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Expression of the tetrahydrofolate-dependent nitric oxide synthase from the green alga *Ostreococcus tauri* increases tolerance to abiotic stresses and influences stomatal development in Arabidopsis

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SUMMARY

Nitric oxide (NO) is a signaling molecule with diverse biological functions in plants. NO plays a crucial role in growth and development, from germination to senescence, and is also involved in plant responses to biotic and abiotic stresses. In animals, NO is synthesized by well-described nitric oxide synthase (NOS) enzymes. NOS activity has also been detected in higher plants, but no gene encoding an NOS protein, or the enzymes required for synthesis of tetrahydrobiopterin, an essential cofactor of mammalian NOS activity, have been identified so far. Recently, an NOS gene from the unicellular marine alga *Ostreococcus tauri* (*OtNOS*) has been discovered and characterized. *Arabidopsis thaliana* plants were transformed with *OtNOS* under the control of the inducible short promoter fragment (*SPF*) of the sunflower (*Helianthus annuus*) *Hahb-4* gene, which responds to abiotic stresses and abscisic acid. Transgenic plants expressing *OtNOS* accumulated higher NO concentrations compared with siblings transformed with the empty vector, and displayed enhanced salt, drought and oxidative stress tolerance. Moreover, transgenic *OtNOS* lines exhibited increased stomatal development compared with plants transformed with the empty vector. Both *in vitro* and *in vivo* experiments indicate that OtNOS, unlike mammalian NOS, efficiently uses tetrahydrofolate as a cofactor in Arabidopsis plants. The modulation of NO production to alleviate abiotic stress disturbances in higher plants highlights the potential of genetic manipulation to influence NO metabolism as a tool to improve plant fitness under adverse growth conditions.

Keywords: nitric oxide, nitric oxide synthase, tetrahydrofolate, *Arabidopsis thaliana*, oxidative stress, stomatal index, abiotic stress.

INTRODUCTION

Since the turn of the century, plant biologists have reported many original findings indicating that nitric oxide (NO) is a central signal molecule with multiple biological functions throughout plant life (Lamattina *et al.*, 2003; Wendehenne *et al.*, 2004; Delledonne, 2005; Besson-Bard *et al.*, 2008; Tun *et al.*, 2008; Wilson *et al.*, 2008). NO promotes seed germination (Beligni and Lamattina, 2000), acts as a non-traditional regulator influencing plant growth and development (Beligni and Lamattina, 2001; He *et al.*, 2004), modulates plant maturation and senescence (Leshem *et al.*, 1998), and regulates abscisic acid (ABA)-induced stomatal closure (García-Mata and Lamattina, 2001; Desikan *et al.*, 2004). NO also mediates key signaling pathways involved in responses to abiotic stresses, such as cold, drought, salt and heat, as well as in disease resistance and apoptotic processes (Delledonne *et al.*, 1998; García-Mata and Lamattina, 2002; Zhao *et al.*, 2007, 2009; Tada *et al.*, 2008). With a few exceptions (see below), most approaches

designed to elucidate NO roles in plant physiology are based on plant infiltration with mammalian enzymes synthesizing NO (Durner *et al.*, 1998), or exogenous application of NO donors and/or scavengers (Lamattina *et al.*, 2003; Besson-Bard *et al.*, 2008).

In animals, NO is produced by the enzyme nitric oxide synthase (NOS; EC 1.14.13.39). NOS catalyzes the formation of NO and citrulline from L-arginine (L-Arg) in a reaction that uses NADPH as an electron donor and O_2 as a co-substrate. The enzyme also exhibits a strict requirement for tetrahydrobiopterin (H_4B), a redox cofactor that is typical of hydroxylases (Werner *et al.*, 2011). The precise role of H_4B in NOS catalytic activity is still not completely understood. It has been postulated that involvement of H_4B determines that the electron flow within the enzyme is directed to L-Arg. The rate of this electron transfer step is important for coupling L-Arg hydroxylation with formation of a heme $Fe^{2+}{\cdot}O_2$ intermediate within the enzyme. Indeed, several biochemical studies have demonstrated that activation of mammalian NOS in the presence of sub-optimal concentrations of H_4B results in uncoupling of oxygen reduction from L-Arg oxidation, thereby generating superoxide anions (Wei *et al.*, 2002). Three NOS isoforms have been described in animals: the constitutive neuronal (nNOS) and endothelial NOS (eNOS), and the inducible NOS (iNOS) (Alderton *et al.*, 2001).

In plants, a series of reductive pathways for NO generation have been postulated (Gupta *et al.*, 2011), including a plasma membrane-bound nitrite:NO reductase (Stöhr *et al.*, 2001) and a peroxisomal xanthine oxidoreductase that reduces nitrite to NO at the expense of NADPH under anaerobic conditions (Corpas *et al.*, 2008). The cytosolic nitrate reductase is considered one of the main sources of NO in plants under aerobic conditions, and has been implicated in NO production during bacterial-induced defense responses (Modolo *et al.*, 2005), disease development in certain plant–pathogen interactions (Shi and Li, 2008), drought stress responses (Freschi *et al.*, 2010), cold acclimation (Zhao *et al.*, 2009), regulation of stomatal aperture (Srivastava *et al.*, 2009), alleviation of iron deficiency (Graziano and Lamattina, 2007), and various developmental processes such as flowering initiation (Seligman *et al.*, 2008) and root growth (Stöhr and Stremlau, 2006). No protein, cDNA or gene with homology to mammalian NOS has been described in higher plants, and the enzymes required for synthesis of H_4B appear to be absent from both plants and algae (Werner-Felmayer *et al.*, 2002; Correa-Aragunde *et al.*, 2013). Despite this unfavorable prognosis, several lines of evidence support the presence of an arginine-dependent NO production pathway in plants (Cueto *et al.*, 1996; Corpas *et al.*, 2009; Talwar *et al.*, 2012).

Two sequences found in the genomes of the unicellular marine algae *Ostreococcus tauri* and *Ostreococcus lucimarinus*, belonging to the plant kingdom, showed homology with canonical NOS enzymes (Derelle *et al.*, 2006). The amino acid sequence of *O. tauri* NOS (OtNOS, EMBL accession number CAID01000017.1) was found to display 34.3, 41.6 and 42.7% similarity with human nNOS, iNOS and eNOS, respectively (Foresi *et al.*, 2010). Sequence-based methods of fold assignment showed that OtNOS folds similarly to the human eNOS isoform (Foresi *et al.*, 2010). Enzymatic characterization of OtNOS confirmed that it displays Arg-dependent NO production activity, with a K_M of 12 μM for L-Arg (Foresi *et al.*, 2010), which is close to the estimated K_M of previously described NOS isoforms (1–22 μM; Bredt and Snyder, 1990; Stuehr *et al.*, 1991; Roman *et al.*, 1995; Gerber *et al.*, 1997). In *O. tauri*, *OtNOS* expression and NO production are increased under high light intensity (Foresi *et al.*, 2010).

As stated above, several lines of evidence have shown increased NO production during (a)biotic stresses and NO involvement in plant responses to such stress. Under certain stress conditions, exogenous NO donors alleviate the symptoms and deleterious effects of the stress condition (García-Mata and Lamattina, 2001; Bai *et al.*, 2011). Moreover, Arabidopsis and *Nicotiana tabacum* L. (tobacco) plants expressing a *Rattus norvegicus* L. (Rat) nNOS under the control of a constitutive promoter displayed increased tolerance to a range of biotic and abiotic stresses, although some contradictory results were also observed (Chun *et al.*, 2012; Shi *et al.*, 2012). Thus, based on knowledge obtained through molecular characterization of OtNOS (Foresi *et al.*, 2010), we hypothesize that regulated expression of the *OtNOS* gene in higher plants may help the host organism to cope with adverse environmental conditions.

To test our proposal, we generated transgenic *Arabidopsis thaliana* plants expressing the *OtNOS* gene under the control of a stress-inducible promoter, and analyzed their abilities to grow, respond and adapt to adverse environmental situations. Transgenic lines expressing *OtNOS* displayed increased basal and/or inducible NO concentrations compared with siblings transformed with the empty vector (EV). These plants exhibited improved abiotic stress tolerance and showed higher growth rates and survival under severe stress conditions. Here we show that *A. thaliana* expressing the *OtNOS* gene has a higher stomatal index, and hence increased stomatal development. The results also indicate that OtNOS uses tetrahydrofolate (THF) instead of H_4B as the essential cofactor for NOS activity *in planta*. These findings extend our knowledge of NO synthesis and functions in plants. In addition, *OtNOS*-expressing lines constitute a valuable tool to investigate fundamental aspects of stress physiology and the role played by NO in stress tolerance.

RESULTS

Generation of transgenic Arabidopsis plants expressing *OtNOS*

Transgenic Arabidopsis lines expressing *OtNOS* were obtained by transforming Col-0 *rdr6* plants with a full-length cDNA encoding OtNOS under the control of SPF_{pro}, an ABA-, drought- and NaCl-responsive promoter of the sunflower HD-Zip gene *Hahb-4* (Gago *et al.*, 2002; Dezar *et al.*, 2005) (Figure 1a). Under standard growth conditions for Arabidopsis, adult plants transformed with SPF_{pro}*:OtNOS* were phenotypically similar to those transformed with the empty vector (pCHF3) (Figure 1b).

To estimate the level of *OtNOS* expression, 5-day-old plants transformed with SPF_{pro}*:OtNOS* or EV were treated with 10 μM ABA or 100 mM NaCl for a further 5 days, and total RNA was extracted for analysis by reverse transcriptase (RT)-PCR and quantitative RT-PCR. Figure 1(c,d) shows that ABA treatment resulted in significant induction of *OtNOS* in lines *OtNOS4*, *6* and *7*, which represent three independent transformation events. *OtNOS* expression was also enhanced by NaCl treatment in four transgenic lines (*OtNOS3*, *4*, *6* and *7*), but line 2 failed to respond during the analyzed time frame (Figure S1a). Induction of the transgene by NaCl was further confirmed for lines *OtNOS4* and *7* by quantitative PCR (Figure S1b). Low levels of *OtNOS* expression were observed in the transformed lines in the absence of ABA or NaCl (Figures 1c and S1a), in agreement with previous reports on the basal activity of SPF_{pro} (Dezar *et al.*, 2005). Figure S2 shows a Western blot analysis of OtNOS expression in transgenic lines *OtNOS4*, *6* and *7* after induction by ABA. A protein of the expected molecular mass that immunoreacted with the specific antibody against OtNOS was observed in leaf extracts of ABA-induced transgenic lines but not in extracts from siblings that were not exposed to the hormone (Figure S2).

Expression of *OtNOS* in Arabidopsis results in increased NO concentration, and tetrahydrofolate is an efficient cofactor of OtNOS

The production of NO in transgenic lines expressing *OtNOS* was evaluated by two methods: (i) detection with the NO-sensitive fluorescent probe 4-aminomethyl-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM-DA), and (ii) by measuring nitrite concentrations using Griess reagent. Figure 2(a,b) shows the effect of NaCl on the NO content in leaves and roots of *OtNOS* and EV plants. Salt treatment led to a general increase in NO concentration throughout roots and leaves from lines *OtNOS6* and *7*. Microscopic observations showed strong punctate fluorescence in mesophyll cells and stomata of these plants after NaCl induction (Figure 2a).

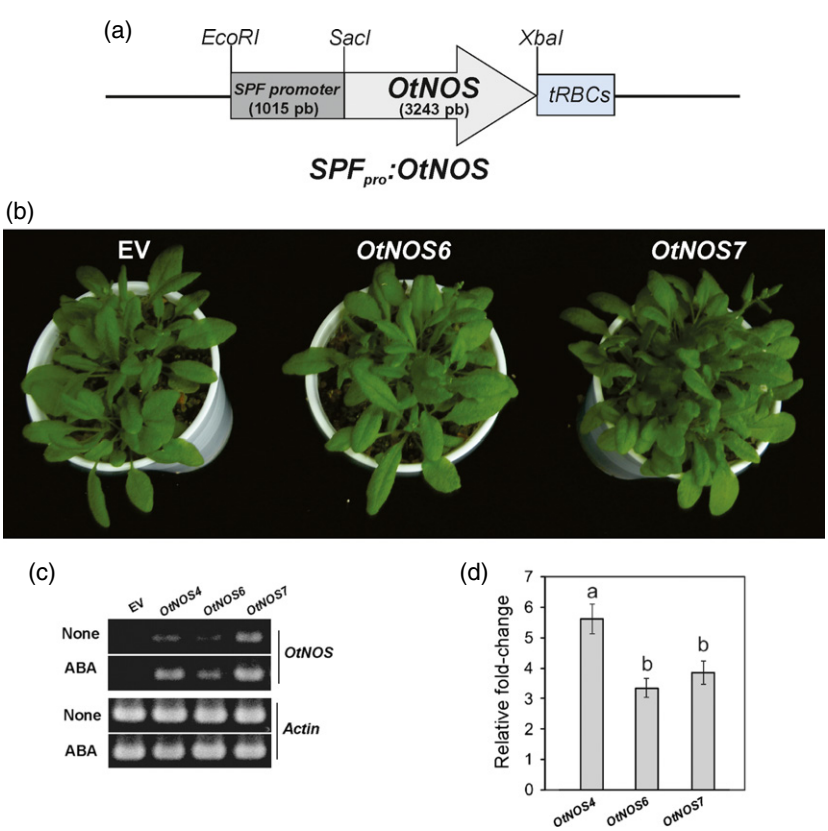

Figure 1. Preparation and characterization of Arabidopsis plants expressing the nitric oxide synthase gene of *O. tauri* (*OtNOS*).
(a) Diagram illustrating the construct used to generate transgenic plants expressing the *OtNOS* gene. A full-length cDNA encoding *OtNOS* was cloned between the SPF_{pro} promoter of the *Hahb-4* gene from *H. annuus* and the pea Rubisco small subunit terminator (tRBCs).
(b) Control plants transformed with the empty vector (EV) and transgenic lines *OtNOS6* and *7* were grown in soil under 16 h light/8 h dark cycles and photographed after 36 days of growth.
(c) RT-PCR analysis of *OtNOS* expression in selected transgenic lines. Five-day-old Arabidopsis seedlings grown on half-strength MS medium were treated or not ('none') with 10 μM ABA for a further 5 days. Actin cDNA was used as a reference for mRNA transcription. Other experimental conditions are described in Experimental Procedures.
(d) Induction of the *OtNOS* gene by ABA was confirmed in lines *4*, *6* and *7* by quantitative RT-PCR. Values are fold changes (means ± SE) relative to non-induced conditions ($n = 3$). In this case, the *PP2A* cDNA was used as a reference for RNA transcription (see Experimental Procedures). Bars marked with different letters indicate values that are statistically significantly different (ANOVA, $P < 0.05$).

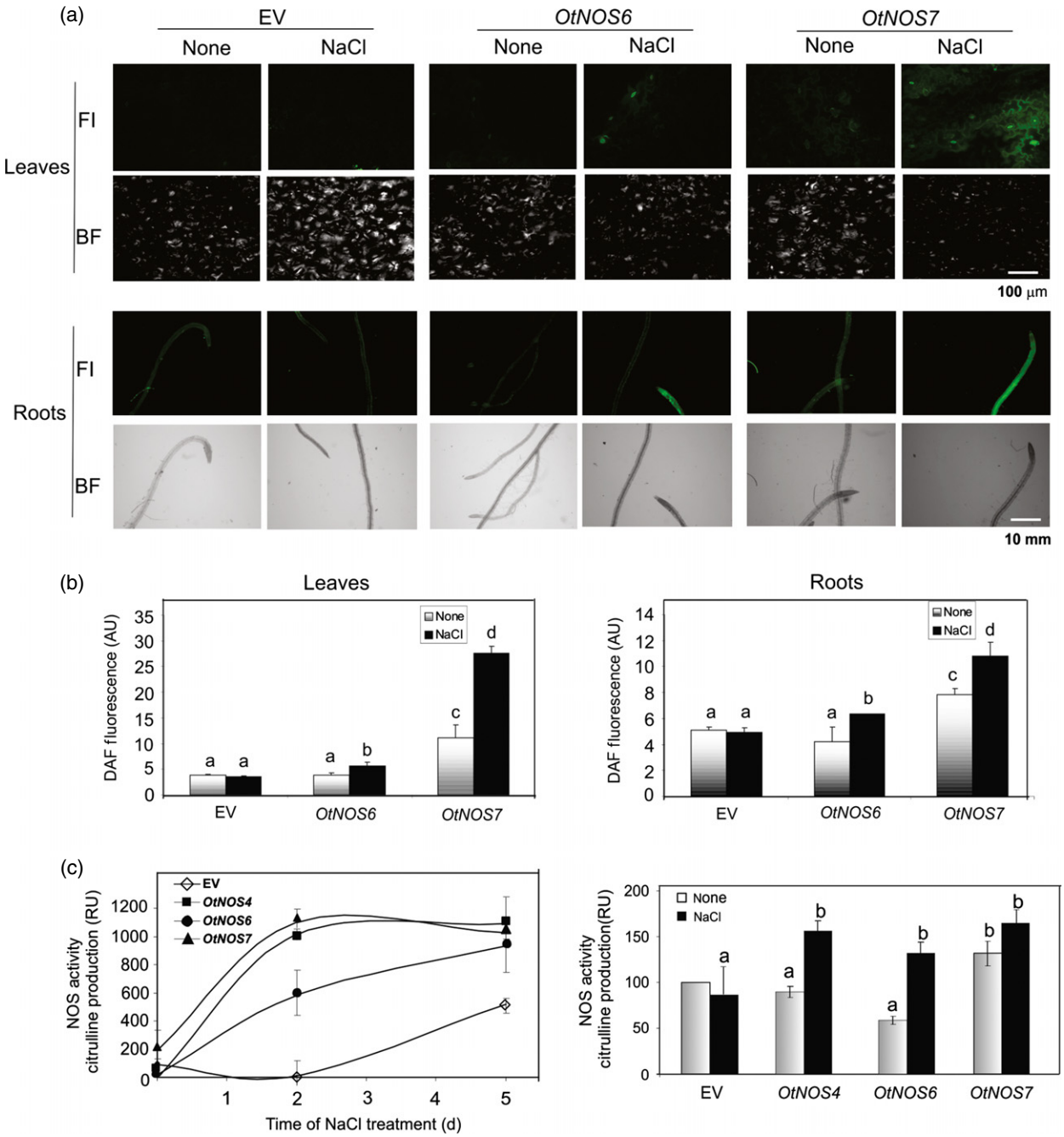

Figure 2. Nitric oxide detection and NOS activity in Arabidopsis leaves and roots of *OtNOS* transgenic lines.
(a) NO contents were determined using DAF-FM-DA and detected by fluorescence microscopy in roots, and confocal laser scanning microscopy in leaves. Five-day-old Arabidopsis seedlings were grown in half-strength MS medium and treated or not ('none') with 100 mM NaCl for a further 5 days (see Experimental Procedures). FI, fluorescence; BF, bright field.
(b) Quantification of DAF-FM-DA fluorescence using ImageJ software. Values are means ± SE (n = 20). Bars marked with different letters indicate values that are statistically significantly different (ANOVA, $P < 0.05$). AU, arbitrary units.
(c) NOS activity was assayed by the citrulline method (Bredt and Snyder, 1990). Five-day-old Arabidopsis seedlings were treated with 100 mM NaCl, and NOS activity was measured in cleared extracts in a time-course experiment (left panel). The initial NOS activity in the EV seedlings was 2.23 pmol mg^{-1} protein min^{-1}, and this was taken as 100 RU (relative units). The right panel shows NOS activity at 5 days with 100 mM NaCl or without ('none'). The NOS activity for the EV seedlings after 5 days in the absence of NaCl was 5.38 pmol mg^{-1} protein min^{-1}, and this was taken as 100 RU (relative units).

Similar results were obtained when NOS activity was measured in leaf extracts by the citrulline method (Figure 2c). Basal NOS activities were 2.23 and 5.37 pmol mg^{-1} protein min^{-1} in cleared extracts of 5- and 10-day-old EV seedlings grown in half-strength MS medium, in the same range as that reported by Zhao *et al.*

(2007) for Arabidopsis leaves analyzed by the same method (4.5 pmol mg^{-1} protein min^{-1}). Both the NO concentration and NOS activities were higher in tissues of the *OtNOS7* plants compared to the other transgenic lines (Figure 2b,c).

Nitrite quantification was used as a complementary approach to estimate NO production. Even though nitrite and nitrate have been shown to be rather reactive species that undergo a series of reactions at acidic pH and in the presence of reductants, nitrite determination using the Griess reaction has been used as an indirect and valuable form of NO determination (Dirsch *et al.*, 1998; Bethke *et al.*, 2004). Table S1 shows that nitrite increased in *OtNOS4* and *6* transgenic lines after 5 days of NaCl treatment. *OtNOS7* plants exhibited increased nitrite contents compared to EV and *OtNOS4* and *6* lines, even in the absence of NaCl.

As mentioned above, whether H_4B is present in plants is controversial (Werner-Felmayer *et al.*, 2002). It has been proposed that H_4B may be replaced in plants by the analogous pterin THF, whose biosynthesis and distribution are well known in plants (Sahr *et al.*, 2005; Corpas *et al.*, 2009). *O. tauri* contains enzymes for THF synthesis (http://www.genome.jp/kegg-bin/show_pathway?ota00790), which may explain why OtNOS is active in this alga (Foresi *et al.*, 2010). Figure 3a shows that recombinant OtNOS expressed in *Escherichia coli* was active with equivalent efficiency in the presence of either THF or H_4B, whereas mammalian iNOS was largely specific for H_4B. Commercial iNOS shows some basal activity without addition of H_4B, presumably due to the presence of the cofactor in the commercialized enzyme, as H_4B is routinely added during purification of the recombinant protein to stabilize it (Figure 3a). Commercial iNOS shows some basal activity without addition of H_4B, presumably due to the presence of the cofactor in the commercialized enzyme, as H_4B is routinely added during purification of the recombinant protein to stabilize it.

Figure 3(b) shows that the roots of transgenic lines expressing *OtNOS* exhibited an increased NO concentration when THF was added to the medium. This result indicates that OtNOS uses the THF cofactor *in vivo*, and that manipulation of folic acid metabolism may represent a tool to increase NO production in higher plants.

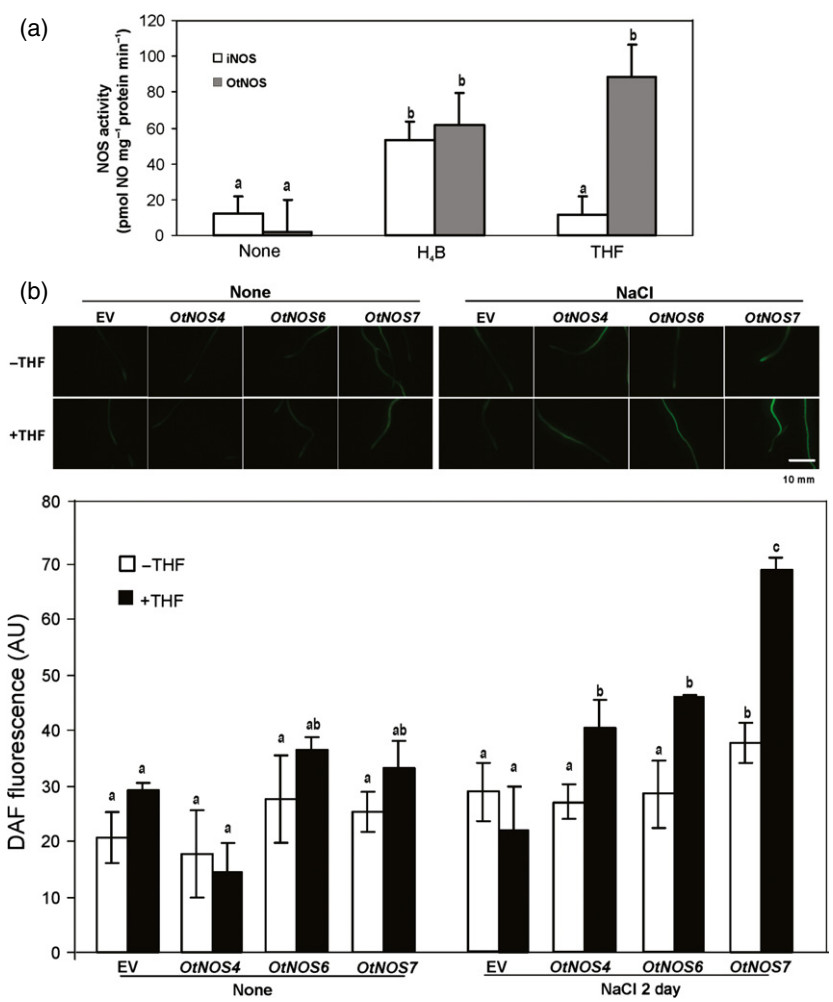

Figure 3. NOS activity of recombinant OtNOS, and NO detection in transgenic *OtNOS* plants assayed in the presence of THF.
(a) NOS activity was determined by following oxyhemoglobin oxidation as described in Experimental Procedures.
(b) NO contents were determined using DAF-FM-DA and detected by fluorescence microscopy in roots. Five-day-old Arabidopsis seedlings were grown in half-strength MS medium and treated with 100 mM NaCl or not ('none') for a further 2 days. Seedlings were incubated with 100 μM THF for 3 h. Quantification of DAF-FM-DA fluorescence was performed using ImageJ software. Values are means ± SE ($n = 10$). Bars marked with different letters indicate values that are statistically significantly different (ANOVA, $P < 0.05$).

Transgenic plants expressing *OtNOS* display enhanced germination rates

Multiple processes are involved in the induction of seed dormancy, as well as in the switch from the dormant to the germinating state (Bentsink and Koornneef, 2008). In addition, several nitrogen-containing compounds have been identified as important inducers of germination, including NO, nitrite and nitrate (Bethke *et al.*, 2006). It has been suggested that these nitrogen-containing compounds affect germination via conversion into NO (Bethke *et al.*, 2007). As *OtNOS*-expressing plants produce higher levels of NO compared to EV siblings, we investigated whether their seeds displayed higher rates of germination. Cold stratification of seeds prior to sowing was omitted, seeds were imbibed on 0.6% w/v agarose (Bethke *et al.*, 2006), and the time course of germination was determined. Figure 4 shows that seeds from transgenic lines *OtNOS6* and *7* exhibited higher germination rates and reached 50% germination at approximately 30 h after imbibition compared with 50 h for EV plants (inset, Figure 4). These results indicate that the OtNOS gene is expressed at an early stage in development of the plants due to basal activity of the SPF_{pro} promoter (see below).

OtNOS expression improves shoot and root development under NaCl stress and ABA treatments

Salinity is a major factor that inhibits plant growth via a combination of ionic toxicity, osmotic stress, nutritional disorder and oxidative damage (Abogadallah, 2010). Plant responses to salt stress are controlled at the molecular level by multiple extracellular and intracellular signals. There is increasing evidence that NO occupies a central position in the signaling network that is necessary to attenuate the undesirable consequences of salinity, possibly acting in conjunction with other signals (Molassiotis *et al.*, 2010).

Plants expressing *OtNOS* were exposed to NaCl or ABA, and their responses were analyzed in roots and aerial parts. In addition to driving *OtNOS* expression, the two treat-

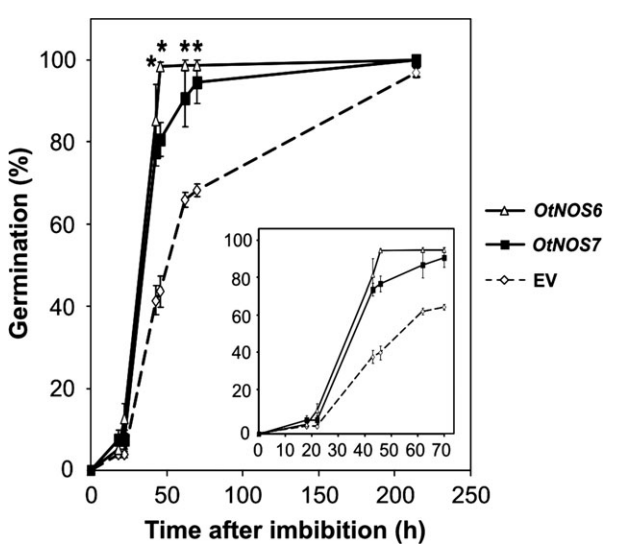

Figure 4. Seeds from Arabidopsis *OtNOS* transgenic lines show increased germination rates.
Time courses of germination for seeds from *OtNOS* and EV plants imbibed in 0.6% agarose without prior cold stratification. The inset shows percentages of germination during the first 70 h. Values are means ± SE of 3–4 experiments. Asterisks indicate statistically significant differences between *OtNOS* and EV plants by ANOVA ($P < 0.05$).

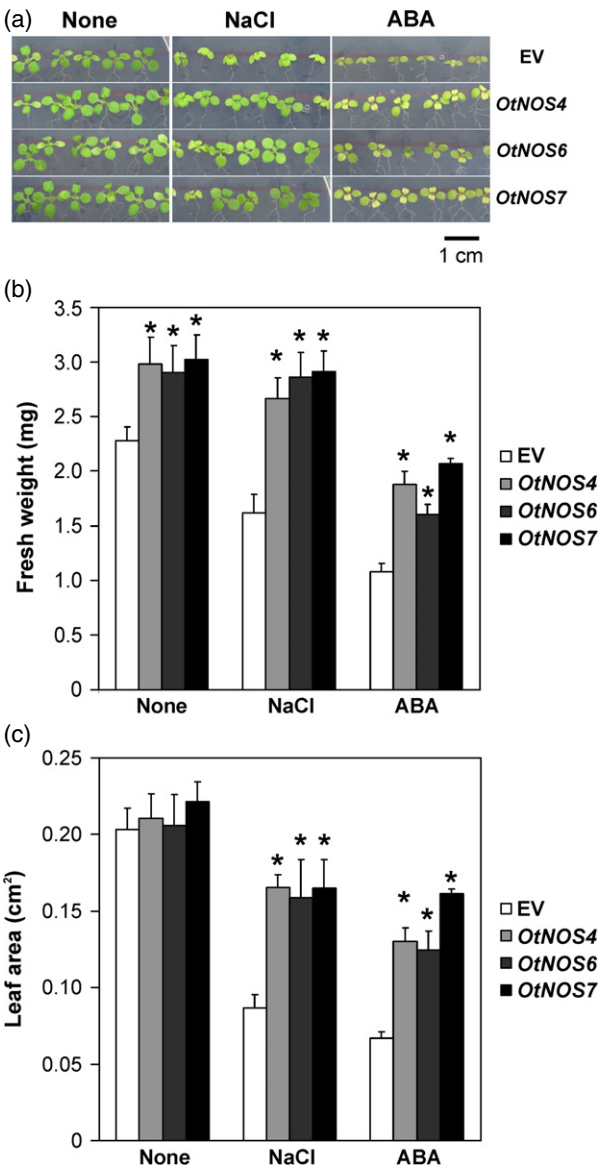

Figure 5. Arabidopsis lines expressing *OtNOS* have a higher fresh weight and leaf area after NaCl and ABA treatments.
(a) Phenotypes of aerial parts of 10-day-old seedlings after 5 days of growth in half-strength MS medium supplemented with 100 mM NaCl or 10 μM ABA or not ('none').
(b, c) Fresh weight (b) and leaf area (c) were determined after these treatments. Values are means ± SE of 8–10 experiments. Asterisks indicate statistically significant differences between *OtNOS* and EV plants (ANOVA, $P < 0.05$).

ments in themselves are stresses for the plant. *OtNOS* lines grew significantly better in NaCl and ABA compared to EV plants (Figure 5a). In the absence of induction ('none'), basal expression of *OtNOS* led to a moderate but significant increase in leaf fresh weight, without changes in leaf area (Figure 5b,c). When exposed to NaCl or ABA for 5 days, the fresh weight of the aerial parts decreased in all lines compared to control conditions ('none'), but this decrease was significantly attenuated in plants transformed with SPF_{pro}:*OtNOS* (Figure 5b). Leaf expansion was also inhibited by NaCl or ABA treatments, with the inhibitions being largely prevented in *OtNOS*-expressing lines (Figure 5c). In contrast, there were no significant differences in chlorophyll and carotenoid levels between *OtNOS* and EV plants after 5 days of treatment with the stressors (Figure S3).

NO has emerged as a key molecule required for root organogenesis (Pagnussat *et al.*, 2002), and especially lateral root formation (Correa-Aragunde *et al.*, 2004), by modulating the expression of cell-cycle regulatory genes (Correa-Aragunde *et al.*, 2006). Therefore, the root architecture of *OtNOS*-expressing lines was analyzed after treatment with NaCl or ABA. In the absence of induction ('none'), *OtNOS* and EV plants showed no differences in primary root length (Figure 6a). However, after 5 days of treatment with NaCl, primary roots were significantly longer in *OtNOS* lines compared with the EV control. A similar trend was observed in plants treated with ABA, although differences were not significant except for the *OtNOS4* line (Figure 6a). The number of lateral roots was reduced after NaCl and ABA treatments (Figure 6b), but this decrease was remarkably reduced in lines expressing *OtNOS* (Figures 6b and S4). The higher lateral root numbers observed in *OtNOS* plants under control conditions may reflect basal SPF_{pro} activity, leading to production of sufficient NO to exert its physiological effects (e.g. lateral root formation). Furthermore, lateral root density, defined as the ratio between the number of lateral roots and primary root length, also showed an appreciable decrease after NaCl or ABA treatment in EV plants, but was only slightly modified in *OtNOS* lines (Figure 6c). The NOS inhibitor N^G-nitro-L-Arg (L-NNA) had little effect on the root phenotype induced by ABA and NaCl in EV plants, but abolished the protection afforded by *OtNOS* expression (Figure S5). The effect of L-NNA correlated with a decrease in NO production in NaCl-treated plants, but not in ABA-treated lines (Figure S5). As anticipated from the reported antioxidant capacity of NO (Beligni and Lamattina, 1999; Beligni *et al.*, 2002), transgenic lines *OtNOS6* and *7* exhibited lower accumulation of reactive oxygen species (ROS) than EV plants upon treatment with 200 mM NaCl (Figure S6).

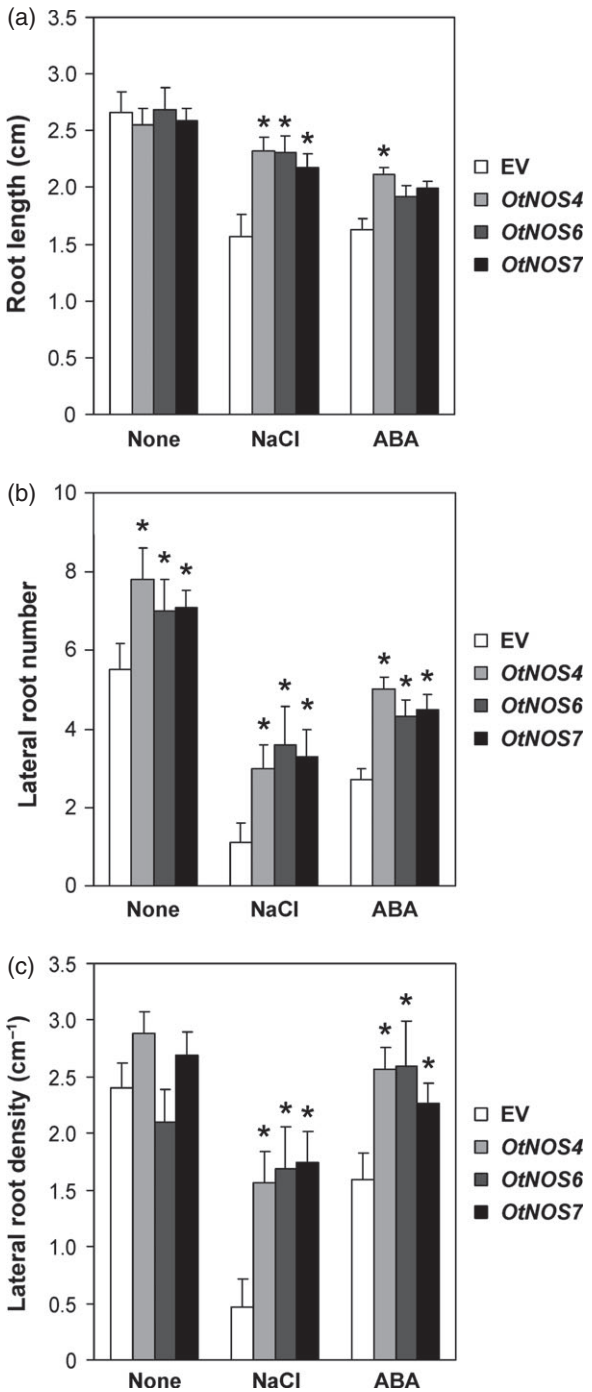

Figure 6. *OtNOS* expression protects Arabidopsis plants from inhibition of root development by NaCl and ABA treatments.
(a) Primary root elongation, (b) lateral root number, and (c) lateral root density were calculated in 10-day-old seedlings as described in Experimental Procedures. Plant growth conditions are described in the legend to Figure 5. Values are means ± SE of 7–10 experiments. Asterisks indicate significant differences between *OtNOS* and EV plants (ANOVA, $P < 0.05$).

Plants expressing *OtNOS* exhibit increased tolerance to oxidative stress

Methyl viologen (MV) is a redox-cycling herbicide that propagates ROS by accepting electrons at photosystem I and transferring them to oxygen to yield superoxide radicals. MV treatment exposes the plant to severe oxidative stress, during which cell ion leakage to intercellular com-

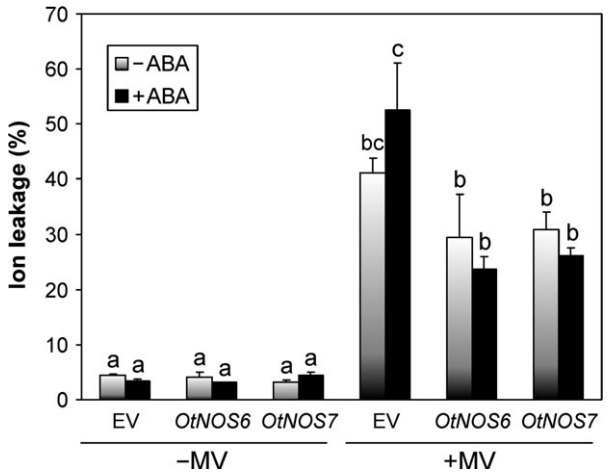

Figure 7. Increased tolerance to methyl viologen (MV) in transgenic Arabidopsis lines expressing *OtNOS*.
Ion leakage was measured on leaf discs pre-treated with 100 μM ABA (+ABA) or not (–ABA) and later incubated with 2.5 μM MV (+MV) or without (–MV), as described in Experimental Procedures. Values are means ± SE of 3–4 experiments. Bars marked with different letters indicate values that are statistically significantly different (ANOVA, $P < 0.05$).

partments occurs as an early step, leading to cell death (Babbs *et al.*, 1989). As NO may protect cells from oxidative damage by acting as a bioactive ROS scavenger (Beligni and Lamattina, 1999) (Figure S6), we tested whether loss of membrane integrity caused by MV is lower in the leaves of plants expressing SPF_{pro}*:OtNOS* than in EV plants. Leaf discs were pre-treated with ABA for 2 h to induce SPF_{pro}, and subsequently exposed to 2.5 μM MV for 3 h. Figure 7 shows that MV increased ion leakage to 52% in discs from EV plants, and this was partially prevented in *OtNOS6* and *7* lines (24 and 26%, respectively). Interestingly, *OtNOS* leaf discs also showed some level of protection against MV even in the absence of ABA. While the differences were moderate and did not show statistical significance, they were systematically observed in all experiments, consistent with basal expression of *OtNOS*.

OtNOS expression increases the stomatal index and improves drought tolerance

ABA is reported to promote NO synthesis in guard cells, and NO, in turn, is required for ABA-induced stomatal closure (García-Mata and Lamattina, 2002; Neill *et al.*, 2002). Moreover, exogenous NO treatment induces stomatal closure, and ABA-promoted NO production confers increased tolerance to severe drought stress (García-Mata and Lamattina, 2001; Ribeiro *et al.*, 2009). In view of these results, the various transgenic lines expressing SPF_{pro}*:OtNOS* were tested for their drought tolerance. Figure 8(a) and Table S2 show that transgenic lines *OtNOS6* and *7* were able to survive for longer periods without watering compared with EV plants. Interestingly, line *OtNOS2* did not show *OtNOS* expression after induction with NaCl at the seedling stage (Figure S1a), and behaved as the EV line (Figure 8a and Table S2). Short-term water loss assays were performed by evaluating the decrease in the fresh weight of detached rosette leaves as described by Verslues *et al.* (2006). Leaves from *OtNOS6* and *7* lines showed reduced water loss under drought (Figure 8b). The *OtNOS6* transgenic line showed lower water loss rates compared to EV plants (Figure 8b), and reached a maximal survival rate after 10 days of water deprivation and re-watering (Table S2).

The aperture of stomatal pores was smaller in *OtNOS* lines than in EV plants even in the absence of induction (Figure 8c), which may result in lower water loss under drought conditions. In addition, ABA treatment did not induce an increase in stomatal closure and NO concentration in lines *OtNOS6* and *7* (Figure 8c,d). ABA promotes NO synthesis in guard cells by the activity of nitrate reductase (NR), an enzyme of the nitrate assimilation pathway (Desikan *et al.*, 2002). NOS-dependent NO formation may also modulate NR activity, suggesting that the increased basal NO levels in guard cells of transgenic lines may mask the effect of ABA treatment (Figure 8d). Figure 8(d) also shows that, as expected, ABA was able to induce an increase of endogenous NO in EV plants.

Stomatal density in the leaf epidermis is regulated by genetic determinants and the environment, resulting in variable stomatal numbers and distribution patterns in mature organs (Bergmann and Sack, 2007; Casson and Hetherington, 2010). However, the stomatal index of transgenic plants expressing *OtNOS* was significantly higher (25%) than that of EV plants (Figure 8e). We also measured the total open surface of pores in stomata per leaf area. The results show that the the total open surface of pores is 9.13 ± 0.8% and 11.7 ± 0.5% lower for *OtNOS6* and *7*, respectively, compared to EV plants (statistically significant by the Mann–Whitney test at $P < 0.03$).

DISCUSSION

Expression of SPF_{pro}*:OtNOS* and production of NO in Arabidopsis, and role of the cofactor THF in OtNOS activity

Almost two decades of intense research trying to unravel functions of NO in plant biology have resulted in the finding of many unexpected pathways modulated by NO. One of the most intriguing aspects of NO biology in plants is its synthetic pathways, especially that dependent on L-Arg and NOS-like activity. This activity has been systematically detected in various plant species, and is blocked by mammalian NOS inhibitors and by factors affecting Arg metabolism (Corpas *et al.*, 2004, 2009; Desikan *et al.*, 2004; Flores *et al.*, 2008). However, the corresponding enzyme(s) remained elusive and were not identified. As a result of this limitation, most approaches aimed at demonstrating

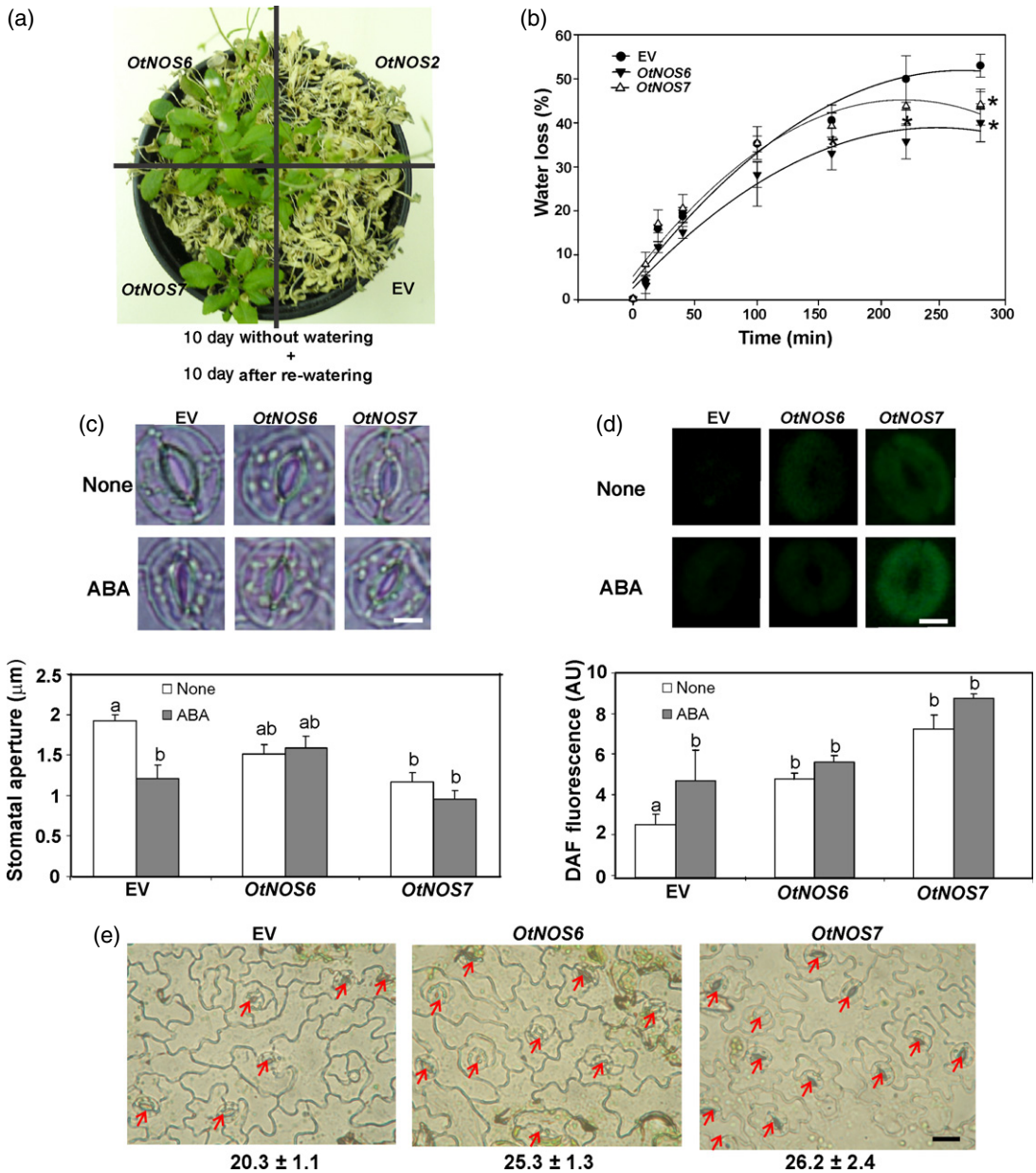

Figure 8. Tolerance to drought stress and determination of stomatal index in transgenic Arabidopsis lines expressing *OtNOS*.
(a) Phenotypes of 3-week-old plants used for drought tolerance assays. Photographs were taken 8 days after interruption of watering (left) and 10 days after re-watering (right).
(b) Rate of water loss. Detached rosette leaves of 3-week-old plants were placed in weighing dishes and incubated at 24°C. Fresh weight was monitored at the indicated times. Water loss was expressed as a percentage of initial fresh weight per leaf area as estimated using ImageJ software. Asterisks indicate statistically significant differences between *OtNOS* and EV plants (Student's *t* test, $P < 0.05$).
(c) Top: images of representative stomata treated with ABA or without ('none'). Strips were pre-incubated for 3 h in opening buffer and then treated with 10 μM ABA. Scale bar = 10 μM. Bottom: stomatal aperture values (means ± SE from at least three independent experiments, $n = 100$).
(d) Top: representative images of epidermal strips pre-incubated for 3 h in stomatal opening buffer. Strips were treated with 10 μM ABA or without ('none') for a further 90 min, and loaded with 15 μM DAF-FM-DA for NO detection. Images were obtained 15 min after loading the probe. Scale bar = 10 μm. Bottom: green fluorescence pixel intensities from three independent experiments. Fluorescent values are expressed in arbitrary units (AU) as means ± SE. Bars marked with different letters indicate values that are statistically significantly different (ANOVA, $P < 0.05$).
(e) Photomicrographs of the abaxial epidermis from Arabidopsis leaves showing stomata. The stomatal index (SI) was calculated for several patches ($n = 80$) using the formula described in Experimental Procedures, and values (means ± SE) are shown below each photograph. Scale bar = 50 μm. Arrows indicate stomata positions.

the role played by NO in plants have been pharmacological, using NO donors and scavengers (Lamattina and Polacco, 2007).

Attempts to express *bona fide* NOS in plants have included transformation of Arabidopsis (Shi *et al.*, 2012) and tobacco (Chun *et al.*, 2012) with a cDNA encoding rat

brain nNOS under the control of the CaMV 35S constitutive promoter. Several Arabidopsis transgenic lines exhibiting increased NOS activity and NO levels were shown to be more tolerant than their wild-type siblings to drought and salt stress, and to infection with a virulent pathogen (Shi *et al.*, 2012). The only transgenic tobacco line assayed by Chun *et al.* (2012) was also resistant to various pathogens displaying various lifestyles, an effect that was mediated by NO-dependent increases in salicylic acid levels. Although the two species (tobacco and Arabidopsis) expressed the same nNOS isoform in the same subcellular compartment, major phenotypic differences were observed by the two research teams (Chun *et al.*, 2012; Shi *et al.*, 2012). Arabidopsis lines were not affected with respect to their vegetative growth, but showed significantly higher numbers of rosette leaves and delayed bolting (Shi *et al.*, 2012). In contrast, the tobacco line exhibited growth arrest in both roots and aerial parts, and developed spontaneous necrotic lesions in leaves of all ages, reminiscent of those that develop during the hypersensitive response (Chun *et al.*, 2012).

Expression of animal NOS in plants raises several questions. First, it is unclear which cofactor is used by nNOS in plants lacking H_4B. The possible use of THF by this enzyme has not been assessed, but Adak *et al.* (2002a,b) assayed the activity of the oxygenase domain of nNOS using THF and did not detect any activity. Furthermore, the negative effects observed by Chun *et al.* (2012) in the tobacco line expressing nNOS emphasize the drawbacks arising from constitutive expression of NO-producing enzymes from a species in a distant kingdom.

We tried to overcome these limitations by generating transgenic Arabidopsis plants that express the NOS gene from *O. tauri* under the control of a stress-inducible promoter, and demonstrated that they display enhanced tolerance to various abiotic stresses (Figures 5–8), without showing phenotypic penalties under normal growth conditions (Figure 1b). *OtNOS* is the only NOS-encoding sequence characterized so far in the plant kingdom, and encodes an active enzyme (Foresi *et al.*, 2010). Expression of *OtNOS* in Arabidopsis represents an interesting approach to study the effects of *in vivo* Arg-dependent production of NO in plants, and provides clear advantages over use of NO donors. In agreement, a recent study showed that manipulating Arg levels and ROS metabolism by arginase expression influences abiotic stress responses in Arabidopsis (Shi *et al.*, 2013).

Here, we show that OtNOS displays high activity using THF as cofactor *in vitro* and *in vivo*. Previous data have demonstrated that THF may be used by the recombinant NOS-like enzyme from the bacterium *Deinococcus radiodurans* to replace H_4B as a redox-active cofactor for NO synthesis (Adak *et al.*, 2002a; Reece *et al.*, 2009). In line with those observations, recombinant OtNOS was shown to be active in *E. coli*, where H_4B is absent (Foresi *et al.*, 2010).

Functional evidence presented here supports *in vivo* use of THF as a cofactor for OtNOS activity in plants (Figures 2 and 3), providing a deeper insight into the mechanism of this enzyme, and adds to understanding of NOS functions in plants. Within this context, our findings may contribute to the understanding of the physiological co-factor used by the enzymatic system responsible for the Arg-dependent NO synthesis.

Our study indicates that there is no strict correlation between NOS activity, NO production (Figures 2 and 3), and stress protection (Figures 5–8). However, NO has been proposed to act as a non-traditional plant hormone, whose biological function relies not only on its threshold levels but also on the pro-oxidant or antioxidant status of the cellular redox metabolism (Beligni and Lamattina, 2001). Some of the transgenic lines described here showed increased basal levels of NO, nitrite and NOS activity, while others showed enhanced induction only after perceiving the stress stimulus. These different behaviors probably reflect position effects during T-DNA integration that influence *OtNOS* expression. Moreover, the NO produced by transgenic OtNOS may affect NR activity, reflecting the complexity of the interactions between various NO sources. Rosales *et al.* (2011) showed that, in wheat leaf segments exposed to the NO donors sodium nitroprusside or *S*-nitrosoglutathione, NR activity was almost completely blocked, but was partially recovered by addition of the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide. Furthermore, the NOS inhibitors N^G-nitro-L-Arg methyl ester (L-NAME) and D-Arg increased NR activity. Hence, OtNOS-dependent NO formation may inhibit NR activity, whereas ABA is expected to stimulate it (García-Mata and Lamattina, 2003; Neill *et al.*, 2008), eventually compensating for the effect of OtNOS-dependent NO production on NR activity. On the other hand, it was also reported that NO stimulates the activity of NR in the roots of *Solanum lycopersicum* (Jin *et al.*, 2009) and *Vicia faba* (Caba *et al.*, 1995). This highlights that the effect of NO on NR activity depends on the plant species and the organ analyzed. Figure S5 suggests that the NO generated by both OtNOS and NR is important in determining root phenotypes in ABA-stressed Arabidopsis seedlings.

We were able to decrease NO production induced by NaCl using the NOS inhibitor L-NNA. In addition, physiological and/or pharmacological treatments of plant tissues with inhibitors analogous to L- Arg, such as L-NAME, L-NNA or L-N^G-monomethyl-Arg monoacetate, have shown decreased NO production, thus corroborating the involvement of L-Arg-dependent NOS activity in the generation of NO. The range of inhibitor concentrations used varied between 1 and 10 mM (Corpas *et al.*, 2009; Gupta and Kaiser, 2010). In *O. tauri*, OtNOS was inhibited at 10 mM L-NNA (Foresi *et al.*, 2010).

Here, we measured the activity of OtNOS by the citrulline and oxyhemoglobin methods. Tischner *et al.* (2007) reported that argininosuccinate interferes with the citrulline-based assay used to measure NOS activity in protein extracts from Arabidopsis. However, although the protein extracts used in those experiments did not contain NOS activity, an active NOS enzyme is present in extracts from the *OtNOS* transgenic lines when estimated in various ways, all of which showed similar results and tendencies. Moreover, the results of the citrulline assay in our work showed a good correlation with the expression levels of *OtNOS* as determined by RT-PCR and quantitative RT-PCR (Figures 1, 2 and S1), and with the estimation of nitrite concentrations by the Griess reaction (Table S1).

NO and seed germination

It has been reported that elevated amounts of nitrogen oxides promote seed germination (Keeley and Fotheringham, 1997). Moreover, increases in NO concentrations due to treatments with either NO donors or NO gas reduced seed dormancy in various plant species (Beligni and Lamattina, 2000; Bethke *et al.*, 2006). Liu *et al.* (2009) reported that the effect of NO is achieved by a rapid decrease in ABA as a result of transcriptional activation of (+)-ABA hydroxylase, leading to ABA catabolism during the first stages of imbibition. The germination rate of *OtNOS* seeds clearly differed from that of EV seeds (Figure 4), probably due to basal levels of *OtNOS* expression and NO accumulation.

***OtNOS* expression attenuates the effect of salinity and increases tolerance against MV-derived oxidative stress**

Salinity is one of the most serious environmental limitations for plant growth, severely restricting the use of salt-affected soils for agriculture. It is known that plants respond to salinity by adapting their cellular metabolism and invoking multigenic defense mechanisms (Zhu, 2001; Maathuis, 2014). Increased susceptibility to salt stress has been demonstrated in Arabidopsis plants that are compromised with respect to NO production due to mutation of the NO-associated gene *AtNOA1* (Zhao *et al.*, 2007). Germination of *atnoa1* seeds and survival of mutant seedlings were shown to be more sensitive to high NaCl concentrations than that of their wild-type siblings. In addition, treatment of wild-type Arabidopsis plants with either NOS inhibitors or NO scavengers exacerbated the effect of NaCl (Zhao *et al.*, 2004; Zhang *et al.*, 2006). NO serves as a signal in salt tolerance by decreasing the Na^+/K^+ ratio through stimulation of plasma membrane H^+-ATPase activity, and promoting the activation of vacuolar H^+-ATPase and H^+-pyrophosphatase, thus providing the driving force for Na^+/H^+ exchange (Zhang *et al.*, 2006). In line with these observations, *OtNOS* transgenic lines showed increased tolerance to salinity, displaying higher primary root growth, lateral root densities, leaf fresh weight and leaf area than EV plants (Figures 5, 6 and S4). Also, the lower ROS accumulation in salt-stressed transgenic lines *OtNOS 6* and *7* compared to EV plants (Figure S6) agrees with previous results in cucumber (*Cucumis sativus* L.) (Shi *et al.*, 2007) and barley (*Hordeum vulgare* L.) (Li *et al.*, 2008).

We also observed that *OtNOS* plants displayed increased tolerance to MV-mediated damage (Figure 7), strengthening the evidence in favor of NO as a potent antioxidant, as previously demonstrated by treatments with NO donors (Beligni and Lamattina, 1999; Beligni *et al.*, 2002).

NO derived from *OtNOS* expression modifies the stomatal index and protects Arabidopsis from water deficit

Many studies have indicated that NO is a pivotal component of the ABA signaling pathway, leading to stomatal closure and control of gas exchange between the plant and the atmosphere (García-Mata and Lamattina, 2001; Desikan *et al.*, 2002; Neill *et al.*, 2002). Within this context, *OtNOS* lines showed improved drought tolerance, a lower stomatal aperture and a higher stomatal index (SI) than EV plants (Figure 8). Plants regulate their gas exchange with the environment through both short-term adaptation involving modulation of stomatal pore aperture and long-term adjustment of the proportion of developing stomata on the leaf surface (Roelfsema and Hedrich, 2005; Casson and Gray, 2008; Casson *et al.*, 2009). No data about the NO effect on SI have been reported previously. Casson *et al.* (2009) showed that mature leaves of Arabidopsis growing at higher photon irradiances showed a significantly increased SI compared to those grown at lower photon irradiances. Interestingly, high levels of irradiance induce NO production in plants and algae (Yamasaki, 2000; Foresi *et al.*, 2010), and hence it will be interesting to further investigate the molecular mechanisms linking NO production and SI. Arabidopsis *OtNOS* plants exhibited partial closure of stomatal pores (Figure 8), which probably results in lower water vapor loss and a higher ratio of carbon gain per water use. The lower stomatal aperture in *OtNOS* lines correlated with increased fresh weight of leaves with no changes in leaf area, suggesting that *OtNOS* transgenic lines possess higher water content. It is therefore proposed that the increase in NO basal concentration in *OtNOS* plants may induce changes in stomatal development leading to increased SI. Interestingly, Yamasaki (2000) reported NO-mediated reduction of CO_2 uptake activity in leaves. In agreement, it has been demonstrated that a continuous increase in atmospheric CO_2 resulted in a decrease of the SI in a number of plant species (Woodward, 1987). Here we report involvement of endogenous NO production in the process regulated by CO_2 concentration that determines stomatal development. It remains to be determined whether the stomatal phenotype is directly associated with the tolerance of the *OtNOS* transgenic lines to longer desiccation periods (Figure 8) and the increased fresh weight of seedlings (Figure 5).

NO has been proposed as a mediator of tolerance mechanisms in plants confronting stress conditions, by keeping cellular redox homeostasis under stress conditions (Lamattina *et al.*, 2003; Wendehenne *et al.*, 2004; Delledonne, 2005; Besson-Bard *et al.*, 2008; Wilson *et al.*, 2008; Yu *et al.*, 2014). Treatments with either NO donors or scavengers suggest that NO is an essential component of plant stress adaptive responses. Here we show that regulation of NO generation in plants expressing the *OtNOS* gene under the control of a stress-inducible promoter provides enhanced tolerance to various abiotic stresses. Several plant traits were taken into consideration, and the results obtained for all of them reinforce the possibility that a controlled and temporally precise bonus of endogenous NO production may assist plants after perceiving a stress situation. NO is a molecule with potential to improve crop agricultural performance, and *OtNOS* manipulation may therefore represent a useful tool in plant genetic engineering programs, even though precautions must be taken with regard to the disadvantages arising from the potential increase in atmospheric NO. Interestingly, Greeg *et al.* (2003) demonstrated that trees grow better in New York City than in rural sites due to the higher concentration of ozone (O_3) in the latter sites, with detrimental effects on plant growth parameters. The NO_x produced by urban activities depletes O_3, resulting in a lower urban concentration of O_3 and a higher plant biomass compared to rural sites. Nevertheless, soil micro-organisms are greater contributors than plants to global NO emissions. Indeed, plants behave mostly as scavengers of NO, due to the activity of non-symbiotic hemoglobins that have been proposed to decrease plant endogenous NO concentrations and even the level of NO in the surrounding environment (Mur *et al.*, 2013).

The possibility that plants themselves generate NO when required under stress situations represents an enormous advantage compared to strategies involving exogenous application of NO donors.

EXPERIMENTAL PROCEDURES

Preparation and characterization of *OtNOS* Arabidopsis transgenic lines

Arabidopsis plants of ecotype Columbia Col-0 *rdr6* (ABRC stock name CS24285; Luo and Chen, 2007) were used for all transformation experiments in order to achieve higher transgene expression by avoiding gene silencing (Butaye *et al.*, 2004). Arabidopsis seeds were stratified at 4°C for 3 days in darkness, and then cultured in half-strength MS medium (Murashige and Skoog, 1962), containing 0.8% w/v agar with 50 μg ml^{-1} kanamycin, at 150 μmol m^{-2} sec^{-1}, 25°C, under 16 h light/8 h dark cycles.

The *O. tauri* DNA sequence encoding NOS was synthesized, sequenced and cloned into the *Xba*I and *Xho*I restriction sites of plasmid pUC57 (Genscript, http://www.genscript.com/). The resulting vector was digested with *Bam*HI and *Xba*I to yield a 3258 bp fragment. The product was purified and sub-cloned into the *Bam*HI and *Xba*I sites of pCHF3 (Jarvis *et al.*, 1998) to give pCHF3-*OtNOS*. Plasmid pCHF3 is a binary vector carrying the CaMV 35S promoter and a pea (*Pisum sativum* L.) Rubisco small subunit terminator. To create *SPFpro:OtNOS*, the *SPFpro* sequence was PCR-amplified using a pBI101.3 construct carrying *SPFpro* as template (Cabello *et al.*, 2007), and cloned into the *Eco*RI/*Sac*I sites of pCHF3-*OtNOS*, in place of the original promoter. The construct was transformed into *Agrobacterium tumefaciens* strain GV3101::pMP90, and introduced into Arabidopsis plants as described previously (Clough and Bent, 1998). Transgenic *SPFpro:OtNOS* lines were selected on the basis of kanamycin resistance, and confirmed by genomic PCR and RT-PCR analyses. The primers used for RT-PCR were OtNOSF (5′-CTACACCATGCGGTTGTTTG-3′) and OtNOSR (5′-CCCTGTGAAGAGTCGAAAGC-3′), amplifying a 600 bp fragment. The primers for actin were actinF (5′-AATCTCCGGCG ACTTGACAG-3′) and actinR (5′-AAACCCTCGTAGATTGGCACA-3′), amplifying a 651 bp fragment. For RT-PCR total RNA was extracted using Trizol isolation reagent (Invitrogen, http://www.lifetechnologies.com/), and treated with RQ1 RNase-free DNase (Promega, http://www.promega.com/). The 1 μg total RNA was used for first-strand cDNA synthesis with an oligo(dT) primer and M-MLV reverse transcriptase (Promega). PCR reactions were performed using 1 μl cDNA, 10 pmol of each primer and 1 μl Taq polymerase (Invitrogen) in a 20 μl reaction volume. Various numbers of amplification cycles (30–38) were tested for the *OtNOS* gene. After 35 cycles for *OtNOS* and 25 cycles for actin with an annealing temperature of 55°C, 10 μl of the PCR products were analyzed by electrophoresis in 1% w/v agarose gels. For quantitative RT-PCR, reactions were performed in a Mastercycler_ep realplex thermal cycler (Eppendorf, http://www.eppendorf.com/) using Platinum Taq DNA polymerase (Invitrogen) and SYBR Green I (Roche, http://www.roche.com/) to monitor the synthesis of double-stranded DNA. Relative transcript levels were determined for each sample and normalized against the levels of *PP2A* cDNA, encoding protein phosphatase 2A (AT1G13320). The primer sequences used were 5′-TGAGACCG CGCTATTATTCC-3′ (OtNOSFw) and 5′-CGTAGCGAGAAAATCTGA GC-3′ (OtNOSRv) for *OtNOS*, and 5′-CCTGCGGTAATAACTGCATC T-3′ (PP2AFw) and 5′-CTTCACTTAGCTCCACCAAGCA-3′ (PP2ARv) for *PP2A*.

Western blotting

The presence of OtNOS protein was determined in cleared extracts obtained from 10-day-old seedlings by SDS–PAGE and immunoblotting. Soluble proteins were resolved on 12% polyacrylamide gels, transferred to nitrocellulose membranes and incubated with polyclonal antibodies raised against the purified OtNOS protein (Genscript). Antigenic polypeptides were visualized using alkaline phosphatase-labeled anti-rabbit secondary antibodies (Sigma-Aldrich, http://www.sigmaaldrich.com/) and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as alkaline phosphatase substrates.

Detection of NO and determination of nitrite concentrations

Endogenous NO levels were estimated by using the NO-sensitive dye DAF-FM-DA (Chandok *et al.*, 2003; He *et al.*, 2004; Moreau *et al.*, 2008). Leaves and roots were observed by confocal fluorescence microscopy and bright-field microscopy using an Eclipse E200 microscope (Nikon, http://www.nikon.com/).

For nitrite determinations, 100 mg seedlings were ground in liquid N_2 and resuspended in 100 mM sodium phosphate. After centrifuging at 10 000 ***g*** for 15 min at 4°C, the supernatant was used for nitrite determination and for estimation of protein concentrations as described by Bradford (1976).

All assays were performed in 96-well microassay plates, and absorbance measurements were performed using a Metrolab 980 microplate reader (Metrolab, http://www.metrolab.com.ar/). First, 50 μl of sample were placed in each well, and then 50 μl sulfanamide in 5% phosphoric acid was added, followed by incubation for 10 min at 20°C, and subsequent addition of 50 μl of 1% w/v *N*-(1-naphthyl)ethylenediamine HCl. The absorbance was read at 550 nm, and nitrite concentration estimated by comparison with a nitrite standard calibration curve (0–100 μM).

Determination of NOS activity

NOS activity was determined in Arabidopsis plant extracts by monitoring the conversion of [^{3}H]-Arg into [^{3}H]-L-citrulline as described by Bredt and Snyder (1990). Enzymatic reactions were performed at 25°C in 50 mM Tris/HCl, pH 7.4, containing 50 mM L-Arg,1 mCi [^{3}H]-Arg monohydrochloride (40–70 Ci/mmol; Perkin-Elmer, http://www.perkinelmer.com/), 100 μM NADPH, 10 μM FAD, 2 mM $CaCl_2$, 10 μg calmodulin and 100 μM H_4B in a volume of 40 μl. Reactions were initiated by adding 20 μg of total plant proteins, and terminated after 30 min by addition of 400 μl of ice-cold 20 mM sodium acetate, pH 5.5, containing 1 mM L-citrulline, 2 mM EDTA and 0.2 mM EGTA (stop buffer). Samples were applied to columns containing 1 ml of Dowex AG50W-X8, Na^+ form (100–200 mesh; Bio-Rad, http://www.bio-rad.com/), pre-equilibrated with stop buffer. L-citrulline was eluted using 2 ml distilled water. Aliquots of eluate (0.5 ml) were dissolved in 10 ml scintillation liquid, and radioactivity was measured using a Beckman, LS 3801 (https://www.beckmancoulter.com/) liquid scintillation system.

The activity of the recombinant OtNOS was assayed by the oxyhemoglobin method as described by Foresi *et al.* (2010). The reaction was initiated by addition of 0.5 μM of purified recombinant OtNOS or commercial iNOS (Sigma-Aldrich). The cofactors THF and H_4B were added at 100 μM. The NO-dependent conversion of oxyhemoglobin to methemoglobin was monitored on an Ultrospec 1100 pro spectrophotometer (Amersham Biosciences, now GE Healthcare, http://www.gelifesciences.com/) by scanning between 380 and 450 nm. An extinction coefficient of 100 mM^{-1} cm^{-1} between the peak at 401 nm and the valley at 420 nm was used to quantify NO production. All chemicals were purchased from Sigma-Aldrich.

Ion leakage assay

Discs (0.9 cm diameter) were punched from fully expanded rosette leaves of plants just prior to bolting (4 weeks old), and vacuum-infiltrated with 100 μM ABA or water. Following a 2 h dark incubation period, discs were floated top side up in 1 ml distilled water containing 0.1% v/v Tween-20 with or without 2.5 μM MV, and illuminated at 400 μmol m^{-2} sec^{-1} for 3 h. Electrolyte leakage was measured as the increase in conductance of the medium as described by Tognetti *et al.* (2006).

Germination assay

Arabidopsis seeds were germinated in 3.5 cm plastic Petri dishes containing 3 ml of 0.6% w/v aqueous agarose, without prior stratification. Seeds (approximately 20–30 per dish) were allowed to germinate under the same growth conditions as described above. Seeds were determined to have germinated when the radicle pierced the seed coat (Bethke *et al.*, 2006). All seeds were harvested from the same group of plants, grown together at the same time and under the same conditions.

NaCl and ABA treatments

To determine the effect of NaCl and ABA on root and aerial part growth, plants transformed with *SPFpro:OtNOS* or the EV pCHF3 were germinated on agar plates containing half-strength MS medium. Five days later, seedlings were transferred to MS plates supplemented with 100 mM NaCl or 10 μM ABA, and the primary root apex was marked. Plates were kept vertical and photographed 5 days after transfer. Primary root elongation was measured using image processing software (Image J, http://imagej.nih.gov/ij/). Lateral root density was calculated as the number of lateral roots divided by the length of the primary root. Aerial parts were photographed and foliar area calculated as above. They were then excised and weighed in order to determine the fresh weight after the treatments. Chlorophyll and carotenoid levels were determined spectrophotometrically in 96% v/v ethanol extracts (Lichtenthaler, 1987).

ROS measurement

Leaves from 30-day-old plants treated or not with 200 mM NaCl for 10 days were ground in liquid N_2 and extracted with H_2O for 30 min in the dark, followed by centrifugation at 10 000 ***g*** for 20 min. ROS were quantified as described by Bellincampi *et al.* (2000) based on the peroxide-mediated oxidation of Fe^{2+} followed by the reaction of Fe^{3+} with xylenol orange (*o*-cresolsulfonephthalein 3,3′-bis(methylimino)diacetic acid; sodium salt). Absorbance of the Fe^{3+}–xylenol orange complex (A_{560}) was detected after 30 min.

Drought stress, water loss, stomatal aperture and NO detection in guard cells

Transgenic Arabidopsis lines expressing *OtNOS* or transformed with EV were grown in soil in the same container, with normal watering at 700 ppm CO_2. Then, water was withdrawn for 10 days, and plants were re-watered and allowed to recover for a further 10 days.

For measurements of water loss rate, rosettes with mature leaves from 3-week-old transgenic and EV Arabidopsis plants were weighed immediately after cutting. The rosette leaves were then kept on filter paper under conditions of 60% humidity and 25°C temperature for varying durations (0, 0.1, 0.2, 0.4, 1, 2, 3, 4 and 5 h), and weighed again. Water loss rate was determined by measuring the percentage of fresh weight loss relative to the initial plant weight per leaf area.

The stomatal aperture experiments were performed using epidermal strips excised from the abaxial side of fully expanded Arabidopsis leaves. Immediately after stripping, peels were floated in opening buffer (10 mM K-MES, pH 6.1, 10 mM KCl) for 3 h. The strips were exposed to various treatments in the same opening buffer. After 90 min, images of stomata were digitalized using a Nikon DS-Fi 1 camera coupled to a Nikon Eclipse Ti microscope. The stomatal aperture width was measured using ImageJ analysis software. NO was detected using the fluorescent probe DAF-FM-DA. Arabidopsis epidermal strips pre-incubated in opening buffer for 3 h in the dark were loaded with 10 μM DAF-FM-DA for 30 min. Strips were washed three times with fresh opening buffer and exposed to various treatments for 15 min. Fluorescent images were obtained using a Nikon DS-Fi 1 digital camera coupled to a Nikon Eclipse Ti epifluorescence microscope (excitation at 495 nm; emission at 515–555 nm). The green fluorescence was quantified as the pixel intensity of a fixed area for all guard cells using ImageJ analysis software. The stomatal index (SI) was

calculated using the formula: SI = [number of stomata/(number of other epidermal cells + number of stomata)] × 100.

Statistical analysis

Results are expressed as means ± SE or median. Statistical significance was determined by analysis of variance (ANOVA) for multiple comparison analyses, or Student's *t* test for pairwise comparisons, using Sigma Statistical software (Jandel Scientific, now Systat software Inc, http://www.sigmaplot.com/).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Analysis of *OtNOS* transcript levels in NaCl-treated Arabidopsis transgenic lines.

Figure S2. Western blot analysis of OtNOS protein levels in ABA-treated transgenic lines.

Figure S3. Levels of chlorophylls and carotenoids in Arabidopsis seedlings transformed with SPF_{pro}*:OtNOS* or EV under NaCl or ABA treatments.

Figure S4. Root phenotypes of Arabidopsis seedlings transformed with SPF_{pro}*:OtNOS* or EV under NaCl or ABA treatments.

Figure S5. The NOS inhibitor L-NNA prevents NO formation in Arabidopsis *OtNOS* transgenic lines.

Figure S6. ROS accumulation in transgenic Arabidopsis *OtNOS* plants under salinity.

Table S1. Nitrite concentration quantified by the Griess assay.

Table S2. Survival of *OtNOS* and EV plants in drought tolerance assays.

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