 2010; Van Ree et al., 2011). These NO-suppressed mutants, however, increased chlorophyll and carotenoids in their leaves on 1/10 MS medium suggesting that they somehow overcome the limitation of NO. Indeed, present study revealed an elevated DAF-2T and Fl2E fluorescence, indices of NO generation (Pluth et al., 2011), in *Atnoa1* and *Atnia1-nia2noa1-2* mutants grown on 1/10 strength MS medium. These mutants, however, produced very limited DAF-2T and Fl2E fluorescence on Hoagland medium indicating deficient NO generation. Abolishment of DAF-2T and Fl2E fluorescence in the presence of MB, a NO scavenger (Graziano et al., 2002; Vandana et al., 2012), suggests that these mutants indeed produced NO in 1/10 MS medium without sucrose under our culture conditions. In contrast *Atnoa1* and *Atnia1nia2noa1-2* mutants did not produce NO in modified Hoagland medium. Similarly reduced levels of NO in *Atnoa1* mutant have previously been reported when this mutant was grown in a media similar to Hoagland medium (Bright et al., 2006; Guo and Crawford, 2005; Guo et al., 2003; Zeidler et al., 2004). However, when exposed to U (25 μM) in Hoagland medium, they induced NO generation similar to wild-type, Col-0, plants. Moreover, U-induced DAF-2T and Fl2E fluorescence was completely abolished in the presence of 200 μM MB. MB application also inhibited plant growth very severely which suggests that NO plays a role plant growth and development. NO generation has already been reported in 25 μM U-exposed *A. thaliana*, Col-0, plants (Tewari et al., 2015). However, role of NOA1 in NO production is still a matter of debate because various conflicting reports are available indicating that sometimes NO can accumulate in *Atnoa1* mutant under various treatment conditions such as ABA (Bright et al., 2006; Kolbert et al., 2008; Lozano-Juste and Leon, 2010), cold stress (Zhao et al., 2009), salicylic acid (Zottini et al., 2007), sucrose (Van Ree et al., 2011), heat stress (Xuan et al., 2010), zeatin (Tun et al., 2008) and cadmium exposure (Han et al., 2014). Double mutant *Atnia1nia2* also produces NO on cadmium exposure (Han et al., 2014). Probable activation of NOS-like activity in NO generation in aforesaid alleged NO-suppressed mutants grown in 1/10 MS medium or Hoagland medium with 25 μM U, cannot be eliminated as NOA1 is not a real NOS.

It has been reported that *Atnia1nia2noa1-2* mutant did not produce NO in basal MS medium with 1% (w/v) sucrose (Lozano-Juste and Leon, 2010; Xie et al., 2013). However, dark-grown etiolated *Atnia1-nia2noa1-2* mutants induced NO comparable to wild-type, Col-0, plants

in their hypocotyl (Lozano-Juste and Leon, 2011). Similar to *Atnoa1*, *Atnia1nia2noa1-2* mutants also produced NO when they were grown on 1/10 strength MS medium without sucrose or on Hoagland medium containing U. Difference in MS salt (particularly of NH_4^+ and NO_3^-) strength compared to Hoagland (first set of experiments on agar plates) did not appear to be responsible for the observed enhanced NO generation as similar NO_3^- and NH_4^+ in Hoagland medium (second set of experiments on hydroponics) did not produce NO in *Atnoa1* and *Atnia1nia2noa1-2* mutants plants. These observations suggest that neither NIA1 and NIA2 nor NOA1 are involved in NO generation in the roots of *Arabidopsis* growing in MS medium and Hoagland medium containing U. The presence of glycine, myoinositol and vitamins (nicotinic acid, pyridoxine and thiamine) in MS medium may somehow affect NO generation in *Atnoa1* and *Atnia1nia2noa1-2* mutants. *Atnoa1* and *Atnia1nia2noa1-2* mutants probably used glycine as a preferential nitrogen source for metabolic function and available NO_3^- and NH_4^+ might have reduced nitrite to NO in MS medium. NO concomitantly enhanced greening of leaves of these mutants. Moreover, an enhanced greening of leaves of U-treated *Atnoa1* and *Atnia1nia2noa1-2* mutants along with enhanced expression of chlorophyll biosynthesis related genes (*POR B*, *POR C* and *CHL D*) indeed suggest an involvement U-induced NO generation in the greening of leaves of aforesaid mutants. It would be interesting to study NO-mediated enhanced nutrient utilization capacity by enhancing chlorophyll biosynthesis in our future studies.

Although NO is diffusible signalling molecule but at the same time it is a free radical (Lancaster, 2015). In present study, we assumed root as primary site of NO generation which is in direct contact of growth medium. However, it is not escape of our notice that NO is also produced in the leaves, particulary, in the chloroplasts (Tewari et al., 2013) and peroxisomes (Corpas et al., 2004). The specificity of DAFs reaction with NO has recently been questioned as DAF-reactive compounds were prevalently identified as reaction products of H_2O_2 plus apoplastic peroxidase and only minor peak was detected for DAF-2T, a reaction product of DAFs with NO (Rumer et al., 2012). On the other hand, O-diamine moiety of DAF-2DA has been shown to be more selective for NO over NO_3^-, $ONOO^-$, H_2O_2, and O_2^- under aerobic conditions and maintains a 5 nM NO detection limit (Pluth et al., 2011). The $ONOO^-$ produced by NO and O_2^- also increased yield of DAF-2T and thus it reflect synthesis of NO. However, excess of O_2^- inhibit DAF-2T formation (Jourd'Heuil, 2002). Measurement of NO by DAF dye may also be perturbed by the presence of dehydroascorbic acid or ascorbic acid (Zhang et al., 2002), glutathione and homocysteine (Pluth et al., 2011). Despite these drawbacks, DAFs dyes are still in frequent use for visualizing NO in biological systems (Corpas and Barroso, 2014; Diao et al., 2017; Han et al., 2014; Hu et al., 2014; Li et al., 2013; Wei et al., 2014) due to ease of its application, selectivity for NO, and detection with generally available instrumentation. Therefore. We confirmed our data with an alternative dye, Fl2E, which has been used for NO detection in biological system and is said to be more selective for NO (McQuade et al., 2010). Results obtained from both of these dyes *i.e.* DAF-2DA and Fl2E are similar and suggest NO generation by *Atnoa1* and *Atnia1nia2noa1-2* mutants under 1/10 MS medium and on exposure with U in Hoagland medium.

In conclusion, mutants *Atnoa1* and *Atnia1nia2noa1-2* are shown here to be conditional NO producers and it appears that NO generation in plants substantially depends on growth medium. Although, present study shows NO generation in alleged NO-suppressed *Arabidopsis* mutants, elucidation of the biosynthetic route for NO generation in *Atnoa1* and *Atnia1nia2noa1-2* needs further investigation. In our future studies, we will try to identify chemical factors (glycine or vitamins) present in MS medium and an involvement of NO synthase-like activity in NO production. Since various media are often being used in plant-NO research, it is recommended to determine NO for each treatment and experimental condition and take utmost care in interpretation of results.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.04.042.

Author contributions

RKT and NH conceived the idea and designed the research. RKT, RN and JW conducted experiments. RKT, RN: Formal analysis, and, MVH: Formal analysis, analyzed the data. RKT: Writing – original draft, and, NH: Writing – original draft, wrote the manuscript. HV helped in preparation and improvement of text. All authors read and approved the manuscript.

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