DIMAS MENDES RIBEIRO

RE-EVALUATION OF THE ROLE OF NITRIC OXIDE AS A COMPONENT OF ABSCISIC ACID SIGNALLING PATHWAY IN GUARD CELLS

Thesis presented to the Universidade Federal de Viçosa as part of the requirement of the Pos-Graduate Program in Plant Physiology for obtention of the degree of *Doctor Scientiae*

VIÇOSA
MINAS GERAIS – BRAZIL
2007

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APROVED: April 13th, 2007

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ACKNOWLEDGMENTS

I thank CNPq (Brazilian Council for Advancement of Science and Technology) for the scholarship conceded and for the financial support granted. I also thank the Universidade Federal de Viçosa and the University of the West of England. My gratitudes are also extended to Prof Steven J. Neill and Dr Radhika Desikan.

BIOGRAPHY

Dimas Mendes Ribeiro was born in Passa Quatro (Minas Gerais State, Brazil) and completed his graduation and MSc degree at the Universidade Federal de Viçosa, Viçosa (MG). He spent ten moths as a doctoral student at the Centre for Research in Plant Science, Faculty of Applied Sciences, University of the West of England, Bristol (UK), before complete his DSc in Plant Physiology at the Universidade Federal de Viçosa.

ABSTRACT

RIBEIRO, Dimas Mendes, D. Sc., Universidade Federal de Viçosa, April 2007. **Re-evaluation of the role of nitric oxide as a component of abscisic acid signalling pathway in guard cells.** Adviser: Raimundo Santos Barros. Co-Advisers: Fernando Luíz Finger, Rolf Puschmann and Steven John Neill

The plant hormone abscisic acid (ABA), synthesized in response to water-deficit, induces stomatal closure via activation of a complex signalling cascade. It has been established that nitric oxide (NO) is a key signalling molecule mediating ABA-induced stomatal closure in several species under well watered condition. However, the function of NO in ABA-induced stomatal closure in response to water deficit has as yet been kept unresolved. The present study provides pharmacological, physiological and genetic evidences that responses of the tissues to NO differ between wilting and turgid conditions in Arabidopsis plants. ABA triggers NO generation, and sequestration of NO by its scavenger 2-phenyl-4, 4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide inhibits stomatal closure in turgid leaves and not in dehydrated leaves. Nitrate reductase (NR)-mediated NO synthesis is required for ABA-induced stomatal closure in turgid leaves but not in dehydrated ones. Moreover, in turgid leaves of the double mutant *nia1 nia2* and *nia1::Ds* mutant both ABA and nitrite failed to induce stomatal closure, but stomatal closure was still induced by SNP (sodium nitroprusside), a NO donor, in these mutants. However, in water-stressed leaves of *nia1 nia2* and *nia1::Ds* mutant stomatal closure was induced by ABA but not by nitrite or SNP, indicating that the ABA-induced stomatal closure was independent on NO synthesis via NR. In agreement with these results, ABA reduced the water loss in *nia1 nia2* and *nia1::Ds* mutants and also in wild type plants, but SNP and nitrite failed in doing the same. In the ABA-insensitive *abi1-1* and *abi2-1* mutants treated either with ABA or NO (applied as SNP) no significant changes in stomatal apertures occurred under both well watered and water stress conditions, suggesting that NO was not able to bypass the effects of *abi1-1* and *abi2-1* mutations. Together, these data indicate that NO is not required for ABA-induced stomatal closure during water stress.

RESUMO

RIBEIRO, Dimas Mendes, D. Sc., Universidade Federal de Viçosa, abril de 2007. **Reavaliação do papel do óxido nítrico como um componente da rota de sinalização do ácido abscísico em células-guardas.** Orientador: Raimundo Santos Barros. Co-Orientadores: Fernando Luíz Finger, Rolf Puschmann e Steven John Neill

O ácido abscísico (AAB), sintetizado em resposta ao défice hídrico, induz o fechamento dos estômatos via ativação de uma complexa cascata de sinalização. Tem-se demonstrado que o óxido nítrico (ON) é um componente chave na cadeia de sinalização que leva ao fechamento dos estômatos induzido pelo AAB em várias espécies, sob boa disponibilidade hídrico. Entretanto, a função do ON no fechamento dos estômatos induzido pelo AAB em resposta ao défice de água não está esclarecida. O AAB induz a formação de ON e a remoção do ON por um seqüestrador inibe o fechamento dos estômatos em folhas túrgidas, mas não nas desidratadas. A síntese de ON induzida pela redutase do nitrato (RN) parece necessária para o fechamento dos estômatos induzido por AAB em folhas túrgidas de Arabidopsis, mas não nas desidratadas. Além disso, as células-guardas de folhas túrgidas do duplo mutante da RN *nia1 nia2* e do mutante simples da RN *nia1::Ds* não sintetizam ON e nem fecham os estômatos em resposta ao AAB ou nitrito, embora os estômatos desses mutantes se feche em resposta ao NPS (nitroprusado de sódio), composto liberador de ON. Entretanto, o fechamento dos estômatos do mutante *nia1 nia2* e *nia1::Ds* foi induzido por AAB, mas não por nitrito ou NPS em folhas mantidas sob défice hídrico, indicando que o fechamento dos estômatos induzido por AAB ocorreu independentemente da síntese de ON mediada pela RN. Confirmando esses resultados, o AAB reduziu a perda de água nos mutantes *nia1 nia2* e *nia1::Ds,* e também em plantas do tipo selvagem, mas NPS e nitrito não diminuíram a transpiração das folhas dos mutantes deficientes na RN e em folhas do tipo selvagem. O tratamento de folhas de mutantes insensíveis ao AAB, *abi1-1* e *abi2-1*, com AAB ou NPS não promoveu qualquer alteração significativa na abertura dos estômatos em folhas tanto sob condições de défice hídrico como em folhas não estressadas, sugerindo que o ON foi incapaz em sobrepor-se à mutação *abi1-1* e *abi2-1*. Assim, o ON não parece necessário para o fechamento dos estômatos induzido pelo AAB em folhas sob condições de défice de água.

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INTRODUCTION

The limitation of water to plants during drought periods causes adverse effects on the growth and productivity of crops. In vegetative tissues, water stress may lead to a reduction in cell turgor which is transduced into enhanced biosynthesis and redistribution of abscisic acid (ABA), resulting in a variety of ABA-mediated responses such as differential gene expression and stomatal closure (Nambara and Marrion-Poll 2005). The role of ABA in stomatal closure is of crucial importance in the response of plants to water deficit, what can be shown by the wilty phenotypes of both ABA-insensitive and ABA- deficient mutants of *Arabidopsis thaliana* (Koornneef et al 1982; Koornneef et al 1984). Recently it has been demonstrated that stomatal responses can be manipulated by modifying guard cell signalling transduction elements to reduce water loss of Arabidopsis plants during water defict periods (Saez et al 2006).

The intracellular signalling cascades by which ABA affects guard cell are complex, with several intermediates being already identified (Hetherington 2001; Himnelbach et al 2003). Amongst the molecules participating in ABA-activated signalling pathways in guard cells leading to stomatal closure, nitric oxide (NO) has been shown to be an important intermediate (Garcia-Mata and Lamattina 2002; Neill et al 2002; She et al 2004). The importance of NO in stomatal aperture size regulation lies in the evidence that SNP (sodium nitroprusside), an NO donor, causes stomatal closure whilst the NO scavenger PTIO (2-phenyl-4, 4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide) impairs ABA-induced stomatal closure in several species (Garcia-Mata and Lamattina 2001; Neill et al 2002). Moreover, ABA-induced NO synthesis in guard cells is shown to be associated with stomatal closure in a number of species (Garcia-Mata and Lamattina 2001, Neill et al 2002, Desikan et al 2004). These studies demonstrate that endogenous NO generated in response to ABA, is required for stomatal closure.

Genetic evidence has confirmed the role of NO-generating enzymes in mediating stomatal closure in response to ABA. The guard cells of

Arabidopsis double mutante (*nia1 nia2*), deficient in the NR apoproteins NIA1 and NIA2 do not close in response to ABA or nitrite (Desikan et al 2002). On the other hand, NO donors promote stomatal closure in the *nia1 nia2* mutant (Desikan et al 2002). This means that nitrate reductase-mediated NO synthesis is required for ABA-induced stomatal closure. In addition, the stomatal closing response to NO also suggests that the deficiencies caused by the *nia1 nia2* mutations lie upstream of NO in the signalling pathway. In general, NO has been identified as an essential component of ABA-mediated stomatal closure in several species under well watered condition. Thus, it is important knowing whether NO is required for the ABA-induced stomatal closure during plant responses to drought.

In a study, Garcia-Mata and Lamattina (2001) found that the SNP reduced transpiration and induced stomatal closure in *Vicia faba*, *Tradescantia* sp., *Salpichroa organifolia* and *Triticum aestivum*. These events closely related to NO responses especially confer an increased tolerance to drought stress via stomatal aperture size regulation. In a subsequent work, Tian and Lei (2006) supported the conclusion that NO, applied as SNP, was able to protect *Triticum aestivum* against abiotic stresses such as water stress. However, water stress tolerance is a complex trait and responses to drought are extremely different depending on the species, growth stage, temperature and the dynamics of drought imposition (Denby and Gehring 2005, Verslus et al 2006). Hence, it is not yet definitely clear whether NO plays a fundamental role in the regulation of plant response to water stress, particularly during stomatal responses to ABA.

When ABA is applied to plants, it causes a rapid stomatal closure, reducing water loss via decreased transpiration (Schroeder et al 2001). Furthermore, NO synthesized in response to ABA seems to control the reduction in stomatal aperture. However, NO has been also found to induce stomatal opening in *Vicia faba* (Sakihama et al 2003). These contradictory results are likely to be due to the high dose (5 mM) of the NO donor *S*-nitrosothiol *S*-nitroso-*N*-acetyl-*DL*-penicillamine (SNAP) employed. In Arabidopsis NO, applied via SNP, in a range of 10-200 µM, causes stomatal closure whilst at higher concentration (0.5-2 mM) stomata remain open and guard cell viable (Bright 2006). This concentration dependence suggests that

NO can exhibit a dualism as for the stomatal movements. Given the convergence, divergence and network of signalling pathways that occur in response to NO in plant cells, it may be that signalling in response to ABA does not depend solely on NO. Stomata of *nia1 nia2* mutant fail to close in response to ABA. Furthermore, NO donors promote closure in the absence of ABA (Desikan et al 2002). However, *nia1 nia2* mutant does not show a wilty phenotype, despite the ABA insensitivity of its guard cells. Thus, although NO seems to play a role in water-stress signalling (Garcia-Mata and Lamattina 2001, Tian and Lei 2006), its function within ABA-related signalling pathways remains unclear. Clearly, a re-evaluation of the mechanism of ABA-induced stomatal closure via NO synthesis is required, in order to elucidate the exact role played by NO in response to ABA and water stress in guard cells.

OBJECTIVES

The objectives of this work were to investigate, at physiological and genetic levels, the role of NO in promoting adaptative responses to water stress in *Arabidopsis thaliana*. It also aims to examine the NO-mediated guard cell responses in Arabidopsis leaves and investigate its contribution to the ABA-induced stomatal closure under water stress conditions.

MATERIAL AND METHODS

Plant material and growth conditions

Seeds of wild type and mutants of either Columbia (Col-0) and Landsberg *erecta* (Ler) ecotypes of *Arabidopsis thaliana* were sown on Levington's F2 compost with sand (Avoncrop, Bristol, UK), and the plants grown under a 16-h photoperiod (60-100 µE m^{-2} s^{-1}), at 20 ^{o}C and 70-80 % relative humidity in the plant growth chambers (Sanyo Gallenkamp, Loughborough, UK), for 4 weeks before use. The *nia1 nia2* (*NR deficient 1,2*; background Col-0), *nia1::Ds* (*NR deficient 1*; background Ler) and *nia2-5* (*NR deficient 2*, background Col-0) mutant seeds were obtained from Dr Nigel Crawford (University of California, San Diego, CA, USA); *abi1-1* (*ABA insensitive 1*), *abi2-1* (*ABA insensitive 2*) and *aba1-1* (*ABA deficient 1*) mutant seeds (background Ler) were obtained from the Nottingham Arabidopsis Stock Centre (Nottingham, UK). In all experiments using mutants, the appropriate background was used for wild type controls.

Stomatal bioassay

Stomatal bioassays were performed on leaves as described by Desikan et al (2006). Leaves detached from 4-week old plants were floated in MES-KCl buffer (5 mM KCl, 10 mM MES, 50 µM $CaCl_2$, pH 6.15) with abaxial side down in Petri dishes (30 mm diameter) and incubated under continuous illumination (60-100 µE m^{-2} s^{-1}) at 20 ^{o}C, for 2.5 h to open the stomata. Following this, leaves were treated with various compounds supplemented in the same buffer medium for a further 2.5 h. Inhibitor compounds, tungstate, L-NAME (N^{ω}-nitro-L-arginine methyl ester) or PTIO, were added to the MES-KCl buffer medium on which leaves were floated 30 min prior to other treatments. The leaves were subsequently homogenized individually with approximately 250 ml water in a Waring blender (Christison Scientific, Gateshead, UK), for 30 s. The epidermal fragments were collected on a 100 µm nylon mesh (Spectra-Mesh, BDH-Merck, Nottingham, UK), transferred onto a glass slide and covered with a glass slip. Stomatal apertures from epidermal fragments were then measured using a calibrated light microscope

coupled to an imaging system (LEICA QWIN Software, Leica, Milton Keynes, UK).

To measure the effect of the various compounds on stomatal closure under water stress condition, detached leaves were floated in MES-KCl buffer medium under light, for 2.5 h to open the stomata, and then the compounds were added to the buffer solution. Thereafter 2.5 h drought stress was imposed by placing detached leaves with abaxial side up on open Petri dishes on paper layer (Whatman number 1) under a direct light source (50 cm from a 60 Watt bulb) on the laboratory bench, for 0.5 h. The leaves were subsequently blended and the resulting epidermal fragments were taken for examination of stomatal aperture immediately, as described above.

Measurement of water loss

Leaves were floated in MES-KCl buffer medium, for 2.5 h, under light conditions (60-100 µE m^{-2} s^{-1}) in a growth chamber. Following this, leaves were treated with ABA, SNP, nitrite or sodium ferricyanide and incubated in the same buffer medium for a further 2.5 h. Water loss of detached leaves was measured by weighing leaves placed abaxial side up on open Petri dishes (30 mm diameter) with one layer of filter paper (Whatman number 1), under a direct light source (50 cm from a 60 Watt bulb), on the laboratory bench. The weight of each set of leaves was determined every 20 min over a period of 3 h. Water loss was expressed as the percentage of the initial weight.

Drought stress in whole plants and relative water content (RWC) measurement

Aerial parts of four-week-old Arabidopsis plants were submerged in MES-KCl buffer medium or in solutions of ABA (50 µM), SNP (50 µM) or sodium ferricyanide (50 µM), prepared in MES-KCl buffer solution, for 5 s every day, for 3 days. Drought was induced by withholding watering of plants maintained in the growth chamber under light (16-h photoperiod, 60-100 µE m^{-2} s^{-1}), at 20 ^{o}C and 70-80 % relative humidity. To minimize experimental variations, Arabidopsis plants were grown single on 0.17-L plastic pots. The evaporation of the soil was minimized by covering pots

with plastic film (Parafilm “M” Chicago, IL, USA) during the water deficit imposition of the plants. Ten leaves from four different plants were removed after different periods of drought and fresh weight (FW) were immediately recorded. Subsequently, leaves were incubated in distilled water for 3 h in the growth chamber, and the turgid weight (TW) was recovered. After drying for 24 h at 80 ^{o}C, dry weight (DW) was recorded. RWC was estimated according to the formula: RWC (%) = [(FW-DW)/(TW-DW)]x 100.

Confocal microscopy

Nitric oxide was visualized using the DAF2-DA (4,5-diaminofluorescein diacetato; Calbiochem, Nottingham, UK). The leaves were homogenized individually with water in a Waring blender (Christison Scientific, Gateshead, UK), for 30 s. The epidermal fragments were collected on a 100 µm nylon mesh (spectra-Mesh, BDH-Merck, Nottingham, UK) and transferred to 2 ml MES-KCl buffer in a Petri-dish. Epidermal fragments were incubated under a direct light source for 2 h. After this step, the epidermal fragments were load with 15 µM DAF2-DA for 15 min, followed by a wash step with MES-KCl buffer, for 20 min. Following this, fragments were treated with various compounds supplemented in the MES-KCl buffer for further 25 min. Confocal laser scanning was used to visualize fluorescence, using an excitation wavelength of 488 nm and a emission wavelength of 515 nm (Nikon PCM 2000; Nikon, Kingston-upon-Thames, UK). Images acquired from the confocal microscope were analysed using SCION IMAGE software (Scion Corp, Frederick, MA, USA). Data represent pixel intensities expressed as a percentage of the control values, from guard cells analyses in different experiments.

Statistical analysis

Data from stomatal apertures assays, confocal, RWC and water loss analyses were subjected to analysis of variance (ANOVA) and P >0.05 was considered as not significant. Differences in stomatal apertures means, confocal and RWC assays were examined by the Tukey test or *t* test ($P \leq 0.05$). Differences in the water loss assays were examined by the *t* test ($P \leq$

0.05). All mean comparisons were performed with SPSS (Statistical Package for the Social Science) 11.0 for Windows Statistical Software Package.

RESULTS AND DISCUSSION

Responses of the stomatal to NO differ between wilting and turgid tissues

Stomatal are known to close in response to drought to reduce water loss by transpiration. During this process, ABA is synthesized and plays a role in closing stomata. The intra-cellular signalling cascades by which ABA affects guard cell resulting in stomatal closure are complex; several new signalling intermediates have already been identified (Hetherington 2001). Among the molecules participating in ABA-activated signalling pathways in guard cell, NO has been identified as a key intermediated in ABA-induced stomatal closure in several species, under well watered conditions (Neill et al 2002, Garcia-Mata and Lamattina 2002, Neill et al 2003). Thus, to study the effect of NO on stomatal closure under drought stress, Arabidopsis leaves previously treated with SNP, a NO donor, or ABA (positive control) were submitted to a time-course cycle of dehydration under the dry atmosphere of laboratory. Following treatment of leaves from wild type plants with buffer solution (control), ABA and SNP, the stomatal apertures were 2.58, 1.33 and 1.39 µm, respectively (Fig 1). Time-course observations demonstrated that within 5 min from the start of drought, the stomata of control plants closed, with stomatal apertures decreasing to 1.53, which corresponded to a 40 % reduction in width. The stomatal closure did not enhance further in every time-course observation in leaves treated with SNP and submitted to dry conditions. However, in leaves treated with ABA the stomatal closure enhanced within 5 min after water stress treatment, with 33 % reduction in width, and being maintained in this condition for at least 60 min. Although the initial stomatal apertures caused by both ABA and SNP treatment were similar, stomata still closed in response to ABA in dry atmosphere.

To test the possibility that NO could cause a loss of viability of guard cell stomatal behaviour was measured in washout experiments. At the concentration tested (50 µM), the effects of SNP and ABA on stomatal aperture were fully reversible both in fully turgid leaves and water stressed leaves (Fig 2). There was no significant difference ($P < 0.05$ by Tukey test)

between the apertures of stomata treated with SNP or ABA for 2.5 h followed by a 2 h washout and those incubated under the same conditions for 4.5 h in the buffer alone (control) (Fig 2 a). This is similar to that reported previously for guard cells of *Commelina communis* and *Vicia faba* treated with H_2O_2 (McAinsh et al 1996, Zhang et al 2001). In previously stressed leaves, the effects of SNP and ABA

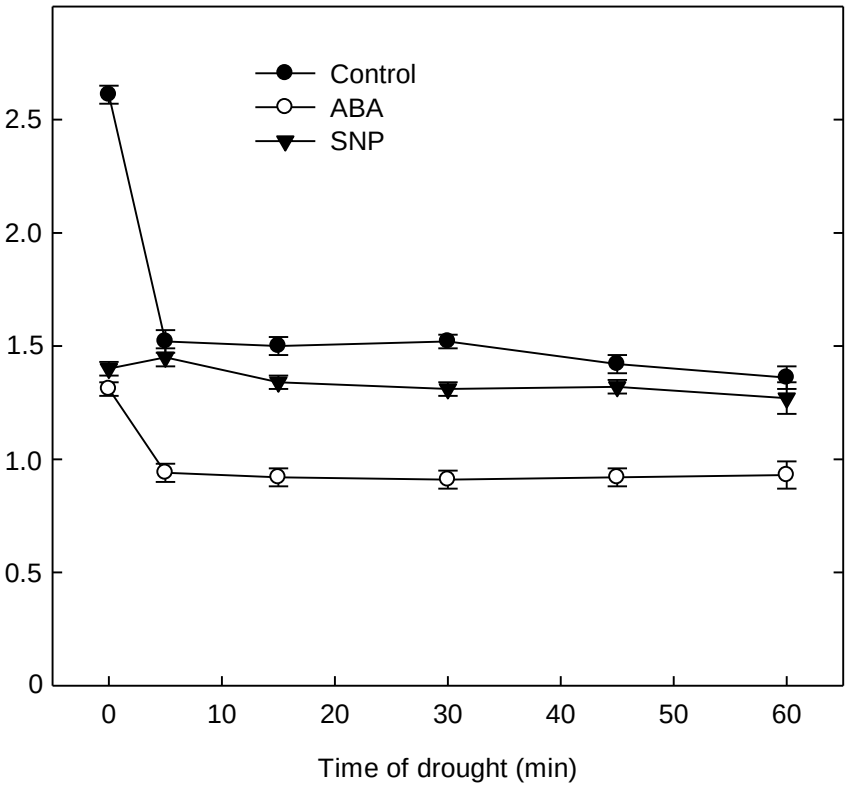

Figure 1 - Time-course changes in stomatal apertures after water stress treatment. Leaves from 4-week-old plants (Col-0) were treated with ABA (50 µM) or SNP (50 µM) and stomatal apertures width were determined after exposing the leaves to a direct light source (50 cm from a 60 Watt bulb) on the laboratory bench. The data are representative of 5 independent experiments with the means of 150 stomata ± standard errors

on stomatal behaviour were also reversed, as their stomatal aperture did not differ significantly from control ((P < 0.05 by Tukey test, Fig 2 b). Together, these data indicate that at the concentration tested, SNP and ABA did not reduce the viability of guard cells and that the plasma membrane remained capable of regulating the transport of ions required for both stomatal opening and closure.

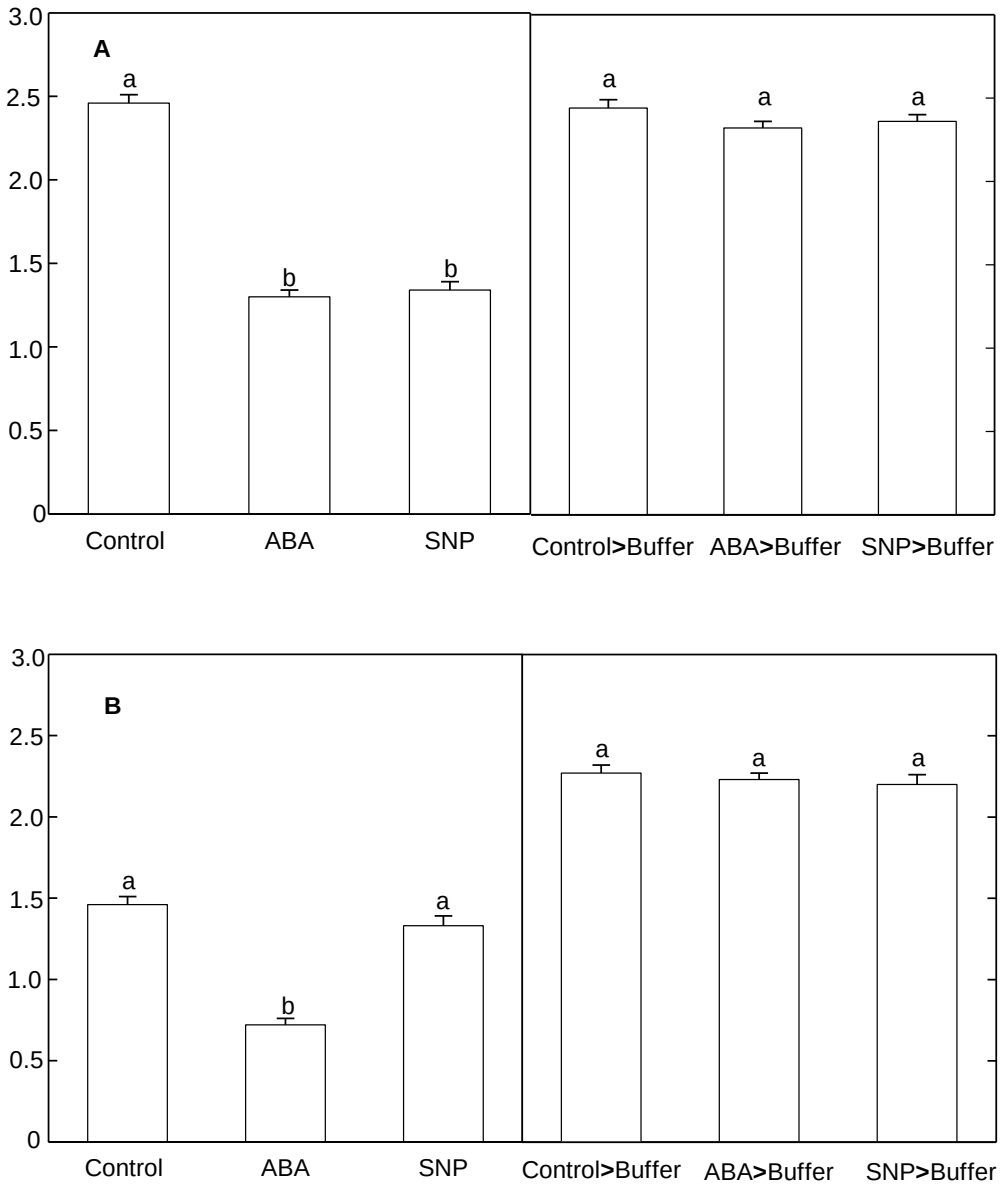

Figure 2 - Reversible inhibition of stomatal closure by ABA and SNP. Wild type Arabidopsis (Col-0) leaves were floated in MES-KCl buffer to induce stomatal opening and then incubated in ABA (50 µM) or SNP (50 µM) for 2.5 h. Turgid leaves (A) and water stressed (30 min under a direct light source) leaves (B) treated with ABA or SNP were then transferred to MES-KCl buffer and incubated for another 2 h under opening conditions. Stomatal apertures were determined at the end of the initial 4.5 h incubation and at the end of the subsequent 2 h wash-out experiments with the means of 100 stomata $\pm$ standard errors

In an attempt to clarify the effect of NO on the stomatal movement, wild type Arabidopsis leaves were treated with increasing concentration of SNP. As expected, SNP induced stomatal closure in a dose dependent manner (Fig 3, white bars), causing a significant reduction in stomatal width

at concentrations as low as 10 µM ($P < 0.05$ by Tukey test). The maximum significant effect induced by SNP occurred at 50 µM when stomatal aperture was reduced by 44 % as compared with control. No further significant reduction in stomatal aperture occurred in the range 100-150 µM. These data support previous studies that demonstrated that NO mediates in a dose-dependent manner stomatal closure in a number of species (Garcia-Mata and Lamattina 2001, Neill et al 2002). To measure the effect of NO on stomatal closure under drought condition, wild type leaves were treated with increasing of SNP amounts and then water stress was imposed by placing leaves under the dry atmosphere of laboratory. After 30 min under water stress, NO applied as SNP failed to induce stomatal closure in leaves stressed by water deficit (Fig 3, striped bars), whereas there was a clear dose-response in the stomatal closure in fully turgid leaves. The insensitivity of wild type stomata under water stress to NO correlated with inability of NO to reduce water loss as evaluated by the decline in fresh weight of detached leaves (Fig 4). The leaves from wild type plants lost about 70 % of their fresh weight within 3 h, regardless of whether they were treated with SNP in a range 10-150 µM. However, Garcia-Mata and Lamattina (2001) found that the NO, administered via SNP (150 µM), reduced transpiration and induced estomatal closure in *Triticum aestivum*. This discrepancy might be then species-specific or might reflect that NO does not protect Arabidopsis plants against water stress.

In an attempt to further analyse stomatal responses to NO in Arabidopsis, the specific NO scavenger PTIO was used. Fig 5 (white bars) shows that upon treatment of leaves with PTIO plus SNP the stomatal apertures remained at the same level of the control leaves through all SNP concentrations tested. This shows that SNP-induced stomatal closure in Arabidopsis leaves, under well watered conditions, might be mediated via NO released from SNP. Importantly PTIO alone at 200 µM caused no effect on stomatal apertures as compared to control ($P < 0.05$ by *t* test). However, under water stress conditions the stomatal apertures in Arabidopsis leaves treated with SNP (50 µM) alone did not differ significantly from control ($P < 0.05$ by *t* test), indicating that NO released from SNP had no effect on guard cell movements (Fig 5, striped bars). Stomatal apertures of leaves treated

with either PTIO or SNP and with PTIO plus SNP remained constant through all tested SNP concentrations, suggesting that NO was not a key component for stomatal closure in Arabidopsis leaves under water stress.

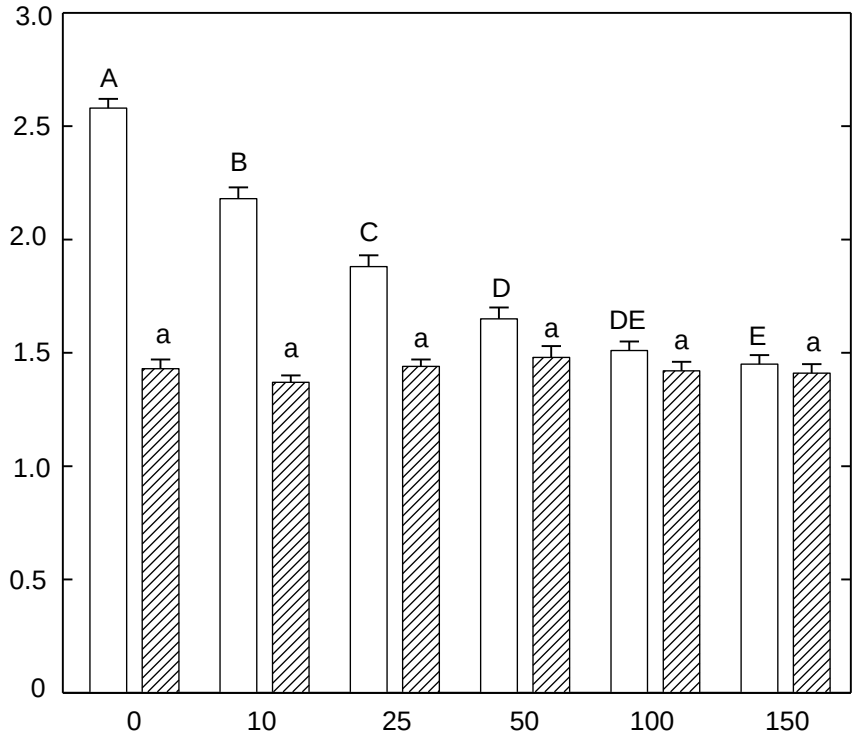

Figure 3 - Negation of the effect of SNP on the stomatal closure in Arabidopsis leaves under water stress conditions. Wild type Arabidopsis (Col-0) leaves were floated in MES-KCl buffer to induce stomatal opening and then incubated in SNP (10-150 µM) for 2.5 h. Stomatal apertures were measured before dehydration (white bars) and 30 min after submitting the leaves the dehydration (striped bars) under a direct light source (50 cm from a 60 Watt bulb) on the laboratory bench. The data are representative of 4 independent experiments with the means of 100 stomata ± standard errors

A major concern over the use of SNP as a NO donor was its concurrent generation of both NO and cyanide. Cyanide was recently shown to break seed dormancy in Arabidopsis as did NO gas and each effect was inhibited by PTIO (Bethke et al 2006). Thus, it was necessary to determine whether cyanide also affects stomatal responses. Cyanide, applied as sodium ferricyanide, did not induce stomatal closure both in turgid and dehydrated leaves (Fig 6). These data provide evidence that SNP induced stomatal closure in leaves under well watered conditions is mediated via NO release and not by the release of any other SNP-related breakdown product.

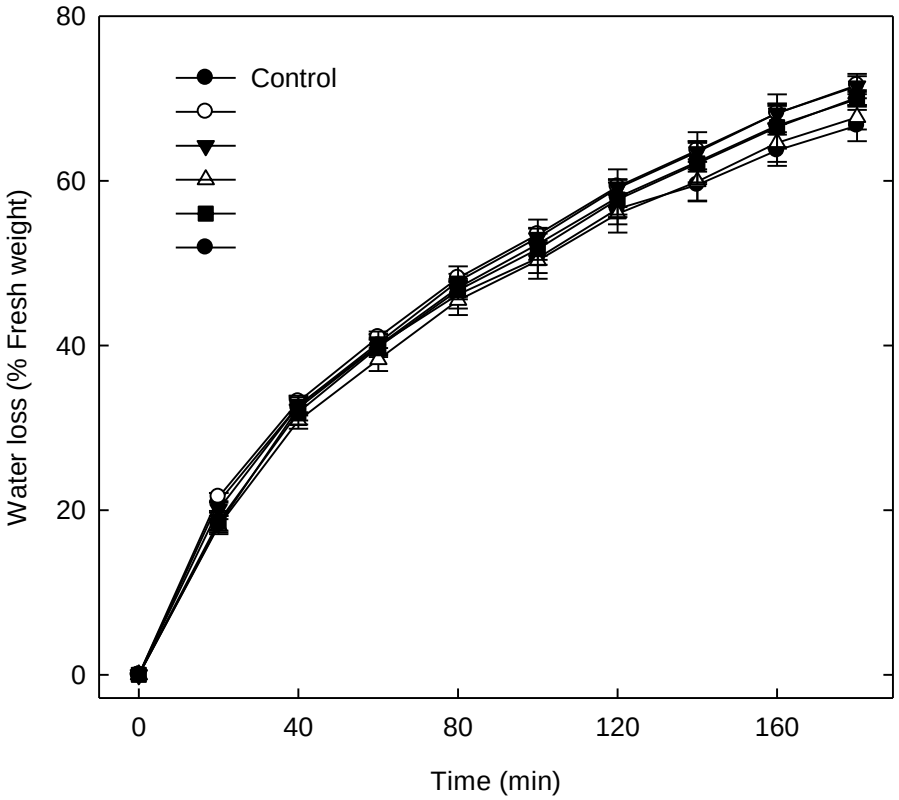

Figure 4 - SNP does not reduce water loss produced by water stress. Leaves at the same developmental stage and size from 4-week-old plants (Col-0) were floated in MES-KCl buffer to induced stomatal apertures. After that, leaves were incubated in SNP (10-150 µM) for 2.5 h. The water loss of detached leaves was measured by weighing fresh leaves exposed to a direct light source (50 cm from a 60 Watt bulb) on the laboratory bench. Water loss was expressed as the percentage of initial weight at each time point. Data are means of two independent experiments with 4 replicates (Petri dishes with 4 leaves) for each treatment ± standard errors

NO is not required for ABA-induced stomatal closure in leaves under water stress conditions

The signalling process in guard cell is highly complex and modulated by many stimuli, most notably ABA (Hetherington 2003, Fan et al 2004). Several works have reported that NO is an important signalling intermediate in ABA-induced stomatal closure (Neill et al 2002, Garcia-Mata and Lamattina 2002, She et al 2004). However, a report that ABA have failed to induce NO synthesis in tobacco cell suspension cultures suggested that it may be restricted to specialized ABA target cells such as guard cells (Tun et al 2001). Moreover, it is possible to exist an NO-independent pathway during

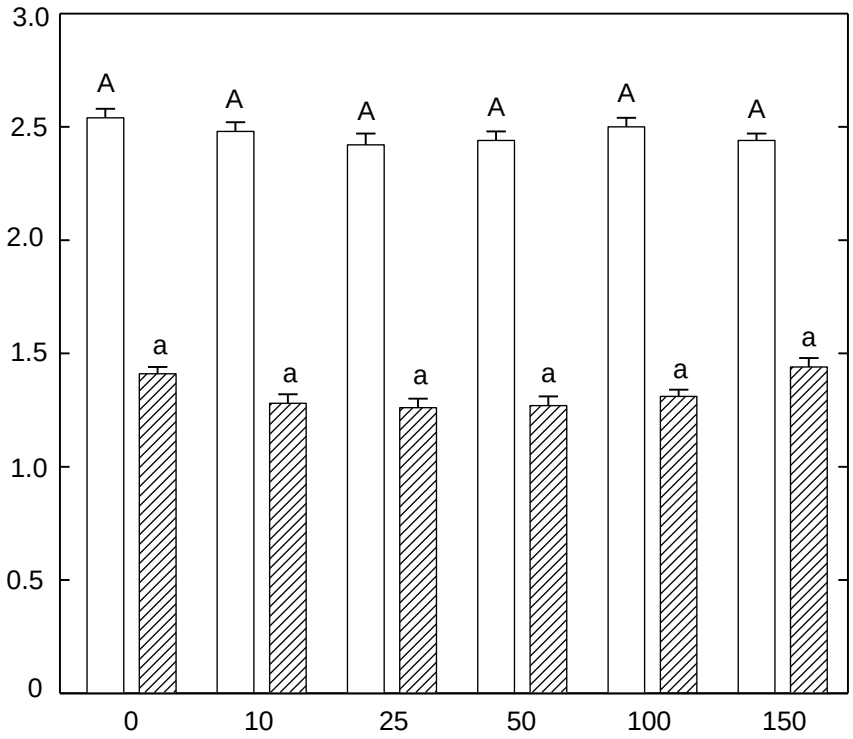

Figure 6 - Sodium ferricyanide does not induce stomatal closure in Arabidopsis leaves. Wild type Arabidopsis (Col-0) leaves were floated in MES-KCl buffer to induce stomatal opening and then incubated in sodium ferricyanide (10-150 µM) for 2.5 h. Stomatal apertures were measured before dehydration (white bars) and 30 min after submitting the leaves the dehydration (striped bars) under a direct light source (50 cm from a 60 Watt bulb) on the laboratory bench. The data are representative of 4 independent experiments with the means of 100 stomata ± standard errors

the process of response to ABA. ABA signalling, by regulating stomatal aperture, plays a crucial role to reduce water loss under water shortage. Different analyses were performed to evaluated response of NO in ABA-induced stomatal closure. Thus, stomatal response to ABA was evaluated by directly measuring stomatal closure in Arabidopsis leaves under water deprivation. ABA induced stomatal closure in a dose-dependent manner, causing significant reduction in stomatal width (Fig 7, white bars) at concentration as low as 0.1 µM ($P < 0.05$ by Tukey test). The dose-dependent manner that ABA-mediated response in Arabidopsis turgid leaves was similar to leaves treated with ABA and submitted to the dry atmosphere for 30 min (Fig 7, striped bars). The maximum significant effect induced by ABA occurred at 10 µM in which stomatal aperture was reduced to 1.48 µm,

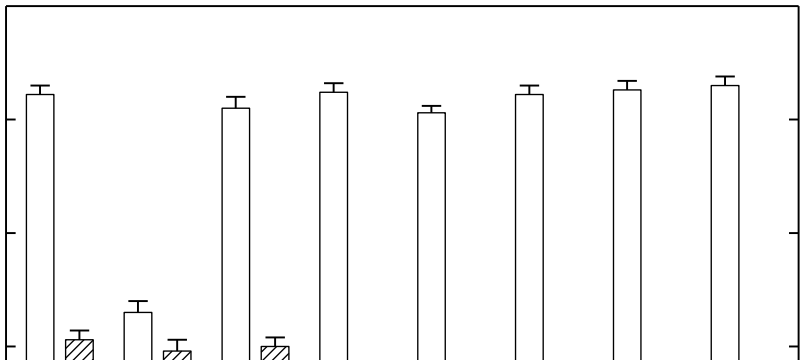

Figure 5 - NO does not induce stomatal closure in Arabidopsis leaves under water stress conditions. Wild type (Col-0) Arabidopsis leaves were floated in MES-KCl buffer under light to induce stomatal opening and then treated with the NO scavenger PTIO (200 µM) in MES-KCl buffer for 30 min prior to treatment with SNP (10-150 µM) for 2.5 h. Stomatal apertures were measured before dehydration (white bars) and 30 min after submitting the leaves to dehydration (striped bars) under a direct light source on the laboratory bench. The data are representative of 4 independent experiments with means of 100 stomata ± standard errors

in turgid Arabidopsis, which correspond to a 42 % reduction as compared to control apertures. On the other hand, in dehydrated leaves the maximum significant effect induced by ABA occurred at 50 µM when stomatal aperture was reduced to 0.88 µM, which correspond to 47 % reduction compared to control apertures. Moreover, ABA induced stomatal closure in a dose-dependent manner that correlated with the ability of ABA to reduce transpiration (Fig 8), that was evaluated by the decline in fresh weight of detached leaves (Verslues et al 2006). The results demonstrated that leaves exposed to ABA (0.1-50 µM) showed an accelerated decrease of the weight loss in comparison to untreated wild type leaves. The maximum significant

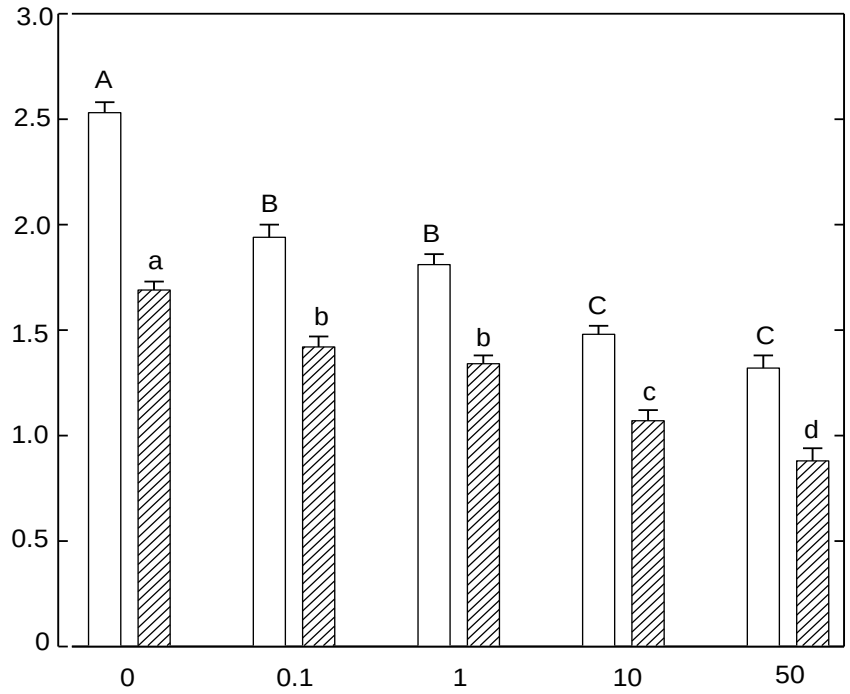

Figure 7 - ABA-induced stomatal closure in wild type Arabidopsis leaves. Wild type Arabidopsis (Col-0) leaves were floated in MES-KCl buffer to induce stomatal opening and then incubated in ABA (0.1-50 µM) for 2.5 h. Stomatal apertures were measured before dehydration (white bars) and 30 min after submitting the leaves to dehydration (striped bars) under a direct light source (50 cm from a 60 Watt bulb) on the laboratory bench. The data are representative of 4 independent experiments with the means of 100 stomata ± standard errors

effect induced by ABA occurred at 50 µM, which led to an approximately 1.3-fold decrease in the water loss within 3 h.

The observation that ABA induces a reduction in stomatal apertures in a concentration-dependent manner in Arabidopsis leaves under water deprivation contrast to the effect of NO, applied as SNP, which failed to induce stomatal closure and to reduce transpiration in leaves under drought stress (Figs 3 and 4). This suggests that NO might not to be required for the ABA-induced stomatal closure during adaptative plant responses to water stress. To determine whether ABA-induced stomatal closure in leaves under water stress does not require NO, Arabidopsis leaves were pre-treated with the NO scavenger PTIO prior to ABA application. PTIO (200 µM) significantly inhibited the effects of ABA in Arabidopsis turgid leaves (Fig 9, white bars; see also Fig 5), as aperture width was not different to control apertures

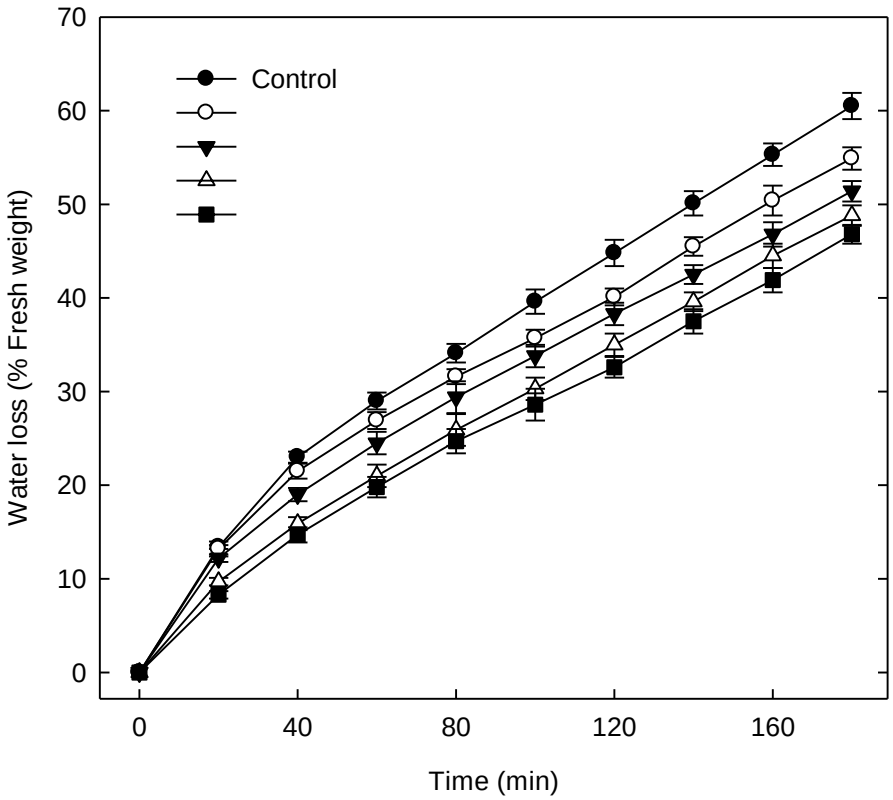

Figure 8 - ABA reduces water loss produced by drought. Leaves at the same developmental stage and size from 4-week-old plants (Col-O) were floated in MES-KCl buffer to induced stomatal apertures. After that, leaves were incubated in ABA (0.1-50 µM) for 2.5 h. The water loss of detached leaves was measured by weighing fresh leaves placed under a direct light source (50 cm from a 60 Watt bulb) on the laboratory bench. Water loss was expressed as the percentage of initial weight at each time point. Data are means of two independent experiments with 4 replicates (Petri dishes with 4 leaves) for each treatment ± standard errors

($P < 0.05$ by Tukey test). These data confirm that NO was required for ABA-induced stomatal closure in non-stressed leaves of Arabidopsis, thereby supporting the findings of previous studies (Neill et al 2002, Garcia-Mata and Lamattina 2002, Desikan et al 2002). On the other hand, ABA-induced stomatal closure was not suppressed by PTIO after submitting the leaves to a drought stress treatment for 30 min (Fig 9, striped bars; see also Fig 5). Furthermore, PTIO alone has no effect on stomatal apertures as compared to controls ($P < 0.05$ by *t* test). These data suggest that NO was not an essential component for ABA-induced stomatal closure in Arabidopsis leaves under drought stress. In keeping with these responses, wild type leaves

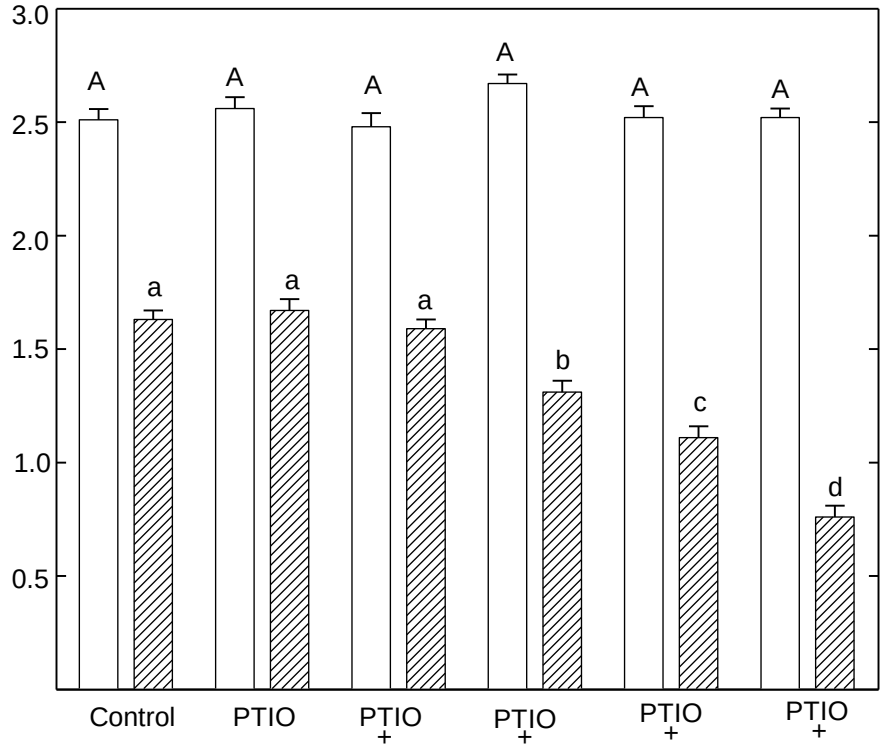

Figure 9 - Negation of the antagonistic effect of the NO scavenger PTIO on ABA-induced stomatal closure in Arabidopsis leaves under water stress conditions. Wild type (Col-0) Arabidopsis leaves were floated in MES-KCl buffer under light to induce stomatal opening and then treated with NO scavenger PTIO (200 µM) in MES-KCl buffer for 30 min prior to treatment with ABA (0.1-50 µM) for 2.5 h. Stomatal apertures were measured before dehydration (white bars) and 30 min after submitting the leaves the dehydration (striped bars) under a direct light source on the laboratory bench. The data are representative of 4 independent experiments with means of 100 stomata ± standard errors

treated with SNP showed reduced relative water content (RWC) as compared to leaves treated with ABA (Fig 10). After 2 h of water stress leaves treated with ABA (50 µM) retain approximately 33 % more water than the control leaves. However, leaves treated with SNP did not exhibit significant difference in the RWC compared with control ($P < 0.05$ by Tukey test). When SNP was added together with ABA, RWC was not significantly different from that measured in leaves treated with ABA alone suggesting that ABA was responsible for inducing tolerance to water stress in Arabidopsis. In agreement with this result, leaves treated with

ABA+SNP+PTIO did not exhibit significantly difference in the RWC as compared with leaves treated with ABA alone (Fig 10). Importantly, RWC in

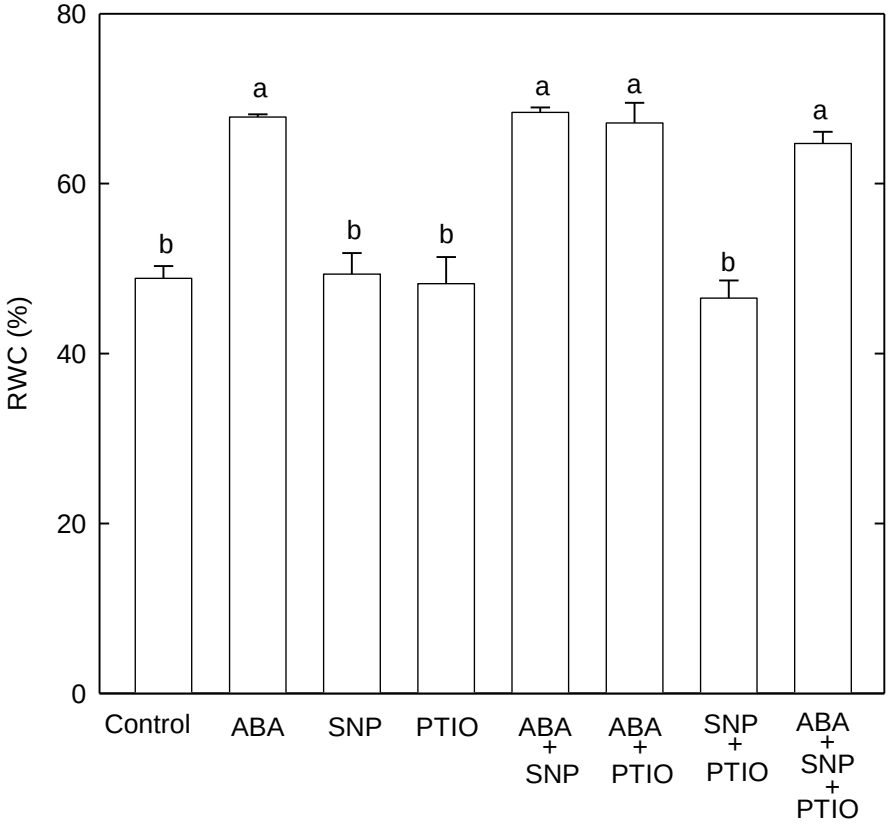

Figure 10 - NO does not protect Arabidopsis leaves against drought. Wild type (Col-0) Arabidopsis leaves at the same developmental stage and size were incubated in 5 ml MES-KCl buffer for 2.5 h under light conditions (60-100 µE m^{-2} s^{-1}) in a growth chamber. After that, leaves were treated with various compounds and incubated in the same buffer for 2.5 h. Drought stress was performed by placing different sets of detached leaves with abaxial side up in open Petri dishes on a white paper under a direct light source (50 cm from a 60 Watt bulb) on the laboratory bench. After 2 h drought stress, RWC measurements were determined, according to the formula: RWC (%) = (FW-DW)/(TW-DW) x 100. The values are average of 4 replicates (Petri dishes with 4 leaves) ± standard errors.

leaves treated with PTIO alone did not differ significantly from control, indicating that alone, PTIO causes no effect on transpiration. In another test of response to ABA, the fresh mass of detached leaves was determined in leaves treated with ABA, ABA+PTIO, SNP and SNP+PTIO during desiccation. Leaves treated with 50 µM SNP did not show a significant reduction in the amount of water loss as compared with control treatment ($P < 0.05$ by *t* test, Fig 11). However, ABA treatment reduced the water loss

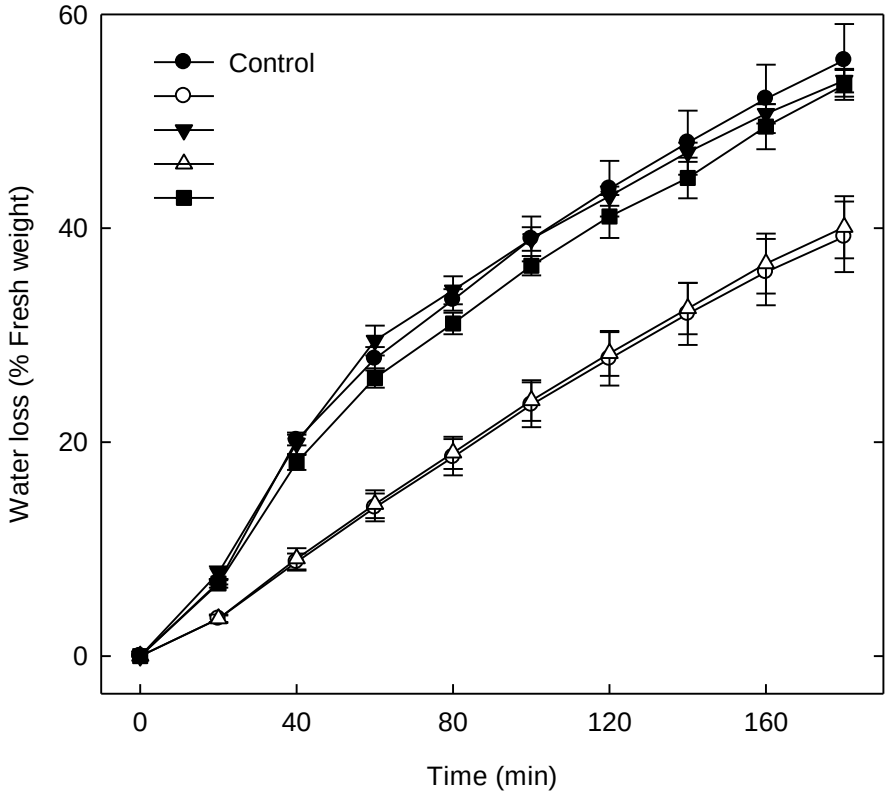

Figure 11 - NO does not reduce water loss caused by water stress. Wild type (Col-0) Arabidopsis leaves at the same developmental stage and size were floated in MES-KCl buffer for 2.5 h under light conditions (60-100 µE m^{-2} s^{-1}) in a growth chamber. After that, leaves were treated with various compounds and incubated in the same buffer for 2.5 h. The transpiration of detached leaves was measured by weighing leaves placed abaxial side up in open Petri dishes with one layer of filter paper under a direct light source (50 cm from a 60 Watt bulb) on the laboratory bench. The weight of each set of leaves was determined every 20 min over a period of 3 h. Water loss was expressed as the percentage of initial fresh weight. The values are the average of 4 replicates (Petri dishes with 4 leaves each) ± standard errors.

in 30 % after 3 h under drought. When combined with ABA, PTIO was not able to prevent the ABA-mediated decrease of the transpiration. Importantly, SNP+PTIO did not cause any effect on transpiration as compared with control leaves. Together, these data suggest that NO is not required for ABA-induced tolerance against water stress.

NO production can be visualized as an increase in fluorescence emission from the cell-permeant probe DAF-2A (4,5-diaminofluorescein diacetate). To demonstrate the effects of ABA on guard cell NO generation, wild type Col-0 epidermal fragments were loaded with DAF-2A and confocal laser scanning microscopy was used to monitor changes in NO-induced

fluorescence in response to treatment with ABA (50 µM) and PTIO. Fig 12 shows that significant NO production occurred at ABA concentration 50 µM, causing a 128 % increase in DAF-2 fluorescence compared to controls. On the other hand, PTIO (200 µM) abolished de DAF-2 fluorescence induced by ABA, significantly reducing NO synthesis to the levels of controls ($P < 0.05$ by Tukey test). Importantly, NO synthesis in epidermal fragments treated with PTIO alone do not differ significantly from controls ($P < 0.05$ by Tukey test) indicating that alone, PTIO has no effect on guard cell NO synthesis (Fig 12). Together, these data show that guard cells generate NO in response to ABA in turgid leaves.

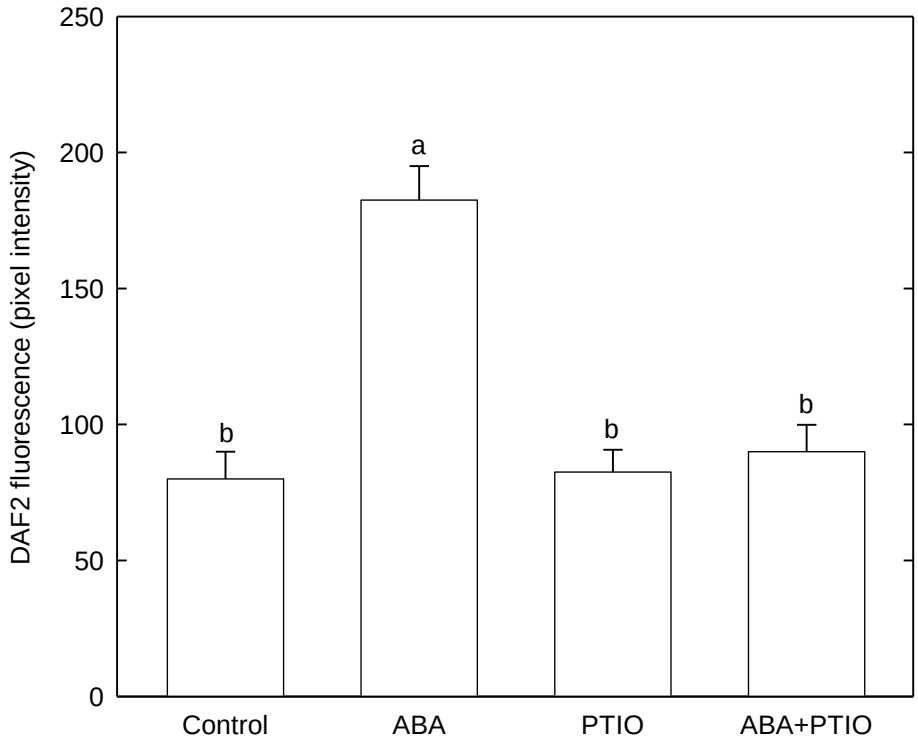

Figure 12 - ABA-induced NO synthesis is abolished by PTIO. Wild type Arabidopsis leaf epidermal fragments were incubated with DAF-2DA in MES-KCl buffer. NO synthesis was monitored 30 min after treatment with 50 µ ABA, 200 µM PTIO, ABA+PTIO and in controls. Confocal microscopy data are displayed as mean pixel intensities of 4 independent replicates $\pm$ standard errors

The observation that ABA-induced stomatal closure in water-stressed leaves does not require NO (see Figs 1, 7 and 9) was investigated in further

nitrate reductase (NR). NOS is a family of well characterized enzymes in mammalian cells that catalyze the conversion of L-arginine to L-citrulline and NO (Alderton 2001). NOS activity was first demonstrated in plant tissues by monitoring the conversion of radiolabelled arginine to radiolabelled citrulline (Cueto et al 1996) and the inhibition of this reaction by the arginine analogues N^6-nitro-L-arginine (L-NNA) and L-NAME (Nimerman and Maier 1996). However, the identified gene and protein (AtNOS1) responsible for arginine-dependent NO synthesis in *Arabidopsis thaliana* shows no sequence similarities to the NOS family of enzymes in animals (Guo et al 2003). NO synthesis in plants has also been attributed to NR. NR is a central enzyme of nitrogen assimilation in plants, catalyzing the transfer of two electrons from nicotinamide-adenine dinucleotide phosphate (NADPH) to nitrate to produce nitrite (Lea 1999). NR also shows the capacity to generate NO from nitrite utilising NADPH as an electron donor (Rockel et al 2002). This function of NR has been demonstrated *in vitro* and *in vivo* in a number of species (Yamasaki and Sakihama 2000, Rockel et al 2002, Dordas et al 2003). Tungstate serves as molybdenum analogue and has been used to block NR activity in plants because NO is thought to be generated by reduction of nitrite at the molybdopterin site (Meyer et al 2005, Deng et al 1989). The NR inhibitors (tungstate) and NOS inhibitor (L-NAME) have been used to demonstrate that NR and NOS are sources of NO in several plant species (Delledonne et al 1998, Garces et al 2001, Neill et al 2002, Bright et al 2006). Incubation of turgid leaves of Arabidopsis with either tungstate or L-NAME alone had no significant effect on the stomatal response (Fig 13, white bars). However, in fully turgid leaves of Arabidopsis ABA-induced stomatal closure was partially inhibited by tungstate. Tungstate (100 µM) caused 27 % inhibition of ABA induced stomatal closure in Arabidopsis leaves under well watered condition (see ABA+Tungstate; Fig 13 white bars). On the other hand, L-NAME had little effect (9 %) on ABA-induced stomatal closure (see ABA+L-NAME) implying that NR can be the predominant source of NO in guard cells exposed to ABA, which is the same as that seen in previous work (Desikan et al 2002, Bright et al 2006). ABA-mediated stomatal closure was not completely abolished by tungstate or L-NAME or by tungstate plus L-NAME. This may be due to the fact that the ABA-induced response is not

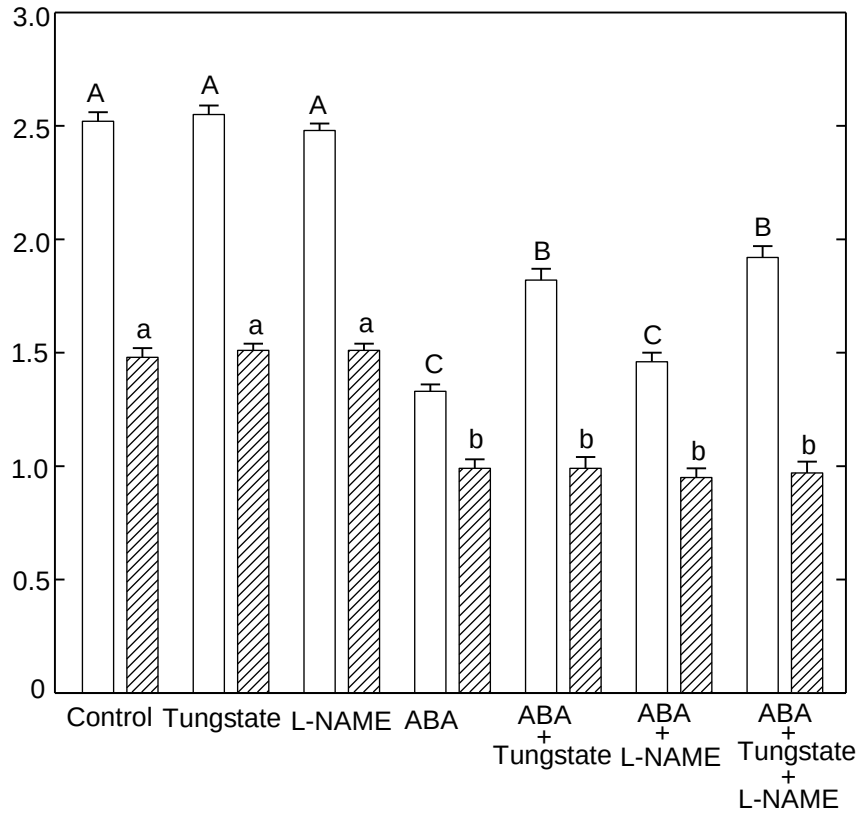

Figure 13 - Negation of the antagonistic effects of tungstate and L-NAME on ABA-induced stomatal closure in Arabidopsis leaves under water stress. Wild type (Col-0) Arabidopsis leaves were floated in MES-KCl buffer under light to induce stomatal opening and then treated with tungstate (100 µM), L-NAME (200 µM) or tungstate plus L-NAME in MES-KCl buffer for 30 min prior to treatment with ABA (50 µM) for 2.5 h. Stomatal apertures were measured before dehydration (white bars) and 30 min after submitting the leaves the dehydration (striped bars) under a direct light source on the laboratory bench. The data are representative of 4 independent experiments with means of 100 stomata ± standard errors

solely dependent on the NR or NOS or, in fact, NO (Figs 13, white bars). Interestingly, the inhibitory effects of tungstate, L-NAME and tungstate plus L-NAME on ABA-induced stomatal closure were completely abolished after submitting the leaves for 30 min to the drying atmosphere of the laboratory, indicating that NO does not play a central role in response to ABA-induced stomatal closure under water stress conditions (Fig 13, striped bars). Taken together, the lack of inhibition by PTIO to ABA-induced stomatal closure in leaves under drought (see Fig 9) and the fact of NO failed to reduce the transpiration in Arabidopsis leaves (see Fig 4) indicate NO as a componet

not required in the ABA signalling cascade in Arabidopsis guard cell under water deprivation.

However, pharmacological experiments could lead to misinterpretations of data due to the possibility of compound-specific side effects that can occur within a biological system. Consequently, a genetic approach was used to substantiate the pharmacologically-based findings.

NR mutations do not affect ABA-induced stomatal closure in leaves under water stress conditions

Two genes in the Arabidopsis genome, *NIA1* and *NIA2*, encode nitrate reductase. NIA2 accounts for 90 % of the total NR activity in seedlings whilst NIA1 accounts for the remaining 10 % (Wilkinson and Crawford 1991); this work, however, relates only to NR reduction of nitrate to nitrite and not to NR-generation of NO. The two genes are approximately 70 % identical in nucleotide sequence and 76 % identical at the amino acid level (Wilkinson and Crawford 1993) The Arabidopsis double mutant, *nia1 nia2*, that has less than 1 % of the NR activity of the wild type (Wilkinson and Crawford 1993), did not emit NO under conditions in which the wild type did (Magalhães et al 2000, Desikan et al 2002). It is also known which of the two *NIA* genes (*NIA1* and *NIA2*) are expressed in guard cells (Bright 2006). The presence of expressed NIA1 and NIA2 further supports the possibility that these enzymes may be involved in guard cell response. Desikan et al (2002) used both pharmacological and genetic approach to show that *nia1 nia2* guard cell did not generate NO in response to ABA or nitrite, nor did *nia1 nia2* stomata closed in response to both stimuli. Importantly, *nia1 nia2* guard cells do respond to SNP indicating that NR lies upstream of NO in the signalling pathway leading to closure. Moreover, Bright (2006) demonstrated that treatment with ABA did not induce NO synthesis in *nia1*::Ds guard cells, suggesting that NIA1 was responsible for NO production and stomatal closure in response to ABA. The guard cells of *nia1::Ds* mutants did not respond to ABA or nitrite whist the guard cells of *nia2-5* mutants responded in the same manner as the wild type Arabidopsis (Bright 2006). As described above, several studies support a major role of NO in modulating the ABA-induced stomatal closure under well watered conditions. However, a general

correlation between ABA-induced stomatal closure, via NO, and drought tolerance has not been well established. Thus, the relationship between ABA and NO in stomatal closure was investigated under drought conditional using NR mutants: *nia1 nia2*; *nia1*::Ds and *nia2-5*.

In guard cells of *nia1 nia2* mutant, which does not produce NO in response to ABA or nitrite (Desikan et al 2002), both ABA and nitrite had little effect on stomatal closure in leaves under well watered conditions (Fig 14). Stomatal apertures were found to be 64 % and 81 % greater in *nia1 nia2* mutants than wild type leaves treated with ABA or nitrite, respectively. On the other hand, in wild type plants ABA and nitrite induced stomatal closure causing a significant reduction in stomatal aperture width ($P < 0.05$ by *t* test). Moreover, incubation in NaCl (as a negative control for sodium nitrite) did not induce stomatal closure, indicating that nitrite effect were not simply ionic (Fig 14). Although it was assumed that nitrate would be converted into nitrite once inside the cell, no stomatal response was observed both in turgid leaves from wild type and *nia1 nia2* plants. One explanation for these results is that nitrate can work as inhibitor of NR-NO activity (Rockel et al 2002). Alternatively, in guard cells, exogenous nitrite is made accessible by NR, whilst nitrate-derivate nitrite may be sequestered into the chloroplast (see Crawford and Forde 2002) before it can be further reduced to NO. To confirm that the insensitivity of *nia1 nia2* stomata to ABA and nitrite was caused by reduced NR activity, leaves were exposed to NO, via incubation in SNP. *nia1 nia2* stomata showed a response similar to wild type stomata (Fig 14). In wild type plants, SNP caused a 49 % reduction in stomatal aperture whereas a 47 % difference in aperture width was observed in *nia1 nia2* plants. To determine if *nia1 nia2* mutant are also altered in ABA-induced stomatal closure in response to desiccation, leaves from wild type and mutant plants were treated with ABA and then placed under dry atmosphere of the laboratory. Both mutant and wild type plants showed similar stomatal closure by ABA (Fig 14), indicating that NR was not required to ABA-induced stomatal closure in dehydrating conditions. In other word, ABA-induced stomatal closure was independent on NO synthesized via NR. This was confirmed because no significant difference ($P < 0.05$ by *t* test) in stomatal closure was observed between wild type and *nia1 nia2* leaves treated with

nitrite, a substrate for NO synthesis (Garcia-Mata and Lamattina 2003, Neill et al 2003). When NO_2^- was replaced by Cl^- or NO_3^- both mutant and wild type plants showed similar stomatal apertures. Moreover, *nia1 nia2* and wild type stomata did not display enhanced stomatal closure when leaves were exposed to NO via incubation in SNP and placed in dry atmosphere (Fig 14), indicating ABA-induced stomatal closure does not requires NO synthesis.

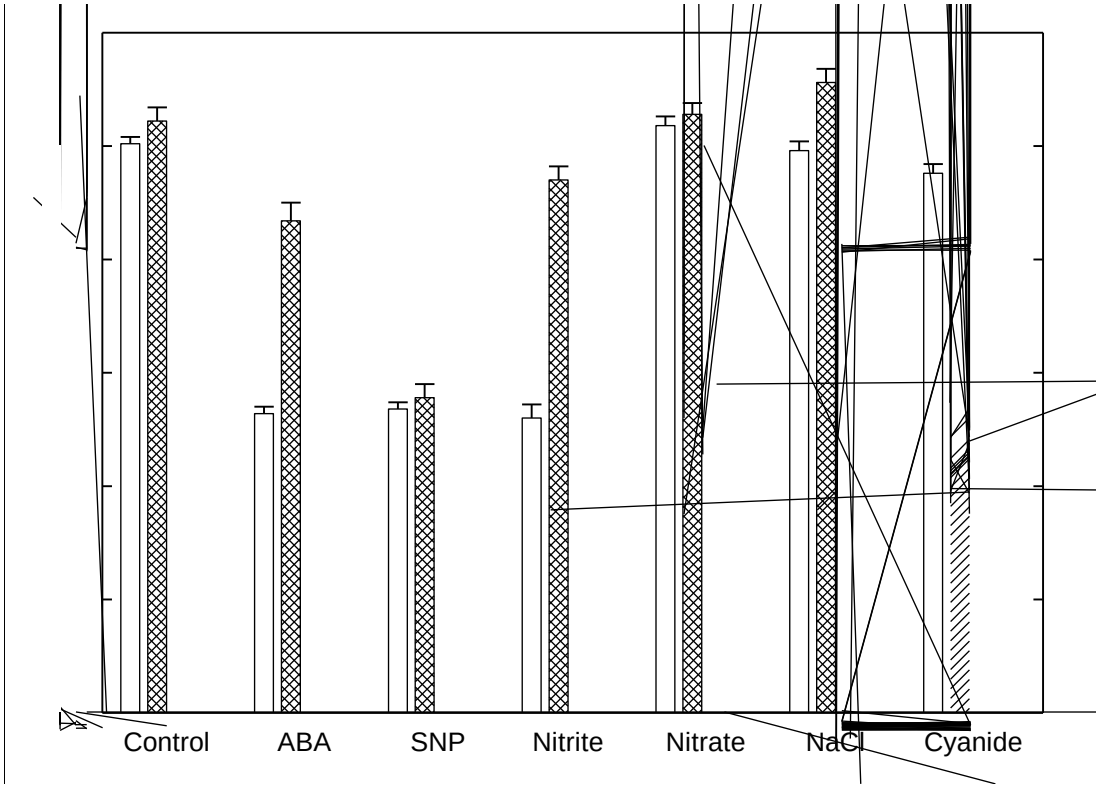

Figure 14 - ABA-mediated stomatal closure is impaired in *nai1 nia2* Arabidopsis turgid leaves but not in leaves under water stress conditions. Leaves from wild-type (Col-0) or *nia1 nia2* plants were floated in MES-KCl buffer under light to induced stomatal aperture. Afterwards, leaves were incubated for 2.5 h in buffer alone (control), 50 µM ABA, 50 µM SNP, 1 mM nitrite, 50 µM sodium ferricyanide, 1mM nitrate or 1mM NaCl. Stomatal apertures were measured before dehydration (white bars, Col-0; checkered bars, *nia1 nia2*) and 30 min after submitting the leaves to dehydration (striped bars, Col-0; black bars, *nia1 nia2*) under a direct light source on the laboratory bench. The data are representative of 4 independent experiments with means of 100 stomata $\pm$ standard errors

Further confirmations that the loss of NR does not suppress stomatal closure induced by ABA in leaves under water stress were obtained with two single NR mutants; a NIA1-null mutant (*nia1*::Ds) which has a stable T-DNA dissociation (*Ds*) element insertion in the *NIA1* gene (Parinov et al 1999) and display no NIA1 activity (Wang et al 2004) and a NIA2-null mutant (*nia2-5*)

which has a y-irradiation-mediated deletion in the *NIA2* gene (Wilkinson and Crawford 1991). ABA-mediated closure was 68 % less effective in *nia1*::Ds leaves compared to the wild type response, under well watered condition (Fig 15). In turgid leaves from wild type plants, nitrite caused a 51 % reduction

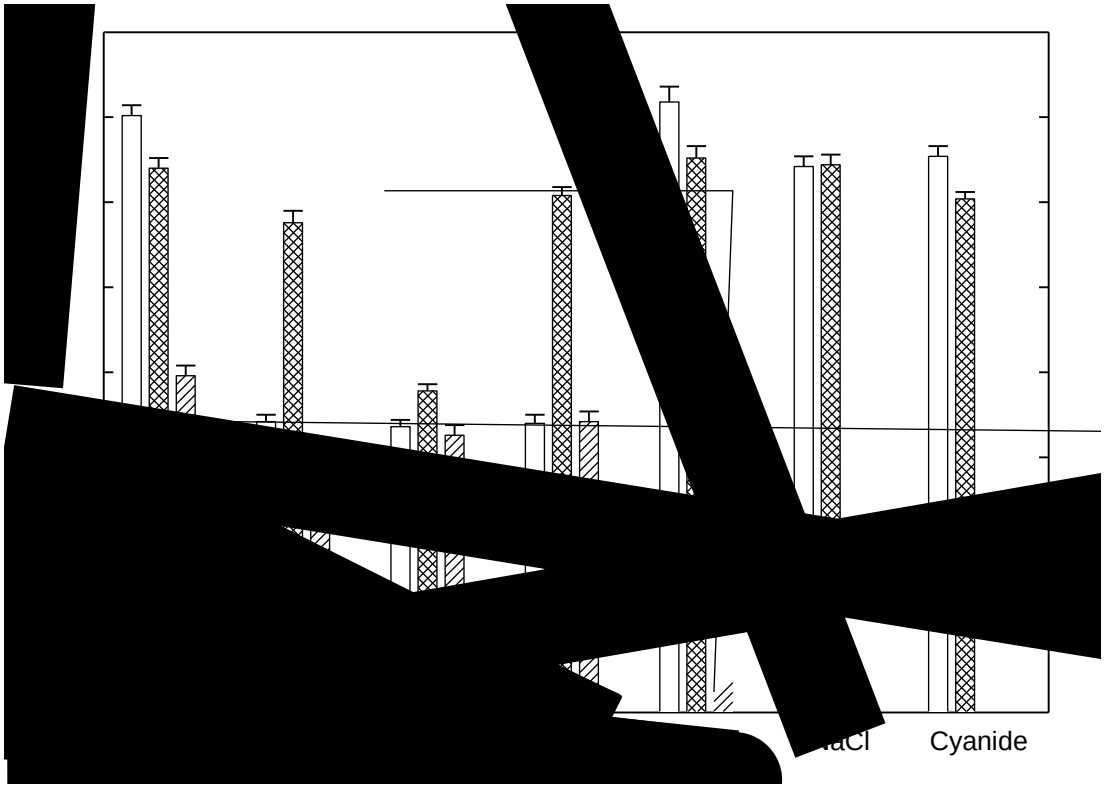

Figure 15 - ABA-mediated stomatal closure is impaired in turgid leaves from *nia1::Ds* plants but not in leaves under water stress conditions. Leaves from wild-type (Ler) or *nia1*::Ds plants were floated in MES-KCl buffer under light to induced stomatal aperture. Afterwards, leaves were incubated for 2.5 h in buffer alone (control), 50 µM ABA, 50 µM SNP, 1 mM nitrite, 50 µM sodium ferricyanide, 1mM nitrate or 1mM NaCl. Stomatal apertures were measured before dehydration (white bars, Ler; checkered bars, *nia1*::Ds) and 30 min after submitting the leaves the dehydration (striped bars, Ler; black bars, *nia1*::Ds) under a direct light source on the laboratory bench. The data are representative of 4 independent experiments with means of 100 stomata $\pm$ standard errors

in stomatal aperture compared to control. When NO_2^- was replaced with Cl^- or NO_3^- no significant difference in stomatal apertures ($P < 0.05\%$ by *t* test) was observed in relation to control indicating that stomatal closure was dependent on nitrite. However, nitrite-induced stomatal closure was abolished in mutant plants such that the mean aperture width observed in *nia1*::Ds plants was not significantly different from control aperture ($P < 0.05$ by *t* test). These data demonstrate that NR1 was required to generate NO

that subsequently mediated stomatal responses. In agreement with this result, stomatal of mutant leaves responded to SNP in the same manner as stomata of wild type suggesting that NR1 lies upstream of NO in the signalling pathway leading to closure in leaves under well watered conditions. Importantly, sodium ferricyanide (as a negative control for SNP) did not induce closure both in wild type and mutant leaves, indicating (Fig 15) again that NO synthesis is required for stomatal closure in non-stressed leaves. In keeping with these data, nitrite treatment did not induce NO generation in *nia1*::Ds guard cells. DAF2 fluorescence did not significantly alter with nitrite treatment compared to control. However, nitrite induced an 80 % increase in DAF2 fluorescence in wild type guard cells compared to control treatment (Fig 16).

The effect of NIA1 mutation was analyzed on ABA-induced stomatal closure under water deprivation. To this end, wild type and *nia1*::Ds Arabidopis leaves were treated with ABA and after submitting the leaves to drying atmosphere of the laboratory for 30 min stomatal closure was assayed in leaves from wild type and *nia1*::Ds plants. Results demonstrated that ABA-induced stomatal closure was not affected by the NIA1 mutation in water-stressed leaves (Fig 15). There was no significant difference between the apertures of stomata in wild type and *nia1*::Ds leaves treated with ABA ($P < 0.05$ by *t* test) suggesting that ABA-induced stomatal response presumably occurred via a NO-independent mechanism. In agreement with this result, nitrite-induced stomatal closure was abolished in stressed leaves of wild-type plants such that the mean aperture width observed in wild type leaves treated with nitrite or NaCl (as a control to nitrite) was not significantly different from control apertures ($P < 0.05$ by *t* test), i.e., wild type leaves treated with buffer alone. Moreover, *nia1*::Ds stomata responded to NO, applied as SNP, and cyanide (as a control for SNP decomposition) in the same manner as wild type stomata of stressed leaves treated with NO or sodium ferricyanide (Fig 15).

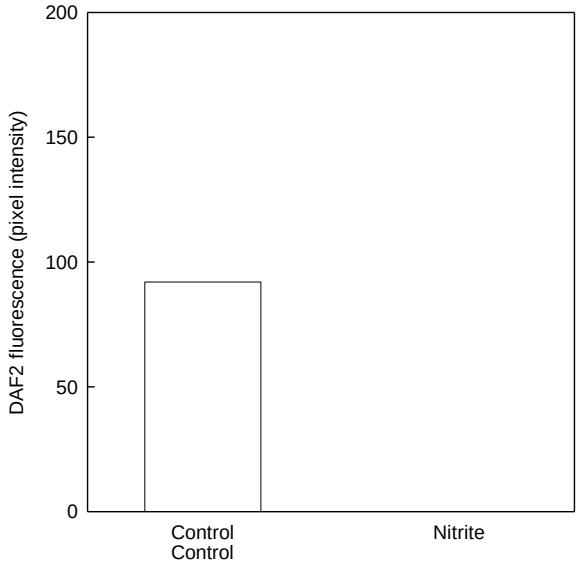

Figure 16 - Nitrite do not induce NO synthesis in *nia1*::Ds guard cells. Wild type (Ler) and *nia1*::Ds Arabidopsis leaf epidermal fragments were incubated with DAF2-DA in MES-KCl buffer. Fragments were treated with 1 mM nitrite. NO synthesis was monitored 30 min after treatment with nitrite and controls. Confocal microscopy data are displayed as mean pixel intensities of 4 independent replicates ± standard errors

ABA-, SNP- and nitrite-induced stomatal closure was not abolished in fully turgid leaves from mutant plants such that the stomatal aperture width observed in *nia2-5* leaves was significantly different ($P < 0.05$ by *t* test) from control apertures (Fig 17), which is consistent with the previous report by Bright (2006). *nia2-5* stomata responded to ABA, SNP and nitrite in the same manner as wild type stomata, indicating that NR2 functions is not required for NO signalling in guard cells, under well watered conditions. Consistent with these findings, nitrate and NaCl (as negatives controls to nitrite) did not induce stomatal closure in leaves of *nia2-5* mutant and wild type indicating that stomatal closure was dependent on NO_2^-. Moreover, sodium ferricyanide (as a negative control for SNP) caused no significant effect on stomatal apertures as compared to controls ($P < 0.05$ by *t* test) showing that NO released from SNP induced stomatal closure (Fig 17).

Under water stress condition, stomata of wild type plants and *nia2-5* mutant did not respond significantly ($P < 0.05$ by *t* test) to SNP and nitrite. However, ABA induced stomatal closure caused a reduction of 34 % and 23 % in stomatal aperture in water stressed leaves of *nia2-5* and wild type plants compared to fully turgid leaves of wild type and *nia2-5* plants treated

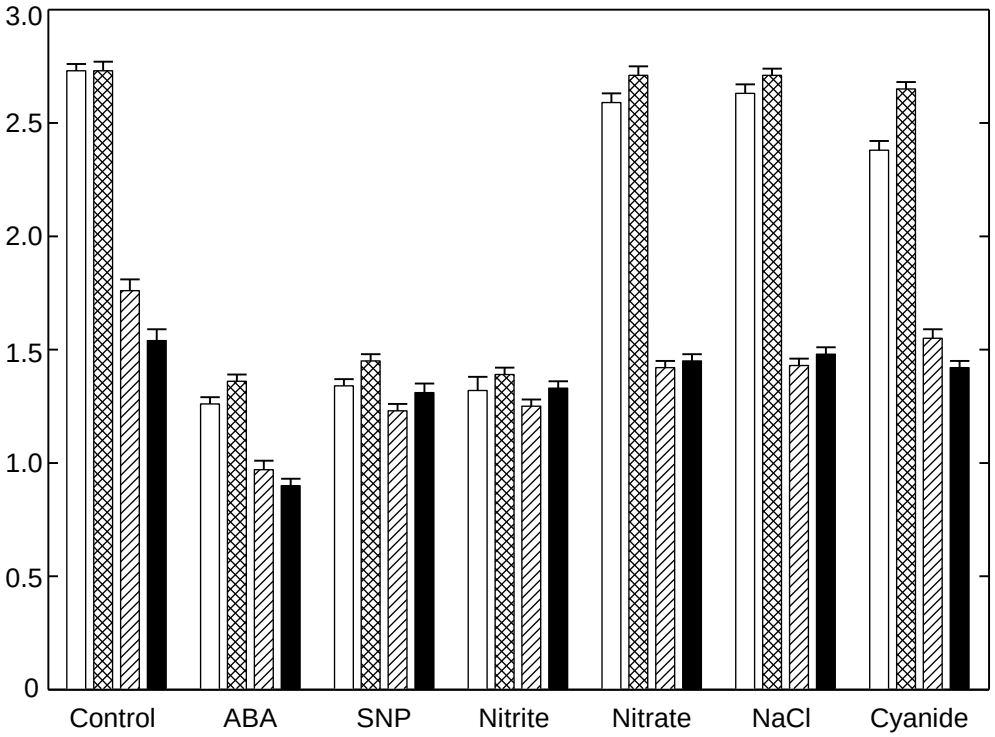

Figure 17 - Negation of the effect of SNP and nitrite on the stomatal closure in leaves from *nia2-5* plants under stress conditions. Leaves from wild-type (Col-0) or *nia2-5* plants were floated in MES-KCl buffer under light to induced stomatal aperture. Afterwards, leaves were incubated for 2.5 h in buffer alone (control), 50 µM ABA, 50 µM SNP, 1 mM nitrite, 50 µM sodium ferricyanide, 1mM nitrate or 1mM NaCl. Stomatal apertures were measured before dehydration (white bars, Col-0; checkered bars, *nia2-5*) and 30 min after submitting the leaves the dehydration (striped bars, Col-0; black bars, *nia2-5*) under a direct light source on the laboratory bench. The data are representative of 4 independent experiments with means of 100 stomata ± standard errors

with ABA. The effects of ABA-induced stomatal closure under water stress on *nia2-5* was similar to those of *nia1*::Ds and *nia1 nia2* double mutant, revealing that a functional NR is not required for ABA-induced stomatal closure in Arabidopsis leaves subject to water shortage. Previous studies have demonstrated that NR-mediated NO synthesis is required for ABA-induced stomatal closure (Desikan et al 2002, Neill et al 2002, Garcia-Matta and Lamattina 2002) under well watered conditions. These results were confirmed here and it was shown that NO likely is not an essential component in guard cell under water deprivation. Other works have also shown no dependence on NR-mediated NO synthesis. NO accumulation in the NR double mutant was similar to wild type during bacterial infection

(Zhang et al 2003). Mechanical stress-induced NO formation in Arabidopsis was reported to be inhibited by NOS inhibitors but not reduced in *nia1 nia2* mutant (Garcês et al 2001). In addition, Modolo et al (2006) reported that the *nia1 nia2* mutant plants displayed reduced levels of nitrite and free amino-acids, particularly L-arginine, other important substrate for NO synthesis. Thus, the effects of L-arginine on stomatal responses were examined in leaves of wild type and *nia1 nia2* mutant plants under well watered conditions.

Nitrite and L-arginine induce stomatal closure in wild type but not *nia1 nia2* mutant

Desikan et al (2002) showed that in the *nia1 nia2* Arabidopsis NR-deficient mutants nitrite did not induce NO synthesis and stomatal closure. Bright (2006) also found that in detached leaves of *nia1 nia2* double mutants, nitrite-induced stomatal closure and NO synthesis was abolished. These results indicated that NR is the predominant source of NO in guard cell. On the other hand, Modolo et al (2006) reported that *nia1 nia2* leaves had reduced levels of free amino acids, with the content of L-arginine (substrate for NO synthesis) being 10-fold lower in double mutants than in wild type plants. Thus, the double mutant was used to determine the effect of L-arginine on stomatal movements. As expected nitrite failed to induce stomatal closure in leaves of *nia1 nia2* mutants, whereas there was a clear dose-response in the wild type plants (Fig 18). The maximum effect induced by applied nitrite occurred at 1 mM, whereas stomatal aperture was reduced to 1.35 µm (Fig 18, white bars; $P < 0.05$ by Tukey test) which corresponds to a 40 % reduction in width compared to control apertures. A similar pattern of changes in stomatal aperture was observed in the wild type plants treated with L-arginine (Fig 19, white bars). Arginine induced dose-dependent stomatal closure in wild type Arabidopsis leaves which was significant at a concentration of L-arginine $\geq$ 0.1 mM ($P < 0.05$ by Tukey test). The maximum promotion of stomatal closure was observed after treatment with 1 mM L-arginine, in which conditions stomatal apertures were 1.33 µm, or 44 % of the control value (Fig 19, white bars). In contrast to wild type plants, *nia1 nia2* stomata were far less sensitive to L-arginine. There was no significant

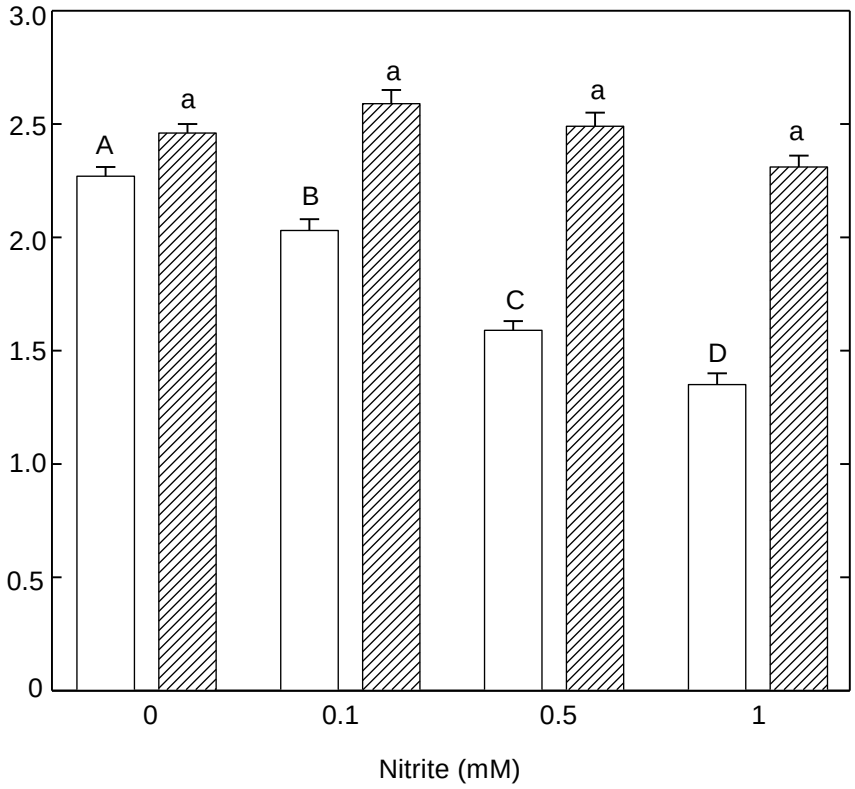

Figure 18 - Nitrite does not induce stomatal closure in *nia1 nia2* NR-deficient mutant. Leaves of either wild type (white bars) or *nia1 nia2* (striped bars) were floated in MES-KCl buffer under light to induced stomatal aperture, and then incubated in nitrite (0.1-1 mM). Stomatal apertures were measured after 2.5 h. The data are representative of 4 independent experiments with means of 100 stomata ± standard errors

difference ($P < 0.05$ by Tukey test) between the apertures of stomatal in *nia1 nia2* leaves treated with L-arginine (0.1-1.0 mM) as compared to control (Fig 19, striped bars). These data suggest that NR might be one of several sources that contribute to NO-mediated stomatal response. In other words, the insensitivity of *nia1 nia2* stomata to arginine and nitrite reflects that the sources that contribute to NO-mediated responses in guard cells of NR-mutant can be classified as nitrite-mediated and arginine-mediated (Crawford 2006). Nitrite-mediated source include NR and the arginine-mediated source would be nitric oxide synthase (NOS). A test of the multiple source models provided evidence for NR and NOS participating in NO production during sorgum germination (Simontacchi et al 2004).

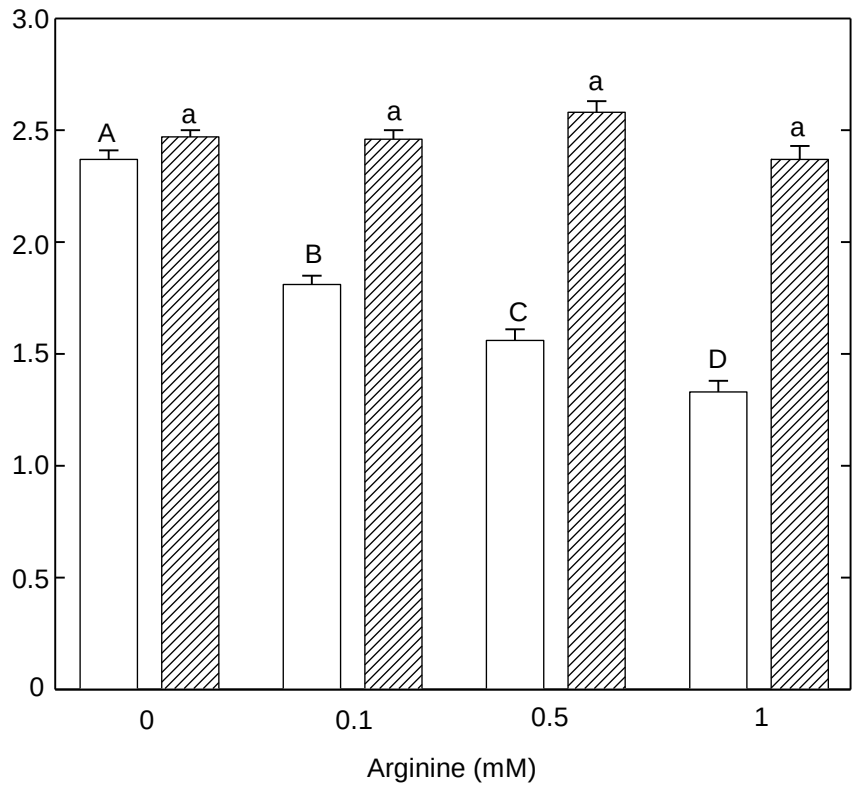

Figure 19 - Arginine does not induced stomatal closure in *nia1 nia2* NR-deficient mutant. Leaves of either wild type (white bars) or *nia1 nia2* (striped bars) were floated in MES-KCl buffer under light to induced stomatal aperture, and then incubated in arginine (0.1-1 mM). Stomatal apertures were measured after 2.5 h. The data are representative of 4 independent experiments with means of 100 stomata ± standard errors

In order to establish that nitrite- and L-arginine-mediated stomatal closure was related to NO synthesis, wild type and *nia1 nia2* leaves were treated with PTIO prior to nitrite plus arginine application. Arginine and nitrite application closed significantly the stomata in wild type plants but not *nia1 nia2* mutant (Fig 20, $P < 0.05$ by *t* test), which is the same as the results reported previously (see Figs 18 and 19). When arginine plus nitrite were applied after treatment with PTIO to the wild type leaves, arginine- and nitrite-induced stomatal closure was suppressed. PTIO (200 µM) significantly inhibited the effects of arginine plus nitrite, as stomatal apertures width were not different from control apertures ($P < 0.05$ by *t* test). On the other hand, arginine plus nitrite failed to induce stomatal closure in leaves of *nia1 nia2* mutants. Treatments of the leaves with arginine plus nitrite together with PTIO did not cause any changes of stomatal aperture in *nia1 nia2* plants (Fig 20, striped bars). Together, these data suggest that arginine and nitrite

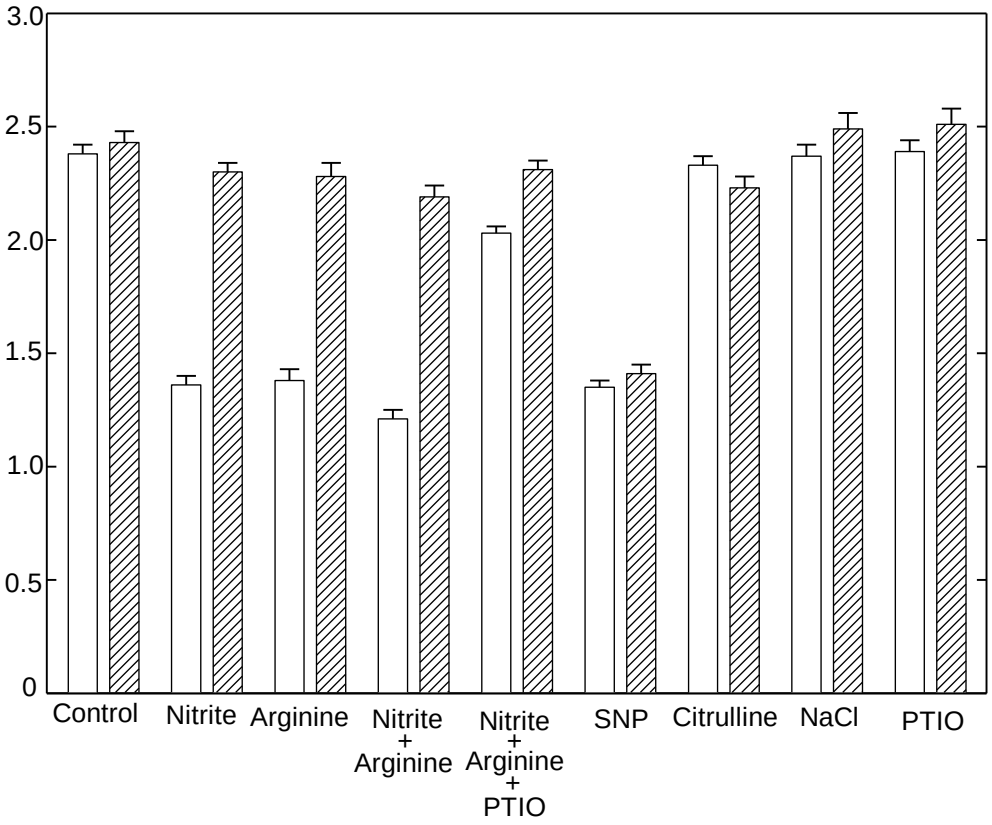

Figure 20 - PTIO treatment impairs stomatal closure induced by nitrite and arginine treatment. Leaves from wild type (Col-0, white bars) or *nia1 nia2* (striped bars) plants were floated in MES-KCl buffer under light to induced stomatal aperture. Afterwards, leaves were incubated in buffer alone, 1 mM nitrite, 1mM arginine, nitrite+arginine, nitrite+arginine+PTIO, 50 µM SNP, 1 mM cintrulline, 1 mM NaCl or 200 µM PTIO and stomatal apertures measured after 2.5 h. The data are representative of 4 independent experiments with means of 100 stomata ± standard errors

promotes stomatal closure in leaves under well watered conditions via a pathway involving NO. To confirm that the insensitivity of *nia1 nia2* stomata to arginine and nitrite reflects their reduced NO synthesis, leaves were exposed to NO via incubation in SNP. *nia1 nia2* stomata showed a response similar to wild type stomata. In wild type plants, SNP caused a 43 % reduction in stomatal aperture whereas a 42 % difference in aperture width was observed in *nia1 nia2* plants (Fig 20). PTIO caused significant inhibition of SNP-induced stomatal closure in both wild type and *nia1nia2* plants, suggesting that insensitivity of *nia1 nia2* stomata to arginine and nitrite correlated with their inability to generate NO. Importantly, incubation in NaCl (as a negative control for sodium nitrite) and citrulline (as a negative control

for arginine) did not induce stomatal closure both in wild type as in the *nia1 nia2* plants indicating that nitrite and arginine are two important substrates for NO synthesis.

Desikan et al (2002) showed that a NR-defective mutant (*nia1 nia2*) is impaired in NO-mediated stomatal closure induced by ABA. Other papers have also supported the involvement of NR-mediated NO synthesis (Xu and Zhao 2003, Simontacchi et al 2004), while others have questioned it (Garces et al 2001, Zhang et al 2003). Under well watered conditions, *nia1 nia2* stomata did not close in response to treatment with nitrite or arginine, even at concentration that induced nearly complete closure in wild plants (Figs 18, 19 and 20). This observation provides evidence for both nitrite- and arginine-mediated NO synthesis in NR double mutant. Thus, cautions are needed about using NR mutants to assess the involvement of NR and nitrite in NO synthesis. Several other nitrite-dependent mechanisms have been reported for NO synthesis. For example, plant mitochondria make NO from nitrite (Tischner et al 2004, Planchet et al 2005). Nitrite is also converted to NO at acidic pH with a reductant compound such as ascorbic acid. Such conditions exist in the apoplasm and have been used to explain NO effects on germinating seeds (Belegni and Lamattina 2000, Bethke et al 2004 a, b).

Phosphorylation events also play a key role in the regulation of NR activity and it is widely accepted that ABA-induced stomatal closure is regulated by phosphorylation-dephosphorylation events (Nilson and Assmann 2007). Arabidopsis *ABI1* and *ABI2* genes encode homologous proteins that belong to the type-2C class of serine/threonine protein phosphatases (PP2C). The action of the two PP2C enzymes, ABI1-1 and ABI2-1, do not seem to be required for NO synthesis, because guard cells of both *abi1-1* and *abi2-1* were able to generate NO in response to ABA, but their somata did not close in response to treatment with ABA or NO (Desikan et al 2002). Thus, the potential role of the PP2C enzymes in ABA-induced NO signalling during stomatal responses was investigated in *abi1-1* and *abi2-1* plants under drought conditions.

ABA and NO signalling in ABA-insensitive mutants

Protein phosphatases type 2C (PP2Cs) were identified as components of ABA signalling pathways in works with the ABA-insensitive *abi1-1* and *abi2-1* mutants (Koornneef et al 1984; Leung et al 1994, 1997, Rodriguez et al 1998). Actually, at least four Arabidopsis (*Arabidopsis thaliana*) PP2Cs, ABI1, ABI2, PP2CA and HAB1 (formerly named AtP2C-HA), are known to regulate ABA signalling. Among Arabidopsis PP2Cs, ABI1 is the best-studied second messenger in guard cells. The first ABI1 mutant to be characterized was the dominant-negative mutant *abi1-1* (Koornneef et al 1984, Leung et al 1994, Meyer et al 1994). This mutant exhibits a strong ABA-insensitive, wilty phenotype, accompanied by elevated ABA-insensitive stomatal conductance (Koornneef et al 1989, Assmam et al 2000). Evidence on role of ABI1 as negative regulator of ABA signalling has been provided by genetic approaches. Intragenic revertant recessive mutants and insertional mutants of ABI1 were isolated (Gosti et al 1999, Mishra et al 2006, Saez et al 2006). These mutants exhibit a moderate ABA sensitivity in stomatal regulation that was strongly enhanced in double mutants created with the related PP2C genes ABI2 or HAB1 (Merlot et al 2001, Saez et al 2004, 2006). In conclusion, the proteins phosphatases 2C play an important role in ABA-induced stomatal movements (Schroeder et al 2001). It has also been shown that in the *abi1-1* and *abi2-1* mutants that are ABA insensitive in response to stomatal closure, NO synthesis still occurs in response to ABA (Desikan et al 2002). Thus, in order to investigate further the importance of ABA-induced NO signalling during stomatal responses, the effects ABA and NO on stomatal responses in wild type (Ler) and *abi1-1*, *abi2-1* mutant were compared.

Under well watered conditions, stomata of both *abi1-1* and *abi2-1* did not close in response to treatment with ABA or

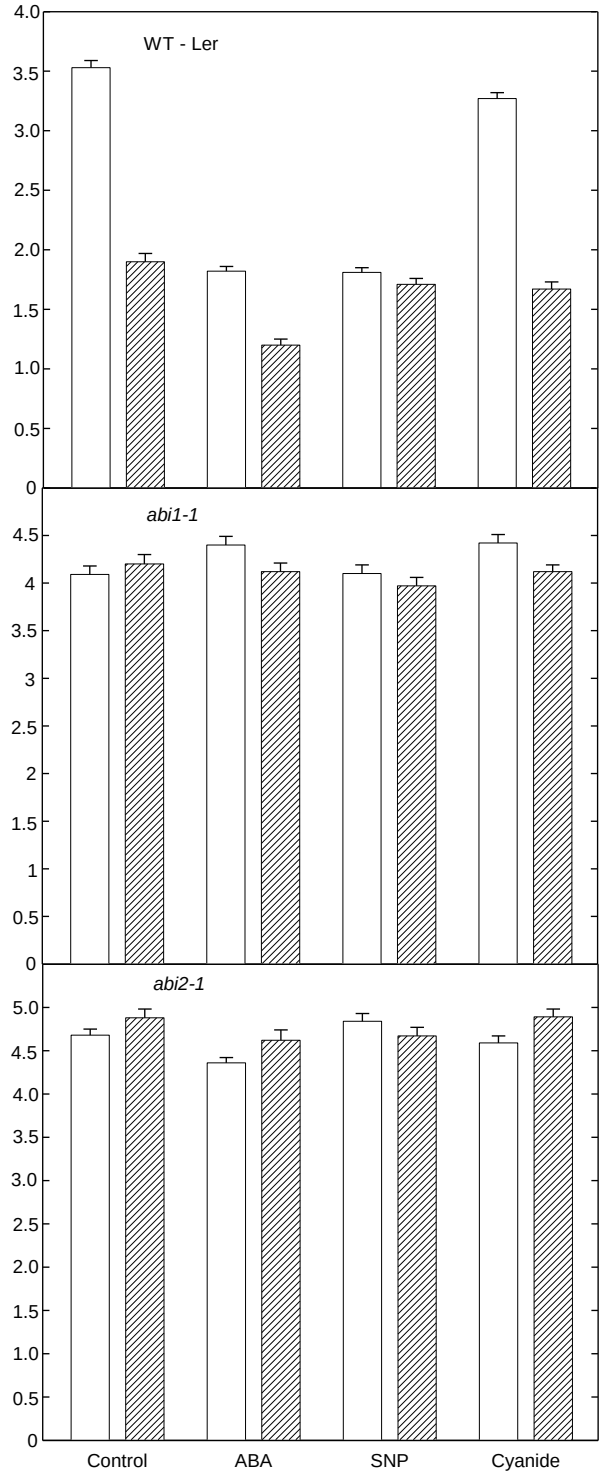

Figure 21 - SNP and ABA-mediated stomatal closure is impaired in leaves from *abi1-1* and *abi2-1*. Leaves from wild-type (Ler), *abi1-1* or *abi2-1* plants were floated in MES-KCl buffer under light to induced stomatal opening and then incubated for 2.5 h in buffer alone (control), 50 µM ABA, 50 µM SNP or 50 µM sodium ferricyanide. Stomatal apertures were measured before dehydration (white bars, Ler, *abi1-1* and *abi2-1*) and 30 min after submitting the leaves to dehydration (striped bars, Ler, *abi1-1* and *abi2-1*) under a direct light source on the laboratory bench. The data are representative of 4 independent experiments with means of 100 stomata ± standard errors

whereas just a 5 % difference in aperture width was observed in response to NO (Fig 21). In wild type plants, stomatal closure responses to ABA and NO under water deprivation were as previously described in this work (see Figs 3 and 7). However, in *abi1-1* and *abi2-1* mutants treated with ABA no significant changes in stomatal aperture occurred under water stress conditions compared to controls ($P < 0.05$ by *t* test). These data suggest that ABA signalling in guard cells operates through of the PP2C enzymes, ABI1-1 and ABI2-1. In agreement with these results, the loss of ABI1 resulted in hypersensitivity to ABA in growth, stomatal closure and induction of ABA-responsive genes (Saez et al 2006). Similarly, *abi1-1* and *abi2-1* stomata were far less sensitive to NO, demonstrating that NO was not able to bypass the effects of *abi1-1* and *abi2-1* mutations.

ABA synthesis is also important for defence signalling against water stress (Hasegawa et al 2000). Consequently, mutant impaired in ABA biosynthesis (*aba1*) was investigated for their guard cell response to NO.

Stomatal response of *aba1-1* mutant to NO and ABA

ABA deficient 1 mutant (*aba1*) in Arabidopsis was identified in a screen for suppressor mutations of the gibberellin-deficient mutant, *ga1* (Koornneef et al 1982). The *aba1-1* mutant is impaired in the production of the epoxy-carotenoid precursors of ABA (Rock and Zeevaart 1991) and has an ABA biosynthetic rate that is approximately 3 % of wild type. Because ABA induces a rapid increase in NO synthesis (Neill et al 2002, García-Mata and Lamattina 2002) and it is known to be involved in plant responses to various environmental stresses (Beligni and Lamattina 2001, Orozco-Cárdenas and Ryan 2002), the availability of *aba1-1* mutant has provided valuable opportunities to investigate the role of NO in plant stress responses.

Under well watered conditions, NO-induced stomatal closure was relatively reduced in *aba1-1* mutant plants compared to control ($P < 0.05$ by *t* test), as NO mediated only a 14% reduction in stomatal aperture in these plants (Fig 22), whereas there was a clear reduction (50 %) in stomatal aperture in wild type leaves treated with SNP, a NO donor. These results suggest that the reduced effects of NO-induced stomatal closure in *aba 1-1* plants resulted from the decreased synthesis of ABA. Importantly, sodium

ferricyanide (as a control for SNP) did not induce closure both in *aba1-1* and wild type plants, indicating that SNP induced stomatal closure was mediated via NO release and not by the release of any other SNP-related breakdown product (Fig 22). Stomatal closure in response to ABA was also observed both in fully turgid leaves of *aba1-1* mutant and wild type plants. ABA induced 45 % decrease in stomatal apertures in *aba1* guard cells as compared to a 55 % decrease in stomatal aperture in wild type plants (Fig 22), showing that ABA-induced stomatal closure was restored by ABA treatment in *aba1-1* mutant. In contrast, SNP-treated *aba1-1* plants showed an NO-induced stomatal closure response similar to control ($P < 0.05$ by *t* test). These data suggest that a functional ABA synthesis was necessary for NO-induced stomatal closure under well watered conditions and apparently, NO did not prime the accumulation of ABA. Priming of ABA biosynthesis can be obtained by direct overexpression of 9-cis-epoxycarotenoid dioxygenase, a key enzyme in the biosynthetic pathway (Iuchi et al 2001, Qin and Zeevaart 2002), or through the use of chemicals that accelerate ABA accumulation (Jakab et al 2005).

In water stressed leaves, stomatal apertures of control treatment were found to be 39 % greater in *aba1-1* mutant than in wild type (Fig 22). For leaves treated with ABA wild type and mutant stomata closed to the same extent, stomatal apertures for wild type and *aba1-1* mutant being 1.39 and 1.37 µm respectively. On the other hand, SNP failed to induce stomatal closure in *aba1* mutant. This deficiency was dependent on NO, because no significant difference ($P < 0.05$ by *t* test) in stomatal aperture was observed between mutant and wild type guard cells if SNP was replaced with cyanide (Fig 22). Together, these data reaffirm the hypothesis that in water stressed leaves ABA-induced stomatal closure does not require NO, but in fully turgid leaves NO may constitute a signal mediating ABA-induced stomatal closure (Neill et al 2002, Garcia-Mata and Lamattina 2002).

Stomata are known to close in response to drought to limit water loss by transpiration. During water stress, ABA levels in plants increase and ABA promotes the closure of opened stomata and inhibits the opening of the closed ones (Wilkinson and Davies 2002). Thus, to assess NO function in

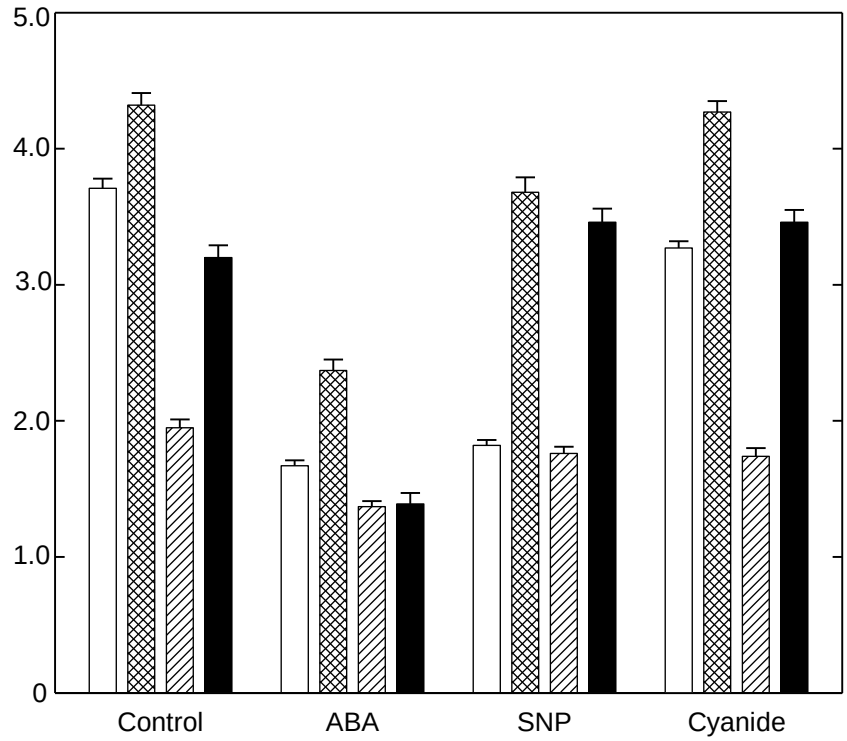

Figure 22 - NO-induced stomatal closure in turgid leaves is inhibited in the *ABA deficient mutant*, *aba1-1*. Leaves from wild-type (Ler) or *aba1-1* plants were floated in MES-KCl buffer under light to induced stomatal opening and then incubated for 2.5 h in buffer alone (control), 50 µM ABA, 50 µM SNP or 50 µM sodium ferrycianide. Stomatal apertures were measured before dehydration (white bars, Ler; checkered bars, *aba1-1*) and 30 min after submitting the leaves to dehydration (striped bars, Ler; black bars, *aba1-1*) under a direct light source on the laboratory bench. The data are representative of 4 independent experiments with means of 100 stomata ± standard errors

this process, water loss was measured in leaves of wild type plants (ecotypes Col-O and Ler) and mutants (*aba1-1*, *abi1-1*, *nia1nia2*, *nia1*::Ds and *nia2-5*) previously exposed to SNP, a NO donor.

ABA-induced water stress tolerance requires functional ABA signalling but no NO signalling

The plant hormone ABA plays a crucial role in plant responses to drought stress. ABA production is increased in vegetative tissues during water stress conditions, and this causes a variety of physiological effects such as stomatal closure and differential gene expression (Finkelstein et al 2002, Nambara and Morion-Poll 2005). Stomatal closure in response to ABA,

which reduces water loss through reduced transpiration, is an important plant survival strategy under water shortage. ABA signalling in guard cells leading to stomatal closure is complex, and several new signalling intermediates have been identified (Pei et al 2000, Zhang et al 2001, Hetherington 2001, Arasimowicz & Floryszak-Wieczorek 2007). One such molecule is NO, a signalling molecule of increasing importance in a number of abiotic stress such as hypoxia, UV-B irradiation, ozone, extreme temperatures and wounding (Lamattina et al 2003, Neill et al 2003). Thus, to determine whether NO-dependent signalling is responsible for ABA-induced drought tolerance different Arabidopsis genotypes affected in NO- or ABA-dependent signalling were treated with ABA, SNP, nitrite or ferricyanide. Short-term water-loss assays were performed by evaluating the decline in fresh weights of detached leaves of wild type and mutants over a different time periods (Verslues et al 2006).

In leaves from wild type plants ecotype Columbia (Col-0) and Landsberg *erecta* (Ler), ABA treatment reduced the water loss to 36 % and 31 % after 3 h under water stress conditions, while following nitrite, SNP and cyanide treatment the water loss remained at the same level as in the control (46 % - 49 %, Fig 23). These data indicate that only ABA was responsible for inducing tolerance to water stress in Arabidopsis. The faster stomatal closure of leaves treated with ABA (see Fig 1) and probably expression of ABA-regulated genes (see Seki et al 2002 a, 2002 b) lead to an enhanced water use efficiency of the plants without necessity of the NO. In agreement with these results, plants impaired in the NO pathway (*nia1::Ds*, *nia2-5*, *nia1nia2* and *Atnos1*) expressed wild type levels of ABA-induced tolerance against drought stress, indicating that NO-dependent signalling is not critical for ABA-induced tolerance to water stress. Importantly, SNP, nitrite and cyanide failed to reduce water loss in leaves of the *nia1::Ds*, *nia2-5*, *nia1nia2*, *Atnos1* mutants and wild type plants (ecotype Col-0 and Ler), indicating that NO was not able to inducing drought tolerance in Arabidopsis plants. Consistent with these findings were results indicating that higher water loss in leaves treated with NO correlate with greater stomatal aperture in mutants impaired in the NO pathway and wild type plants pre-treated with NO under drought conditions (see Figs 14, 15 and 17). Mutants impaired in ABA signalling

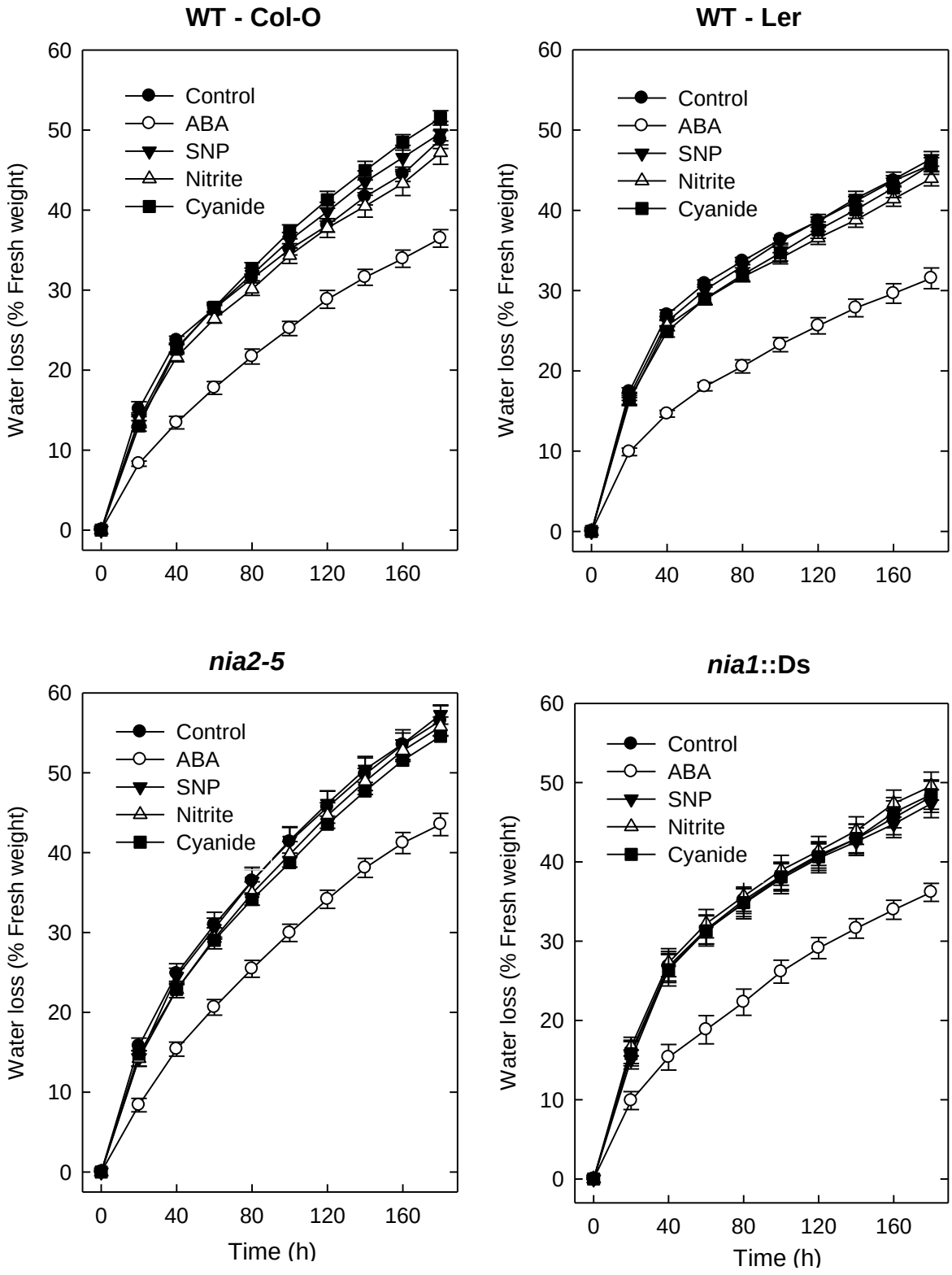

Figure 23 - (see continuation next page)

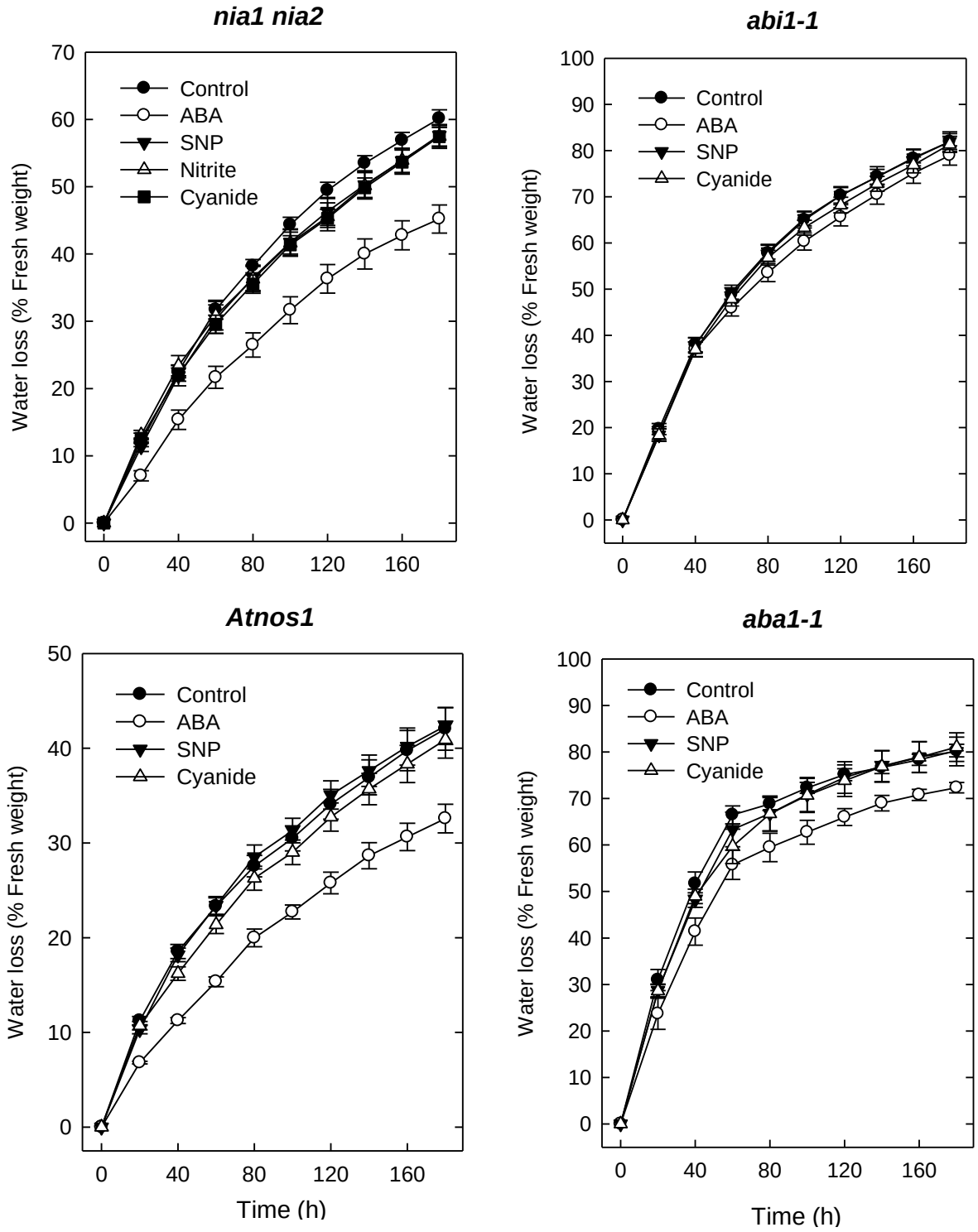

Figure 23 - NO does not diminish water loss produced by water stress. Leaves of either wild-type (Col-0 and Ler), nia 2-5, nia1::Ds, nia1nia2, Atnos1, aba1-1 or abi1-1 plants were floated in MES-KCl buffer under light to induced stomatal aperture. Afterwards, leaves were incubated for 2.5 h in buffer alone (control), 50 µM ABA, 50 µM SNP, 1 mM nitrite. The water loss of detached leaves was measured by weighing freshly leaves placed under a direct light source on the laboratory bench. Water loss was expressed as the percentage of initial weight at each time point. Data are means of two independent experiments with four replicates (Petri dishes with 4 leaves) for each treatment + standard errors

(*abi1-1*) completely lost their ability to react to ABA treatment demonstrating that water stress tolerance was based on ABA-dependent mechanisms (Fig 23). Moreover, treatment of the leaves of *abi1-1* plants with SNP or cyanide did not reduce the water loss indicating that NO was not required for ABA-induced protection against water stress.

ABA synthesis is important for defence signalling against drought stress (Morion-Poll and Leung 2006). Thus, mutant impaired in ABA biosynthesis (*aba1-1*) was used to confirm that NO was not required for ABA-induced tolerance against drought. The leaves from *aba1-1* plant treated with buffer alone (control), SNP or cyanide lost about 81 % of their fresh weight in 3 h of the course of experiment (Fig 23), while leaves from *aba1-1* plant treated with ABA had a little reduced water loss (72 %). These data indicate that NO was not required for ABA-induced tolerance to water stress in Arabidopsis.

For each treatment, the most rapid loss of water occurred during the first 60 min after submitting the leaves of the wild type plants and mutants to dry atmosphere (Fig 24). The maximum rate of water loss for each treatment was observed around 20 min upon transferring leaves to the dry atmosphere, which is consistent with previous report by Qin and Zeevaart (2002). After 20 min under drought conditions, the leaves from wild type (ecotype Col-0 and Ler) and mutants impaired in the NO pathway had a similar reduction in the rate of water loss when treated with ABA (the reduction in the rate of water loss for Col-O, Ler, *nia2-5*, *nia1*::Ds, *nia1nia2* and *Atnos1* were 45 %, 43 %, 41 %, 38 %, 41 % and 39 %, respectively). However, reduction in the rate of water loss was not observed in leaves from wild type plants and mutants impaired in the NO pathway treated with SNP, nitrite or cyanide. As with *nia2-5*, *nia1*::Ds and *nia1 nia2*, *Atnos1* leaves were insensitive to SNP and cyanide with respect to reduction in the rate of water loss, indicating that water stress tolerance observed in leaves treated with ABA was based on a NO-independent mechanism (Fig 24). Rate of water loss was also determined in *abi1-1* and *aba1-1* mutant. The leaves from *abi1-1* showed a similar rate of the fresh weight when treated with buffer (control), ABA, SNP or cyanide. However, after 20 min under drought conditions the rate of the

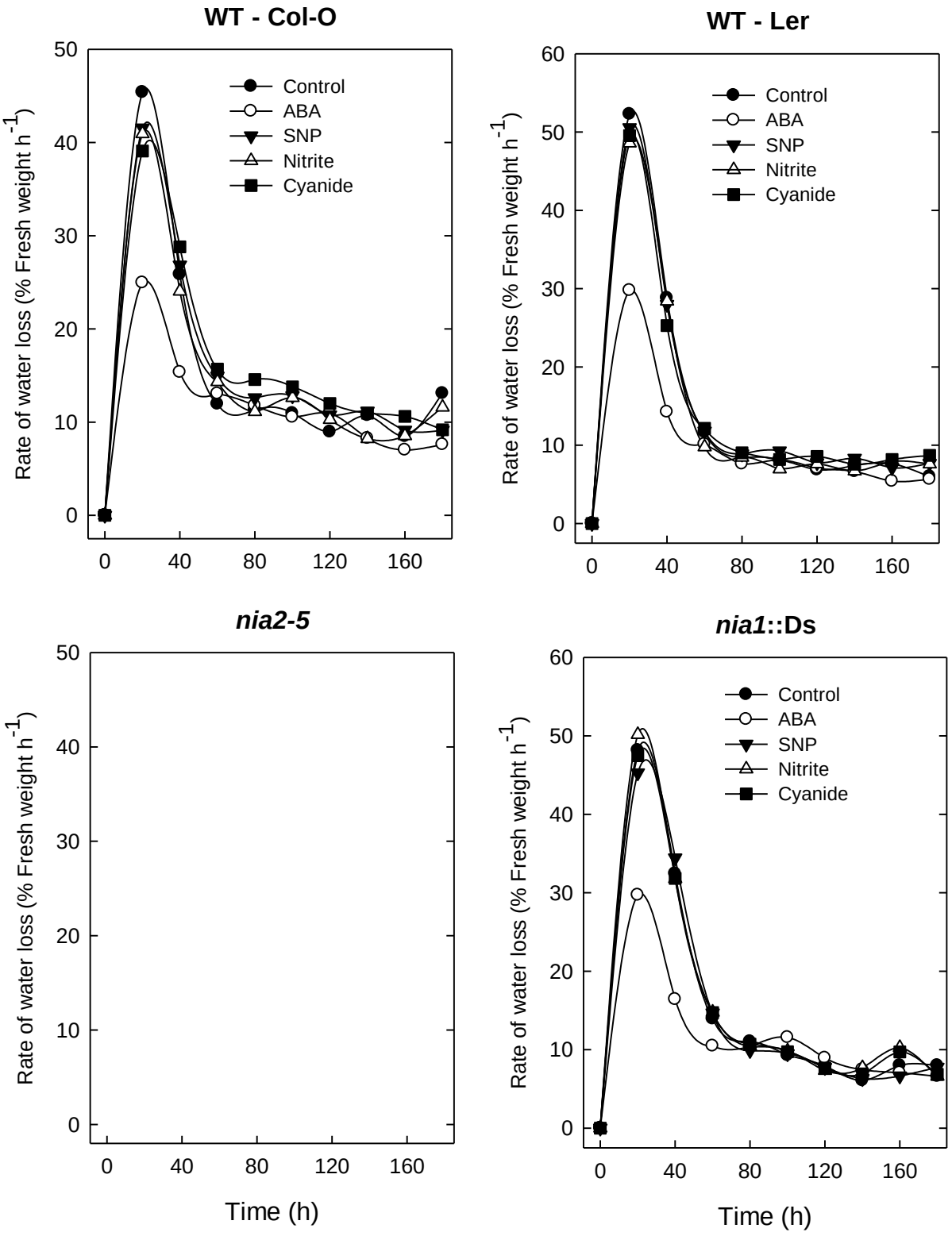

Figure 24 - (see continuation next page)

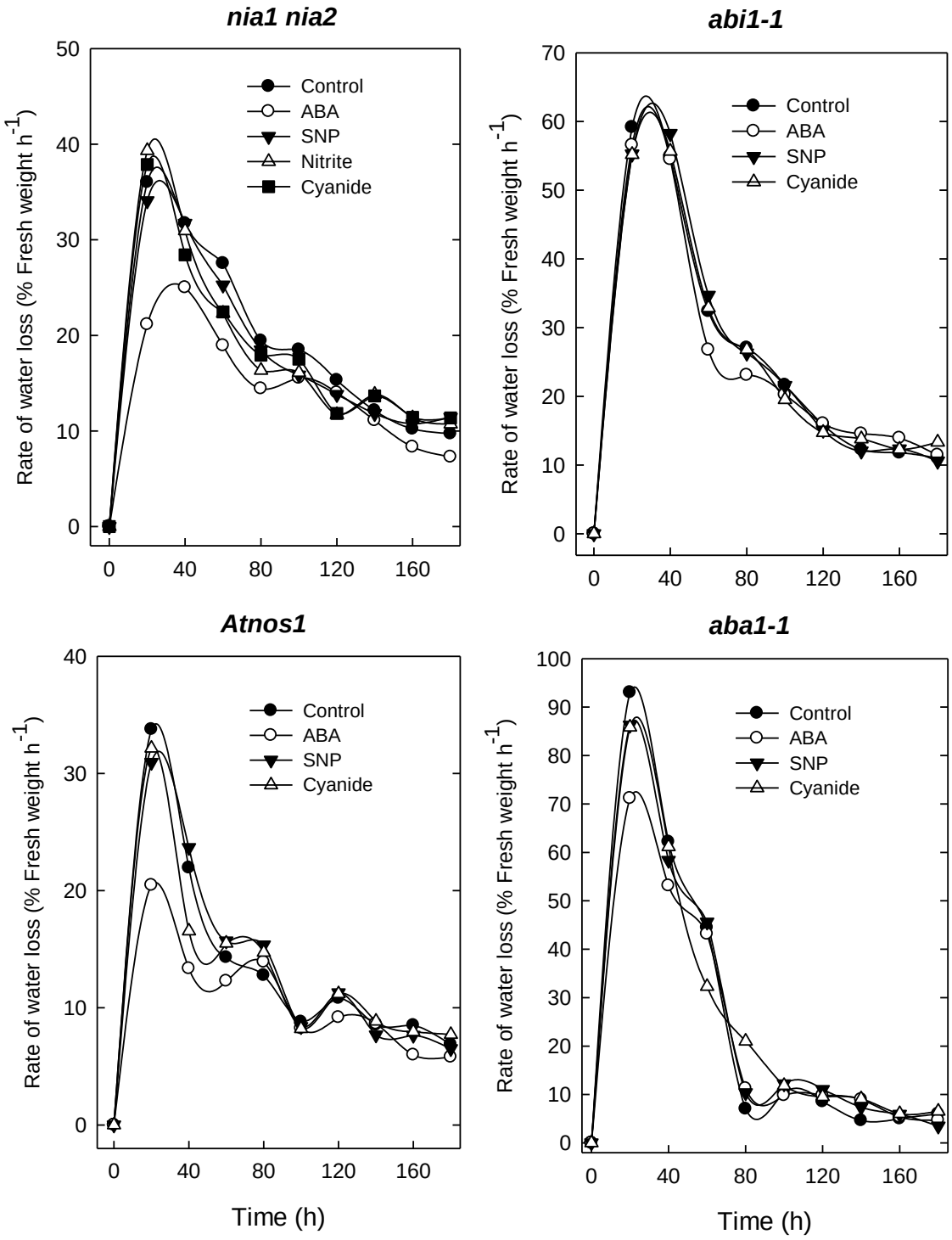

Figure 24 - NO does not decrease rate of water loss produced by drought stress. Arabidopsis leaves from Col-0, Ler, *nia2-5*, *nia1*::Ds, *nia1nia2*, *Atnos1*, *abi1-1* and *aba1-1* were floated in MES-KCl buffer under light to induced stomatal aperture. Afterwards, leaves were incubated for 2.5 h in buffer alone (control), 50 µM ABA, 50 µM SNP, 1 mM nitrite or 50 µM ferricyanide. The water loss of detached leaves was measured by weighing fresh leaves placed under a direct light source on the laboratory bench. Water loss was expressed as the percentage of initial weight at each time point. Data are means of two independent experiments with four replicates (Petri dishes with 4 leaves) for each treatment ± standard errors

fresh weight decreased 23 % in *aba1-1* leaves treated with ABA but not in leaves treated with SNP or cyanide. Together, these data are in agreement with the negation of NO-induced stomatal closure in leaves under drought stress (see Figs 3, 5 and 14), and demonstrate that NO signalling is not required for ABA-induced stomatal closure under water deprivation. However, Garcia Mata and Lamattina (2001) reported that NO, applied as SNP, reduced transpiration in detached leaves from *Triticum aestivum* plants, which was reversed by co-incubation of drought stressed leaves with PTIO. Thus, it is possible that in fact guard cell responses differ between Arabidopsis and *Triticum aestivum*. Moreover, detached-leaf water-loss assays could likely be not sensitive enough to detecting variations in the water loss in Arabidopsis leaves, which are apparent after long periods of drought. Thus, water-loss data were also examined in entire plants, kept under controlled-environment growth chamber (16-h photoperiod 60-100 µ E m^{-2} s^{-1} at 22 ^{o}C and 70-80 % relative humity), after exposing 4-week-old plants to drought stress by completely withholding irrigation and minimizing soil evaporation by covering pots with plastic film.

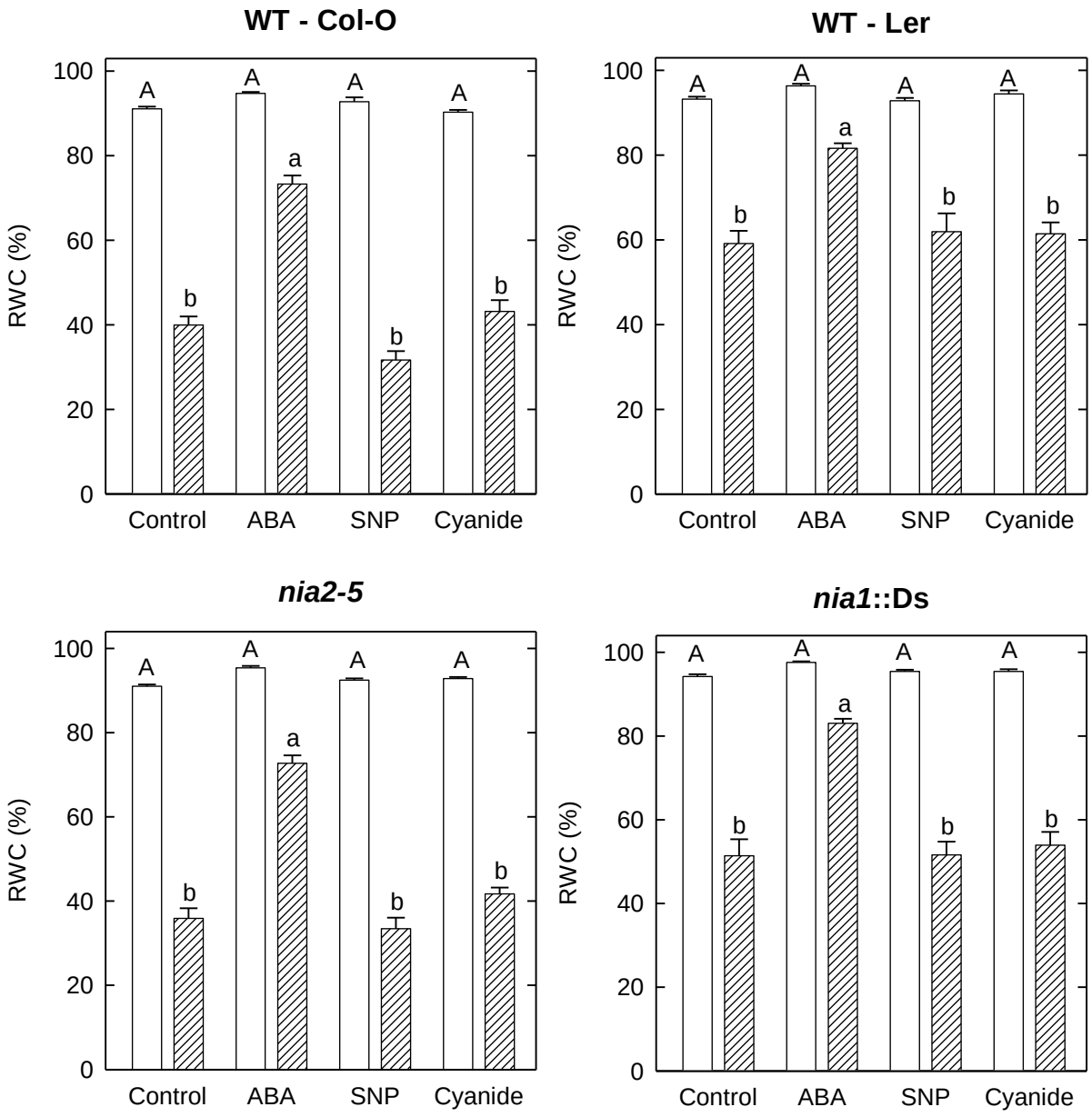

Figure 25 - NO does not induce water stress tolerance in *nia1*::Ds, *nia2-5* and wild type intact plants. Days without watering: 0 d - white bars and 14 d - striped bars. Aerial parts of four-week-old *nia1::Ds*, *nia2-5* and wild type Arabidopsis plants were submerged in solution of ABA (50 µM), SNP (50 µM), cyanide (50 µM) prepared in MES-KCl buffer or in MES-KCl buffer (control) for 5 s during 3 days, prior to the start of drought stress. Water stress was induced by stopping watering in plants maintained in the growth chamber conditions under light (16-h of photoperiod, 60-100 µE m^{-2} sec^{-1}), at 20 ^{o}C, and 70-80 % relative humidity. Ten leaves from four different plants were removed after different periods of water stress and fresh weight (FW) were immediately recorded. Subsequently, leaves were incubated in distilled water for 3 h in the growth chamber and the turgid weight (TW) was recovered. After drying for 24 h at 80 ^{o}C, dry weight (DW) was recorded. RWC was calculated according to the formula: RWC (%) = [(FW-DW)/(TW-DW)]x 100. The data are representative of 3 independent experiments

for NO decomposition) did not exhibit significant differences in the RWC compared with respective controls ($P < 0.05$ by Tukey test). RWC was also determined in the *nia1*::Ds (Crawford et al 1997). After 14 days of water deprivation wild type plants (Ler) and *nia1*::Ds mutant (Background Ler) treated with ABA retain approximately 27 % and 38 % more water than control plants (Fig 25). On the other hand, wild type plants and *nia1*::Ds mutant treated with SNP or ferricyanide did not exhibit significant differences in the RWC compared with respective controls ($P < 0.05$ by Tukey test). Together, these data indicate that NO is not able to protect Arabidopsis against water stress. To further analyze stomatal response to ABA, SNP or cyanide, stomatal bioassay were performed in the wild type plants (Col-0 and Ler) and mutant plants (*nia2-5* and *nia1*::Ds).

NO-induced stomatal closure is not enhanced in Arabidopsis intact plants under water stress

In intact plants after a long drought period, both wild type plants (ecotype Col-0 and Ler) and the *nia2-5*, *nia1*::Ds mutants treated with SNP showed reduced RWC as compared to wild type plants and mutants treated with ABA (see Fig 25). This demonstrated that only ABA was responsible for inducing tolerance to water stress in Arabidopsis. The rapid reductions of stomatal apertures under water stress conditions seem to constitute the basis for ABA-induced tolerance to drought stress. Thus, the effects of ABA and NO on stomatal response were examined in leaves of wild type and mutants from intact plants.

Under well watered conditions (0 day without watering) stomatal closure was induced in *nia2-5* as in the wild type by ABA and SNP (Fig 26 white bars, left side). ABA caused a reduction of 64 % and 68 % in stomatal aperture width to wild type and *nia2-5* plants, respectively. On the other hand, SNP induced a 55 % and 43 % decrease in stomatal aperture width in wild type and *nia2-5* plants as compared to controls treatment. Cyanide (as a control for SNP decomposition) failed to induce stomatal closure in leaves from intact plants of the wild type and *nia2-5* mutants, indicating that NO released from SNP induced stomatal closure. In fully turgid plants of wild type and *nia2-5* plants (0 day without drought), ABA induced stomatal closure that

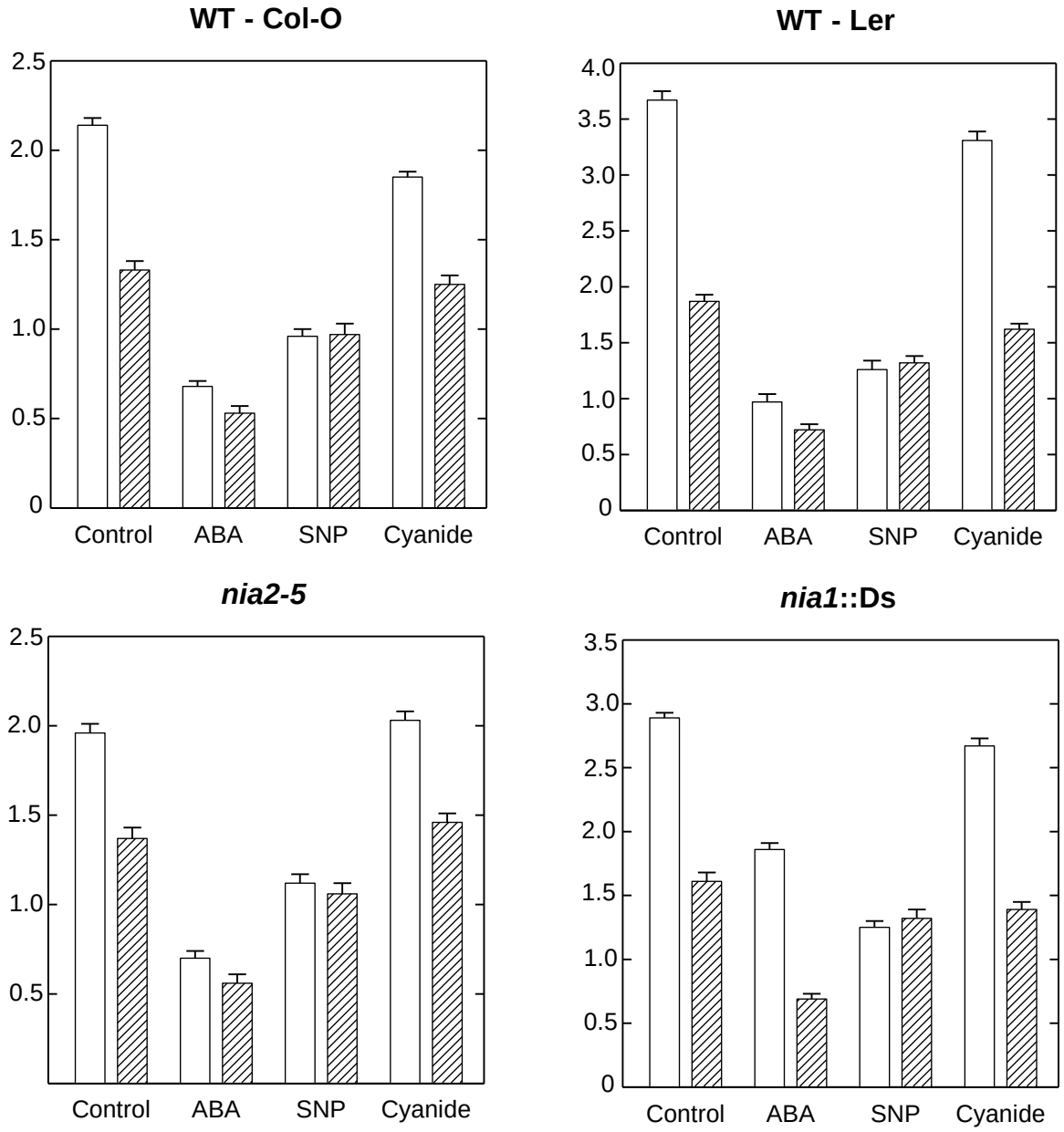

Figure 26 - NO-induced stomatal closure is not enhanced in Arabidopsis intact plants under water stress. Days without watering: 0-white bars and 7-striped bars. Aerial parts of four-week-old *nia1::Ds*, *nia2-5* and wild type Arabidopsis plants were submerged in solution of ABA (50 µM), SNP (50 µM), cyanide (50 µM) prepared in MES-KCl buffer or in MES-KCl buffer (control) for 5 sec during 3 days, prior to the start of drought stress. Water stress was induced by stopping watering in plants maintained in the growth chamber conditions under light (16-h of photoperiod, 60-100 µE m^{-2} s^{-1}), at 20 ^{o}C, and 70-80 % relative humidity. To minimize experimental variations, Arabidopsis plants were grown in single plastic pots. The evaporation of the soil was minimized by covering pots with plastic film after stopping the watering of plants**.** Stomatal apertures were measured in Arabidopsis leaves before to the start of drought stress (0 day of drought stress) and 7 days after starting of drought stress. The data are representative of 3 independent experiments with the means of 120 stomata $\pm$ standard errors

was significantly different to that induced by SNP ($P < 0.05$ by *t* test). These data indicate that NO cannot prime for ABA accumulation in intact plants. Priming of ABA biosynthesis can be obtained with chemicals that accelerate ABA accumulation, for example the non protein amino acid β-aminobutyric acid (Jakab et al 2005).

After 7 days of water stress, stomatal apertures in wild type plants (ecotype Col-0) treated with ABA were 60 % more closed than wild type control apertures (Fig 26 striped bars, left side). In *nia2-5* plants pre-treated with ABA stomatal apertures were 59 % more closed than stomatal apertures in *nia2-5* plants treated with buffer alone (control). On the other hand, stomatal apertures in wild type plants pre-treated with SNP were 27 % smaller than in wild type plants treated with buffer alone (control). In *nia2-5* plants pre-treated with SNP, stomatal widths were 22 % smaller compared to *nia2-5* stomata treated with buffer (control). Thus, the smaller stomatal aperture observed in leaves from wild type and *nia2-5* mutants treated with ABA explain the reduced water loss by transpiration in these plants (see Fig 25 left side).

In wild type plants (ecotype Ler), under well watered conditions, ABA caused a 74 % reduction in stomatal aperture as compared to control whereas a 35 % difference in mean aperture width was observed in *nia1*::Ds plants (Fig 26 white bars, right side). ABA-mediated closure was 39 % less effective in *nia1*::Ds plants compared to the wild type response. Importantly, *nia1*::Ds stomata responded to SNP in the same manner as wild type stomata (apertures for wild type plants and *nia1*::Ds were 1.26 µm and 1.25 µm, respectively) and sodium ferricyanide (as a control for SNP decomposition) failed to induce stomatal closure in leaves from wild type plants and *nia1*::Ds mutants. Bright (2006) reported that ABA when used at concentrations that induced NO synthesis in wild type guard cells did not induce NO synthesis in *nia1*::Ds guard cells. Thus, the above results indicated that functional NR1 is required for ABA-induced NO synthesis in guard cells and stomatal closure in *nia1*::Ds plants, under well watered conditions. However ABA induced a significant stomatal closure (36 %) in *nia1*::Ds plants compared to their controls (Fig 26, right side). This may be

due to the fact that the ABA-induced stomatal closure is not solely dependent on NR1.

After 7 days under water stress, the ABA-induced stomatal closure was restored in mutant plants such that the stomatal apertures width observed in *nia1*::Ds plants was not significantly different ($P < 0.05$ by *t* test) from wild type apertures (Fig 26 striped bars, right side). These data suggest that a functional NR1 protein was not required for the induction of stomatal closure. Stomatal apertures width in wild type and *nia1*::Ds plants treated with sodium ferricyanide did not differ significantly from control and SNP. Together, these data suggest that NO was not an essential component for ABA-induced stomatal closure in Arabidopsis plants under water stress. The decrease in stomatal apertures ensured increased water use efficiency in ABA-treated plants, explaining their enhanced tolerance to water stress. However, NO was not able to protect Arabidopsis against water stress, because NO did not induce stomatal closure in leaves under water shortage. In summary, in contrast to the requirement for NO synthesis during ABA-induced stomatal closure in well hydrated leaves, stomatal closure during wilting may result from ABA signalling that bypasses the need for NO synthesis and perception.

ABA biosynthesis and subsequent action is a key plant response to water-deficit stress. Previous studies have shown that hydrogen peroxide (H_2O_2) is an essential signalling intermediate in ABA-induced stomatal closure in several plant species under well watered conditions (Pei et al 2000, Murata et al 2001, Zhang et al 2001, Mustilli et al 2002, Bright et al 2006). Thus, H_2O_2-induced changes in stomatal behaviour were monitored in water-stressed leaves of *Arabidopsis thaliana*.

H_2O_2 is not an essential signal for ABA-induced stomatal closure in water stressed leaves

In fully turgid leaves, H_2O_2 induced stomatal closure in a dose-dependent manner causing a significant reduction in stomatal width at concentrations as low as 10 µM ($P < 0.05$ by Tukey test; Fig 27, white bars). The maximum significant effect induced by applied H_2O_2 occurred at 100 µM in which stomatal apertures was reduced to 1.28 µM that corresponds to a

48 % reduction in width compared to control apertures (Fig 27, white bars). Previous studies have also shown that H_2O_2 induces a dose-dependent stomatal closure in a number of species (McAinsh 1996, Pei et al 2000, Zhang et al 2001, Desikan et al 2002). However, H_2O_2-induced stomatal closure was abolished in water-stressed leaves such that the stomatal apertures width observed in leaves treated with H_2O_2 (10-100 µM) was not significantly different from control apertures ($P < 0.05$ by Tukey test; Fig 27, striped bars). Under drought stress conditions, NO also failed to induced dose-dependent stomatal closure in Arabidopsis leaves (see Figs 3 and 5).

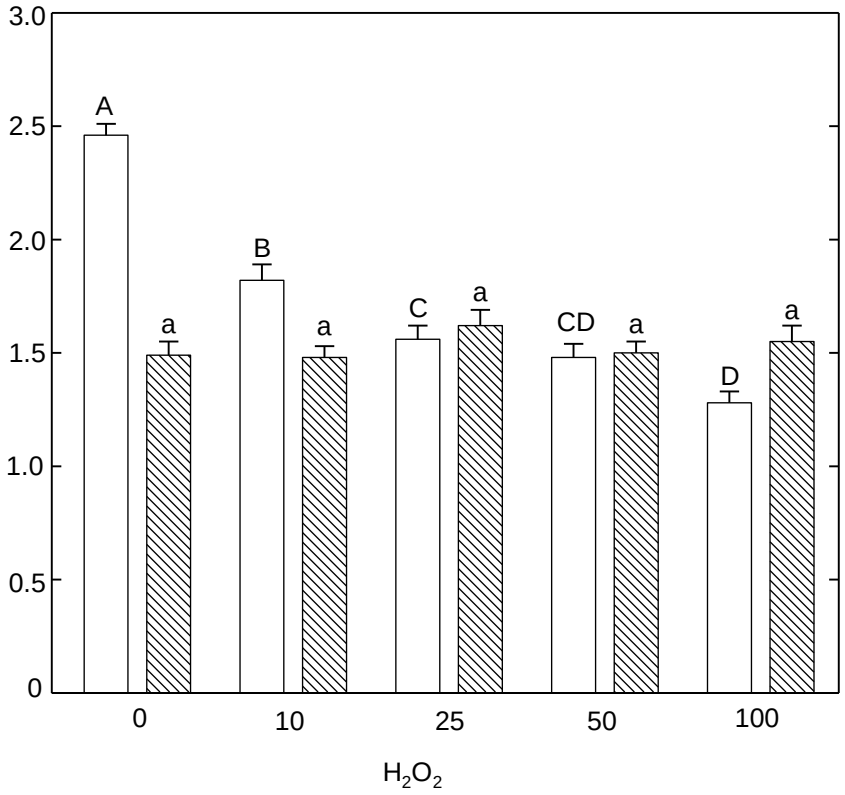

Figure 27 - Negation of the effect of H_2O_2 on the stomatal closure in Arabidopsis leaves under water stress conditions. Wild type Arabidopsis (Col-0) leaves were floated in MES-KCl buffer to induce stomatal opening and then incubated in H_2O_2 (10-100 µM) for 2.5 h. Stomatal apertures were measured before dehydration (white bars) and 30 min after submitting the leaves to dehydration (striped bars) under a direct light source (50 cm from a 60 Watt bulb) on the laboratory bench. The data are representative of 4 independent experiments with the means of 100 stomata $\pm$ standard errors

These results suggest a distinct difference between the mechanisms of stomatal closure in fully turgid leaves and leaves under drought stress conditions.

A role for H_2O_2 has previously been established in ABA-induced stomatal closure (Pei et al 2000, Murata et al 2001, Zang et al 2001). Furthermore, under stress conditions, ABA and H_2O_2 are commonly generated in many biological systems (Assmann and Shimazaki 1999). Thus, ABA-induced stomatal closure was measured in water-stressed leaves treated with the antioxidant N-acetylcisteine (NAC) or ascorbic acid (ASC) (Noctor and Foyer 1998). PTIO, a specific NO scavenger, was also used to investigate the relationhips between H_2O_2 and NO in ABA-induced stomatal closure (Bright et al 2006). Treatment of leaves with NAC inhibited ABA- and H_2O_2-induced stomatal closure (Fig 28, white bars). NAC (1 mM) caused 33 % inhibition of ABA-induced stomatal closure (see ABA+NAC) and 58 % of H_2O_2-induced stomatal closure (see H_2O_2+NAC). ASC had also a marked effect on the inhibition of stomatal closing caused by H_2O_2. ASC caused 65 % inhibition H_2O_2-induced stomatal closure (see H_2O_2+ASC). On the other hand, ASC had little effect (12 %) on ABA-induced stomatal closure (see ABA+ASC). Incubation of leaves with NAC and ASC alone had no effect on stomatal apertures as mean apertures did not differ significantly from those of controls ($P < 0.05$ by *t* test). Together, these data suggest that H_2O_2 synthesis was required for ABA-induced stomatal closure in fully turgid leaves of Arabidopsis, which is the same as that seen in previous works (Pei et al 2000, Zhang et al 2001, Bright et al 2006). ABA- and SNP-induced stomatal closure were greatly reduced in the presence of PTIO, as shown previously in other reports (Garcia-Mata and Lamattina 2002, Neil et al 2002, Desikan et al 2002) and in this study (see Figs 5 and 9). PTIO, a specific NO scavenger, also caused over 68 % inhibition of H_2O_2-induced stomatal closure, similarly to PTIO inhibition of ABA- and SNP-induced stomatal closure (see Figs 5 and 9), indicating a requirement for NO in H_2O_2 induced stomatal closure in leaves under well watered conditions. Moreover, although treatment of leaves with NAC or ASC inhibited H_2O_2-induced closure, it did not affect SNP-induced closure, suggesting that NO did not required H_2O_2 generation to initiate stomatal closure in fully turgid leaves (Fig 28, white bars). In summary, the accumulated evidence suggests that H_2O_2 can be generated in guard cells (Pei et al 2000, Zhang et al 2001, Bright et al 2006) and it appears that synthesis of H_2O_2 is essential for ABA-induced stomatal

closure in various species (McAinsh et al 1994, Desikan et al 2004). However, these works did not deal with the mechanisms of stomatal closure under stress such as water deficit and high-intensity light, during which an oxidative burst had been found in non-stomatal tissues (Auh and Murphy 1995). To examine the role of H_2O_2 in ABA-induced stomatal closure, stomatal apertures were examined in water stressed leaves.

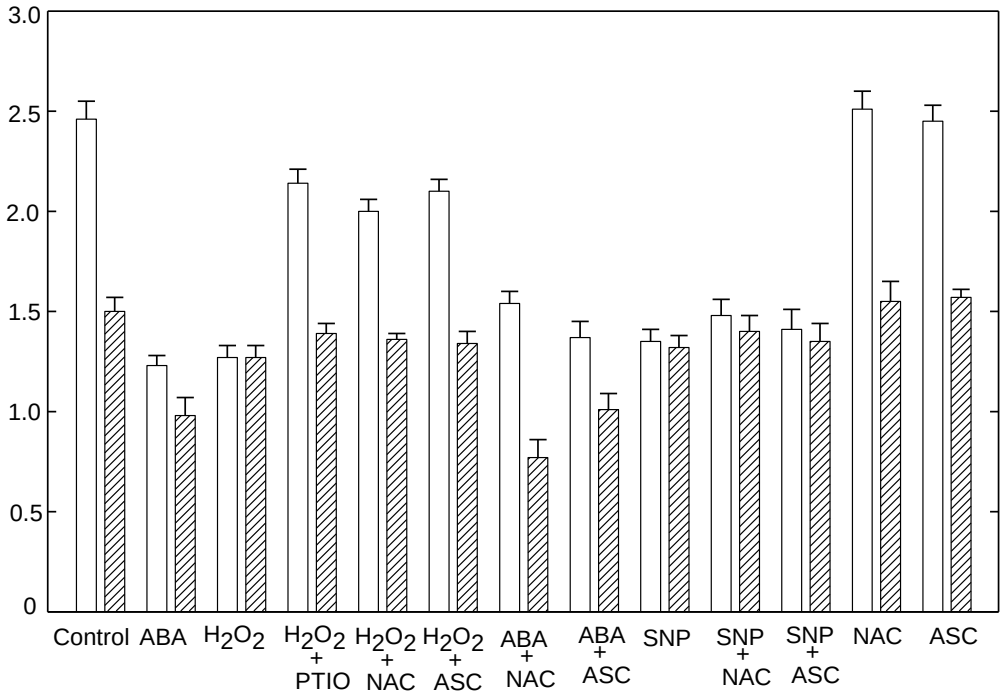

Figure 28 - Negation of the antagonistic effect of the H_2O_2 scavenger NAC and ASC on ABA-induced stomatal closure in Arabidopsis leaves under water stress conditions. Wild type Arabidopsis (Col-0) leaves were floated in MES-KCl buffer under light to induce stomatal opening and then incubated for 2.5 h in buffer alone (control), 50 µM ABA, 100 µM H_2O_2, H_2O_2+200 µM PTIO, H_2O_2+NAC, H_2O_2+ASC, ABA+NAC, ABA+ASC, 50 µM SNP, SNP+NAC, SNP+ASC, 1mM NAC, 1 mM ASC. Stomatal apertures were measured before dehydration (white bars) and 30 min after submitting the leaves to dehydration (striped bars) under a direct light source on the laboratory bench. The data are representative of 4 independent experiments with means of 100 stomata $\pm$ standard errors

Under water stress conditions, stomata of wild type plants did not respond to H_2O_2 and SNP, although they did close in response to ABA (Fig 28, striped bars). ABA-induced stomatal closure was not inhibited in leaves treated with ASC or NAC suggesting that H_2O_2 synthesis was not required for ABA-induced stomatal closure under water shortage. H_2O_2- and SNP-

induced stomatal closure was abolished in wild type plants such that the mean aperture width in leaves treated with H_2O_2 and SNP was not significantly different from control apertures ($P < 0.05$ by *t* test). Importantly, H_2O_2+PTIO-, H_2O_2+NAC-, H_2O_2+ASC- and SNP+NAC-, SNP+ASC-mediated stomatal response did not also differ ($P < 0.05$ by *t* test) from the control response. Together, these data suggest that ABA signalling in leaves under drought stress does not operate through NO and H_2O_2. In agreement with

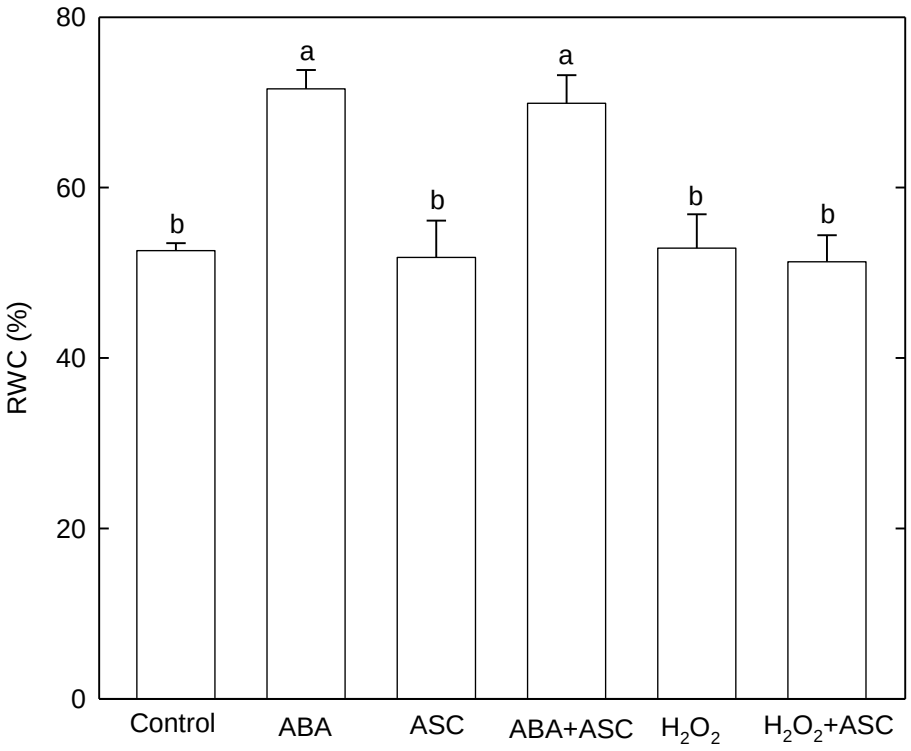

Figure 29 - H_2O_2 is not required for ABA-induced water stress tolerance. Wild-type Arabidopsis (Col-0) leaves were floated in MES-KCl buffer under light to induce stomatal opening and then incubated for 2.5 h in buffer alone (Control), 50 µM ABA, 1mM ASC, ABA+ASC, 100 µM H_2O_2 or H_2O_2+ASC. Drought condition was performed by placing detached leaves with abaxial side up on open dishes under a direct light source on the laboratory bench. RWC values were determined after 2 h of water stress condition. Data are means of two independent experiments with four replicates (Petri dishes with 4 leaves) for each treatment ± standard errors

these results, wild type leaves treated with H_2O_2 showed decreased RWC as compared to leaves treated with ABA (Fig 29). After 2 h droughted leaves treated with ABA or ABA+ASC retain approximately 27 % and 25 % more water than control plants. However, leaves treated with H_2O_2 or H_2O_2+ASC did not exhibit significant differences in the RWC compared with control ($P <$

0.05 by Tukey test). Together, these data suggest that H_2O_2 was not able to protect Arabidopsis against drought. However, H_2O_2 was recently reported to be involved in the protection of plants against salt stress (Wahid et al 2007). Application of H_2O_2 also increases chilling tolerance by enhancing the glutathione level of mung bean seedlings (Murphy et al 2002).

FUTURE WORK

Development of techniques to monitor the generation of NO in response to ABA is essential to discern the spacial and temporal coordination of events leading to stomatal closure. Application of developed genetic screens to monitor water loss, such as thermo-imaging (Mustilli et al 2002), may be useful in revealing novel targets of ABA in guard cells. Experiments using thermographic imaging may be a valuable tool to analyse *in plant* effects of various hormones and NO on transpiration in wild type and background mutant.

It is clear that although a number of key signalling intermediates of ABA-induced stomatal closure have been identified in previous works, much of the ABA-signalling network remains as yet to be elucidated. Thus, identification of downstream targets of ABA should be advanced using proteomics analysis of guard cells from the wild type and from *abi1* or *abi2*. This technology should reveal novel targets for both H_2O_2 and NO in guard cells of plants under well watered conditions.

Recent work has shown that respiratory burst oxidase homologue (Rboh) NADPH oxidases generate H_2O_2 during stomatal closure in response to ABA in Arabidopsis, and that AtrbohD and AtrbohF are highly expressed in guard cells (Kwak et al 2003). Consequently, it will be of interest to determine whether AtrbohD and AtrbohF is a branch of ABA-dependent signalling in plants under stress such as drought.

In this study, it was observed that NO did not protect Arabidopsis plants against water stress. Thus, future work shall also include emphasis on cloned genes, for example, the expression patterns of marker genes for the ABA pathway (*RAB 18*, *RAB 19*, *NCED*) to confirm whether NO does not prime for ABA-dependent signals.

CONCLUSIONS

The data present in this work have demonstrated that stomatal response to NO differ between wilting and turgid tissues of *Arabidopsis thaliana*. NO was confirmed as a signalling component for ABA-induced stomatal closure in turgid leaves. However, NO seemed not required for ABA-induced stomatal closure during drought stress. Thus, another novel aspect of guard cell NO signalling was revealed in the present study.

This study provided also evidence that ABA-induced drought tolerance requires functional ABA-signalling, but no NO-signalling, indicating that NO-dependent signalling may only represent one of the branches of the ABA signalling network. Hetherington and Woodward (2003) proposed that guard cell signalling displays properties similar to a scale-free network, a system that consists of hubs and nodes. These authors explain that removal of a central hub such as an increase in cytosolic Ca^{2+}, an effector that is induced by a range of ABA-induced signals, results in the failure of the system and a loss of the stomatal response. However, removal of signalling components, described as nodes, can be overcome by the robustness of the system and the continuance of signalling to central hubs. This property is not surprising given that control of stomatal apertures in such a crucial process for plants health and survival. The reality is that NO seems to be just one of a large number of signalling components operating in signal transduction, but one that does not act as an essential hub-like component of ABA signal transduction in guard cells. Both NO and H_2O_2 failed to induce stomatal closure in water-stressed leaves of Arabidopsis. The data suggest that H_2O_2 also seems not to be a hub component of the ABA signalling network.

At least, NO synthesis occurs via NR, and/or possibly other enzymes with NOS-like activity. Lack of active NR reduces stomata responses to ABA in turgid plants, but not in water-stressed plants. Thus plants lacking NR was able to retain functional guard cell ABA response indicating that NO does not protect Arabidopsis plants against drought.

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