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Nitrate reductase mutation alters potassium nutrition as well as nitric oxide-mediated control of guard cell ion channels in *Arabidopsis*

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Summary

- Maintaining potassium (K^+) nutrition and a robust guard cell K^+ inward channel activity is considered critical for plants' adaptation to fluctuating and challenging growth environment. ABA induces stomatal closure through hydrogen peroxide and nitric oxide (NO) along with subsequent ion channel-mediated loss of K^+ and anions. However, the interactions of NO synthesis and signalling with K^+ nutrition and guard cell K^+ channel activities have not been fully explored in *Arabidopsis*.
- Physiological and molecular techniques were employed to dissect the interaction of nitrogen and potassium nutrition in regulating stomatal opening, CO_2 assimilation and ion channel activity. These data, gene expression and ABA signalling transduction were compared in wild-type Columbia-0 (Col-0) and the nitrate reductase mutant *nia1nia2*.
- Growth and K^+ nutrition were impaired along with stomatal behaviour, membrane transport, and expression of genes associated with ABA signalling in the *nia1nia2* mutant. ABA-inhibited K^+_{in} current and ABA-enhanced slow anion current were absent in *nia1nia2*. Exogenous NO restored regulation of these channels for complete stomatal closure in *nia1nia2*.
- While NO is an important signalling component in ABA-induced stomatal closure in *Arabidopsis*, our findings demonstrate a more complex interaction associating potassium nutrition and nitrogen metabolism in the *nia1nia2* mutant that affects stomatal function.

Introduction

Potassium (K^+) is one of the most abundant macronutrients in plants consisting of up to 10% of plant dry mass. Long-term K^+ deficiency reduces photosynthesis, plant growth and stress tolerance (Armengaud *et al.*, 2004; Chen *et al.*, 2005; Marschner, 2012). Potassium can accumulate up to a few hundred mmol in guard cells during stomatal opening (Blatt, 2000; Hills *et al.*, 2012). Early reports showed that stomatal movement is quantitatively related to K^+ transport while other major elements remain largely unchanged (Fischer, 1968; Humble & Raschke, 1971). Reducing stress-induced K^+ loss (Shabala & Cuin, 2008) and maintaining a robust K^+ inward channel activity at the guard cell plasma membrane (Lebaudy *et al.*, 2008) have been proposed to determine plant adaptation to abiotic stresses and adverse environment. The inwardly rectifying K^+ (K^+_{in}) channels KAT1, KAT2, AKT1, AKT2 and KC1 and high-affinity K^+ transporters (HAKs, HKTs) are responsible for mediating K^+ uptake into guard cells in *Arabidopsis* (Szyroki *et al.*, 2001; Ward *et al.*, 2009; Chen *et al.*, 2012b; Hills *et al.*, 2012; Wang *et al.*, 2012, 2013). Disruption of K^+_{in} channels appears to have more detrimental effects than that of outwardly rectifying K^+ (K^+_{out}) channels in guard cells (Hosy *et al.*, 2003; Lebaudy *et al.*, 2008). Moreover, stomatal closure and/or opening are regulated by some key transcription factors (TFs), including ABA-responsive kinase substrates/basic helix–loop–helix (bHLH) TFs (*AKS*s). *AKS1* was found to directly enhance the transcription of K^+_{in} channel gene *KAT1* (Takahashi *et al.*, 2013).

Nitrogen is a key element of DNA, RNA and proteins, and its transport and metabolism are critical for the survival of living organisms. Nitrate is the major nitrogen source for most plants (Ho *et al.*, 2009; Léran *et al.*, 2015). Nitrogen and potassium nutrition are closely interconnected, and depleting one component will affect the availability of the other (Schachtman & Shin, 2007; Amtmann & Blatt, 2009). K^+ starvation affects nitrate reductase activity, NO_3^- uptake and nitrate transporters (NRTs) in *Arabidopsis* (Armengaud *et al.*, 2004, 2009), while *Hv*HAKs and *Hv*HKTs are regulated by ammonium in barley roots

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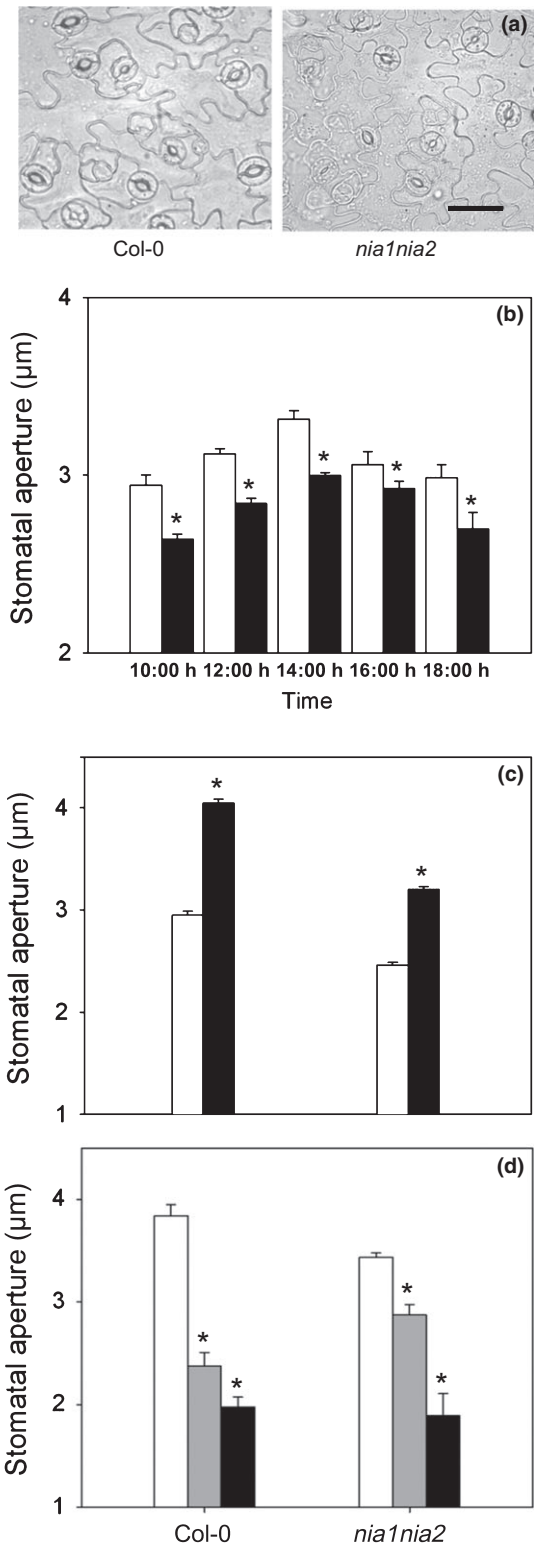
(Santa-María *et al.*, 2000). Interestingly, the calcineurin B-like Ca^{2+} sensor 1 and CBL-interacting protein kinase 23 (CBL1-CIPK23) complex is found to be the common regulator of AKT1 (Xu *et al.*, 2006) and NRT1.1 for potassium and nitrogen nutrition in *Arabidopsis* (Léran *et al.*, 2015). Therefore, *Arabidopsis* mutant with altered nitrogen nutrition, such as the *nia1nia2*, is ideal for investigating K^+ nutrition and stomatal behaviour.



Abscisic acid and nitric oxide (NO) are two extremely important signalling molecules controlling many aspects of plant physiology. Both influence a wide range of signal transductions and regulate the expression of genes and proteins (Lamattina *et al.*, 2003; Garcia-Mata & Lamattina, 2013). Although the intracellular pathways mediating ABA signalling are complex, significant progress has been made (Kim *et al.*, 2010; Boursiac *et al.*, 2013). Guard cells respond to ABA by elevating hydrogen peroxide (H_2O_2) and NO, to increase cytosolic free $[Ca^{2+}]$ ($[Ca^{2+}]_{cyt}$) (Blatt, 2000; Ward *et al.*, 2009). NO is essential in this process as a factor that alters the $[Ca^{2+}]_{cyt}$ sensitivity of intracellular Ca^{2+} stores to promote $[Ca^{2+}]_{cyt}$ elevation (Desikan *et al.*, 2002; Garcia-Mata *et al.*, 2003; Sokolovski *et al.*, 2005; Garcia-Mata & Lamattina, 2007, 2013). The effects in elevating $[Ca^{2+}]_{cyt}$ result in the suppression of currents at the plasma membrane through the K^+_{in} channel to prevent K^+ influx and activation of K^+_{out} and anion channels for ion efflux (Garcia-Mata *et al.*, 2003; Ward *et al.*, 2009).

The possible sources of NO production can be categorized as enzymatic or nonenzymatic (Besson-Bard *et al.*, 2008; Gupta *et al.*, 2011). Orthologues of NO synthase (NOS) genes in animals are yet to be identified in plants and NOS inhibitors have variable effects in plants (Gupta *et al.*, 2011). Nitrate reductase activity, by contrast, is widely accepted as a source of NO production. In *Arabidopsis*, nitrate reductases are encoded by two genes, *NIA1* and *NIA2* (Wilkinson & Crawford, 1993; Wilson *et al.*, 2008). These enzymes play a central role in nitrogen assimilation and catalyse the reduction of nitrite to NO (Yamasaki & Sakihama, 2000). Molybdenum-containing enzymes such as sulphite oxidase, aldehyde oxidase and xanthine dehydrogenase are also likely to produce NO in plants (Gupta *et al.*, 2011). Moreover, there is some evidence in other species of apoplastic NO production. The root-specific nitrite-NO reductase in *Nicotiana tabacum* and rapid nonenzymatic NO production from nitrite in *Hordeum vulgare* aleurone layers both showed apoplastic reduction of nitrite to NO (Bethke *et al.*, 2004). However, the relevance of these NO sources in guard cells is unclear. Nitrate reductase-mediated NO synthesis is required for ABA-induced stomatal closure in *Arabidopsis* and the *nia1nia2* double mutant that has been reported to show a greatly attenuated response to ABA (Clarke *et al.*, 2000; Desikan *et al.*, 2002; Wang *et al.*, 2004). Although the *nia1nia2* mutant showed much reduced NO synthesis and ABA insensitivity, stomata of the mutant responded to exogenous NO (Desikan *et al.*, 2002; Wang *et al.*,

Fig. 1 Stomatal opening and closing properties in Columbia-0 (Col-0) and *nia1nia2* of *Arabidopsis thaliana*. (a, b) Representative images (bar, 20 μm) (a) and stomatal aperture ($n = 35$–45) (b) at different times of the day from leaf epidermal peels of Col-0 (open bars) and *nia1nia2* (closed bars) under normal growth conditions. (c) Stomatal aperture of Col-0 and *nia1nia2* under normal growth conditions (open bars) and after 2 h in an opening buffer (closed bars) ($n = 55$–63). (d) Stomatal aperture after 2 h in an opening buffer (white bars), followed by 1 h treatments in ABA (grey bars) and nitric oxide (NO) (black bars) ($n = 30$–48). Data are means ± SE. *, $P < 0.05$ as compared with Col-0 or the control.

2004). These observations and electrophysiological studies in *Vicia faba* guard cells (Garcia-Mata *et al.*, 2003; Sokolovski *et al.*, 2005) indicate that stomatal behaviour of the *nia1nia2* mutation could be affected, simply because of a loss of NO synthesis. However, the characteristics of major guard cell ion channels and their response to ABA and NO have not been demonstrated directly in *Arabidopsis*.

We hypothesized that eliminating *NIA1* and *NIA2* impose regulations on stomatal behaviour, CO_2 assimilation, ion channel activity, gene expression, and long-term K^+ nutrition in the *Arabidopsis nia1nia2* mutant. Therefore, the main objective of this study was to determine the interaction associating potassium nutrition and nitrogen metabolism in the *nia1nia2* mutant, which affects K^+ nutrition and has unexpected consequences for stomatal function and ion channel regulation.

Materials and Methods

Plant materials

Wild-type Columbia-0 (Col-0) and the *nia1nia2* (*nia1-1/nia2-5* (Wilkinson & Crawford, 1993; Wang *et al.*, 2004) in the Col-0 background) double mutant of *Arabidopsis thaliana* (L.) Heynh were sown in pots with a potting mixture (60% compost, 20% perlite, and 20% vermiculate) and grown under a 12 : 12 h, day : night conditions (100 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR)) and 60% humidity. *Arabidopsis* plants were irrigated weekly with half-strength Hoagland's solution containing additional 5 mM NH_4NO_3. Plants were grown for at least 4 wk before electrophysiological experiments, stomatal aperture assay, gas exchange analysis, and quantitative real-time PCR

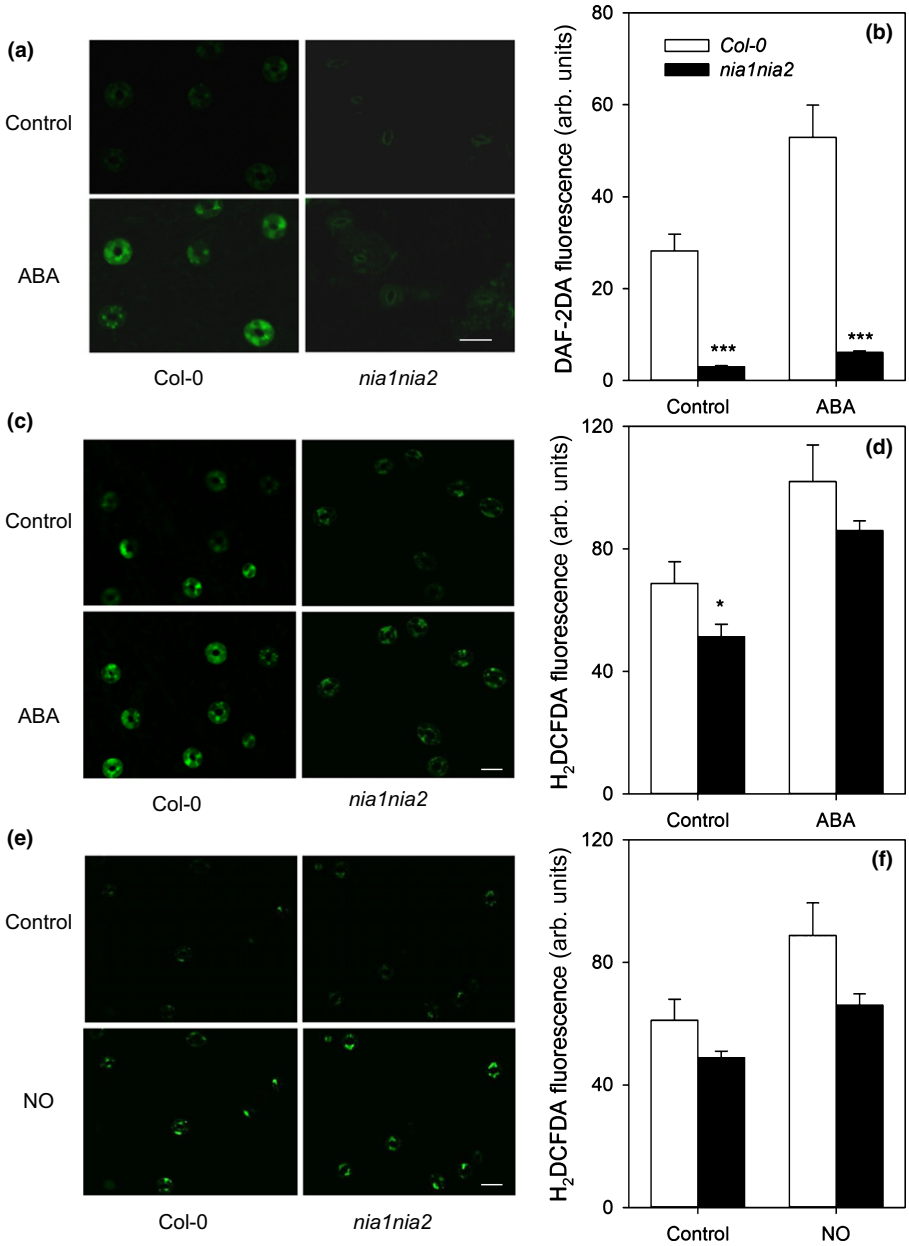


Fig. 2 Effects of ABA on nitric oxide (NO) synthesis and ABA and NO on hydrogen peroxide (H_2O_2) production in stomatal guard cells of Columbia-0 (Col-0) and *nia1nia2* of *Arabidopsis thaliana*. (a) Representative diaminofluorescein diacetate (DAF-2DA) fluorescence images in the control and after 30 min in ABA. (b) Corrected total stomatal fluorescence (CTSF) of DAF-2DA for ABA-induced NO production. (c, e) Representative 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) fluorescence images in the control and after 30 min in ABA (c) and NO (e). (d, f) CTSF for ABA- (d) and NO-induced (f) H_2O_2 production. Bars, 20 μm. Data are means ± SE ($n = 20$–35). *, $P < 0.05$; ***, $P < 0.001$ (as compared with Columbia-0 (Col-0)).

(qPCR). Plants for tissue K^+ measurements and shoot DW were grown to specific ages before sampling.

Stomatal aperture assay

Stomatal aperture assay was carried out essentially as described in Eisenach *et al.* (2012). To compare the stomatal aperture of Col-0 and *nia1nia2* plants in normal growth conditions, leaves were collected at 10:00, 12:00, 14:00, 16:00 and 18:00 h and stomatal images were taken immediately after peeling the epidermis. In the experiments with ABA and NO treatments, epidermal peels were pretreated for 2 h in an opening buffer (50 mM KCl and 5 mM 2-(*N*-morpholino)propanesulfonic acid (MES) at pH 6.1 with NaOH) before adding 50 μM ABA or 100 μM sodium nitroprusside (SNP) to a standard buffer (SB1 = 10 mM KCl, and 5 mM MES at pH at 6.1 with $Ca(OH)_2$).

Confocal microscopy

The NO and H_2O_2 production in guard cells was determined using the fluorescent indicators diaminofluorescein diacetate (DAF-2DA; Calbiochem, Nottingham, UK) and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA; Life Technologies, Melbourne, Australia), respectively. Epidermal peels were prepared as for stomatal aperture assays. The epidermal strips were pretreated with the opening buffer for 2 h before loading 20 μM DAF-2DA or H_2DCFDA for 20 min in the dark, followed by a 20 min flush in SB1 to remove excess dye. The strips were subsequently incubated in SB1 alone or in SB1 containing 50 μM ABA or 100 μM SNP for up to 70 min with a 10 min sampling interval under confocal microscopes (Zeiss and Leica, Wetzlar, Germany). The fluorescence images were collected with excitation at 488 nm and emission at 515–560 nm for DAF-2DA and excitation at 496 nm and emission at 505–525 nm for H_2DCFDA. Corrected total stomatal fluorescence (CTSF) intensity was estimated using ImageJ software (NIH, Bethesda, MD, USA) according to Bonales *et al.* (2013). The stomatal fluorescence signal was first measured as the sum of the intensity of the pixels from the whole stomatal area. The background signal was determined from the empty region of the similar size to the stomata and subtracted from the stomatal signal to obtain CTSF values.

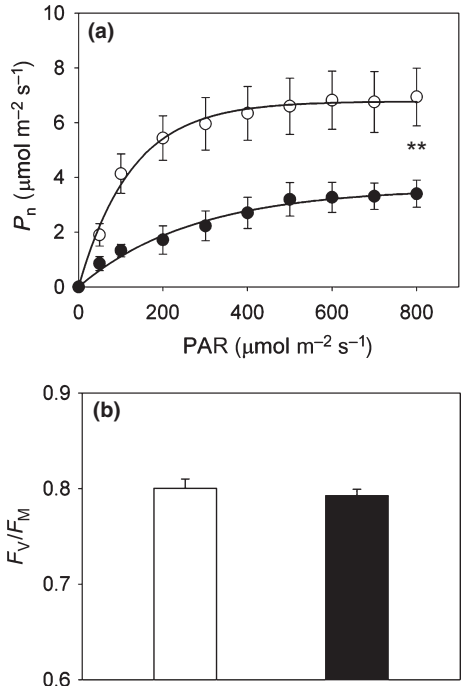


Fig. 3 Photosynthetic characteristics of Columbia-0 (Col-0) and *nia1nia2* of *Arabidopsis thaliana*. (a) Light response of net CO_2 assimilation (P_n) in Col-0 (open circles) and *nia1nia2* (closed circles). Plants were measured with a series of light photosynthetically active radiation (PAR). Data were fitted with single exponential equations, resulting in $PAR_{1/2}$ = 90.8 and 176.4 μmol m^{-2} s^{-1} for Col-0 and *nia1nia2*, respectively (n = 6). (b) Current photochemical capacity of photosystem II (F_V/F_M) from dark-adapted leaves of Col-0 (open bar) and *nia1nia2* (closed bar) plants (n = 12). Data are means ± SE. **, P < 0.01 for P_n at 100 or larger PAR.

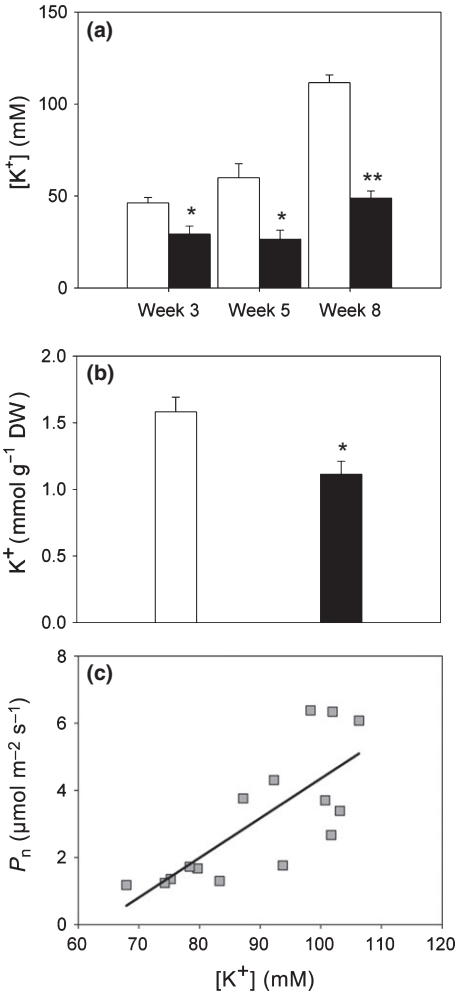


Fig. 4 Leaf potassium (K^+) analysis in Columbia-0 (Col-0) and *nia1nia2* of *Arabidopsis thaliana*. (a) Leaf sap K^+ concentration in Col-0 (open bars) and *nia1nia2* (closed bars) at 3, 5 and 8 wk after sowing (n = 6). (b) Leaf K^+ content in Col-0 (open bars) and *nia1nia2* (closed bars) on a DW basis (n = 6). Data are means ± SE. *, P < 0.05; **, P < 0.01 (as compared with Col-0). (c) Linear correlation between CO_2 assimilation and leaf sap K^+ concentration (r^2 = 0.58, P < 0.01). Each data point represents one plant subjected to whole-plant gas exchange measurements followed by K^+ concentration assay.

Photosynthetic and Chl fluorescence measurements

Net CO_2 assimilation (P_n), stomatal conductance (g_s) and transpiration rate (E) were determined on 4-wk-old plants in an *Arabidopsis* leaf chamber mounted on an infrared gas analyser (LI-6400XT; Li-Cor, Darmstadt, Germany). The conditions in the measurement chamber were controlled at a flow rate of 400 mol s^{-1}, PAR of 100/500 μmol m^{-2} s^{-1}, CO_2 concentration in the sample chamber at 400 mmol mol^{-1}, relative humidity of 50%, and light : dark air temperature of 24 : 20°C. Light response curves were measured with PARs at 0, 50, 100, 200, 300, 400, 500, 600, 700 and 800 μmol m^{-2} s^{-1} for 5 min with 2 min darkness between each PAR. Chl*a* fluorescence measurements of current photochemical capacity of photosystem II (PSII) (F_V/F_M) were conducted on dark-adapted 4-wk-old *Arabidopsis* plants using a PAM 2000 Fluorometer (Walz, Effeltrich, Germany) as described in Chen *et al.* (2005).

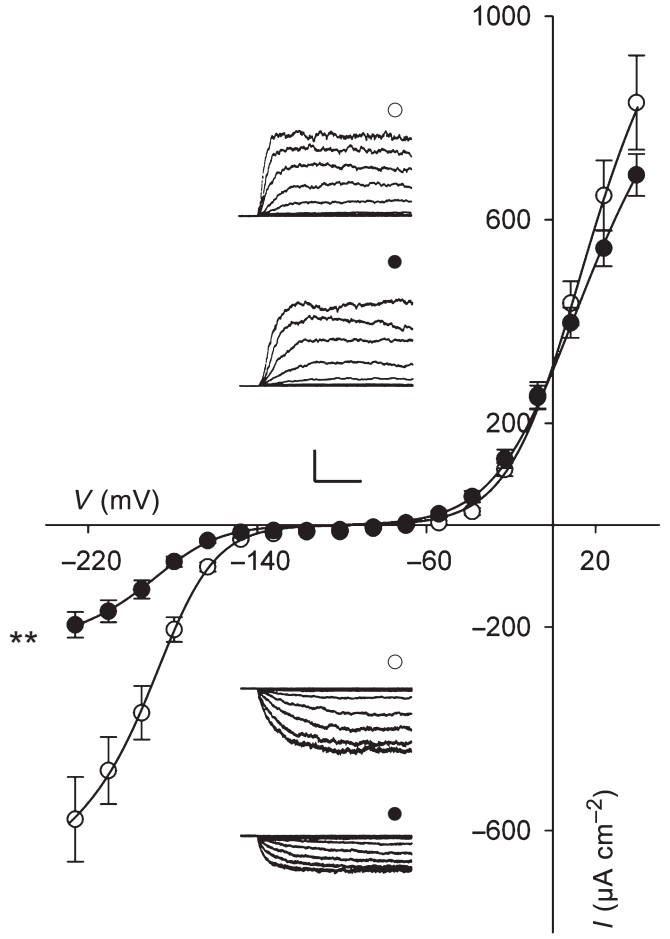


Fig. 5 Characteristics of K^+_{out} and K^+_{in} channels in guard cells of Columbia-0 (Col-0) and *nia1nia2* of *Arabidopsis thaliana*. Mean steady-state *I*/*V* curves were extracted from K^+_{out} and K^+_{in} measurements in an opening buffer for 2 h from both Col-0 (open circles) and *nia1nia2* (closed circles) ($n = 14$ for Col-0 and 38 for *nia1nia2*). Data are means ± SE. **, $P < 0.01$ for $I_{K,in}$ at < −160 mV. Voltage protocols were designed to hold at −100 mV for 2 s and stepped to + 40 mV (10 steps for $I_{K,out}$) and to −230 mV (nine steps for $I_{K,in}$) for 4 s. Representative current traces for K^+_{out} and K^+_{in} channels were measurements from guard cells of both Col-0 and *nia1*/*nia2* and cross-referenced with symbols in the *I*/*V* curves. All the data of $I_{K,out}$ and $I_{K,in}$ were fitted individually to a Boltzmann function for Col-0 and *nia1nia2*, yielding conductance maxima, g_{max}, of 2.7 ± 0.4, 0.9 ± 0.1 ($I_{K,in}$) and 4.9 ± 0.2, 4.1 ± 0.3 ($I_{K,out}$), half-maximal voltages for gating, $V_{1/2}$, −183 ± 12, −183 ± 13 ($I_{K,in}$) and 5 ± 0.5, 0.2 ± 0.05 ($I_{K,out}$), and gating charges, δ, of 1.8 ± 0.2, 1.7 ± 0.2 ($I_{K,in}$) and 1.5 ± 0.2, 1.3 ± 0.1 ($I_{K,out}$). Scale: 200 μA cm^{-2} (vertical) and 2 s (horizontal).

Tissue K^+ and Ca^{2+} measurements

Leaf sap K^+ was determined by a rapid freeze–thaw method (Chen *et al.*, 2007). Plant leaves were sampled at 3, 5 and 6 wk. Three topmost fully expanded leaves of a plant was collected in 1 ml tubes and sealed with Parafilm. Tubes containing leaf samples were frozen in −80°C and left to thaw at room temperature, and this was repeated a few times. Samples were centrifuged twice to release the leaf sap. Ten microlitres of leaf sap from each sample were diluted in distilled H_2O and K^+ was determined by a flame photometer (Sherwood 410, Cambridge, UK). Also, leaf K^+ and Ca^{2+} content were measured after acid digestion of dry leaves using flame photometry (Chen *et al.*, 2005).

Quantitative real time-PCR

Quantitative real-time PCR was essentially as described in Liu *et al.* (2014). Five-week-old plants were treated by spraying 50 μM ABA or 100 μM SNP at the lower epidermis of fully expanded leaves for 1 h. Total leaf RNA was extracted using Trizol reagent (Life Technologies, Australia) following the manufacturer's procedure, and the residual genomic DNA was removed with amplification grade DNase I (Ambion). First-strand cDNA was synthesized with the SensiFAST Kit (Bioline, Alexandria, Australia). Transcript abundances of the target genes were determined by the SensiFAST SYBR No-ROX Kit (Bioline, Australia) with gene-specific primers (Supporting Information Table S1) using a Rotor-Gene Q6000 (Qiagen). qPCR conditions consisted of three-step cycling: polymerase activation at 95°C for 2 min; 40 cycles were set up for 5 s denaturation at 95°C, 10 s annealing at 63°C, 15 s extension at 72°C; SYBR green signal data were acquired at the end. RNA Polymerase II subunit (*RPB1*) was used as the reference for normalization of relative gene expression. Data are averages of three to five independent biological experiments and each has two technical replicates.

Electrophysiology

Electrophysiological recordings using double-barrelled microelectrodes were essentially as described in Blatt & Armstrong (1993) and Chen *et al.* (2012a). For inwardly rectifying K^+ current ($I_{K,in}$) and outwardly rectifying K^+ current ($I_{K,out}$) measurements, electrodes were filled with 200 mM K-acetate at pH 7.5 and SB1 was used as bathing solution. For anion channel current (I_{anion}) measurements, electrodes were filled with 200 mM CsCl (pH 7.5) and a second standard buffer (SB2) containing 15 mM CsCl, 15 mM (tetraethylammonium) TEA-Cl, 5 mM Ca^{2+}-MES at pH 6.1 was used as bathing solution. Quantities of 50 μmol ABA, 100 μM SNP, or 100 μM 2-(4-carboxyphenyl)-4,4,5,5-tetramethyllimidazoline-1-oxyl-3-oxide (c-PTIO) were added to

the standard buffers for ABA, NO, and c-PTIO treatments, respectively. For the $[Ca^{2+}]_{cyt}$ buffering experiments, 150 mM K-acetate and 50 mM of 1,2-bis(o-aminophenoxy)ethane-N,N,N′, N′-tetraacetic acid (BAPTA) (titrated to pH 7.5 with KOH) was used to fill the electrodes. Surface area and volume of impaled guard cells were calculated assuming a spheroid geometry using Henry's EP suite software (Y-Science, University of Glasgow, Glasgow, UK). The current density was then calculated according to the cell surface area for all the measurements.

Statistical analysis

Pearson correlation analysis and statistical significance between genotypes and treatments (Student's *t*-test) were examined using SPSS 20 (IBM, New York, NY, USA). Statistical significance was marked as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. All data are means with SE.

Results

nia1nia2 mutation constitutively affects stomatal opening and ABA-induced stomatal closure

Under normal growth conditions, the stomatal aperture of the *nia1nia2* mutant immediately after peeling the epidermis was found to be significantly smaller than those in the Col-0 throughout the course of the day (Fig. 1a,b). On average, the stomatal aperture of the *nia1nia2* mutant was 25% smaller than that of Col-0 regardless of the pretreatment in the opening buffer (Fig. 1c). Moreover, stomata of *nia1nia2* plants closed only partially in response to ABA compared with Col-0, but presented a full response to the NO donor SNP with respect to Col-0 (Fig. 1d). The requirement of NO production for ABA-induced stomatal closure was validated by confocal microscopy using the DAF-2DA dye. In the control, the NO production in guard cells of *nia1nia2* was significantly ($P < 0.001$) lower than that in Col-0. ABA had little effect on the NO production in *nia1nia2* guard cells in contrast to a significant increase in the Col-0 wild-type (Fig. 2a,b). By contrast, the production of H_2O_2 in guard cells was not altered in the *nia1nia2* mutant and showed a significant increase of H_2O_2 after 30 min in ABA and NO treatments (Fig. 2c–f).

nia1nia2 plants are impaired in CO_2 assimilation and K^+ nutrition

The *nia1nia2* mutant gained an average of only 35% of the wild-type shoot DW over an 8 wk growth period (Fig. S1). The significantly lower DW in *nia1nia2* might be associated with its inability to fully open the stomata (Fig. 1), assuming the lower stomatal aperture was a limiting factor for photosynthesis.

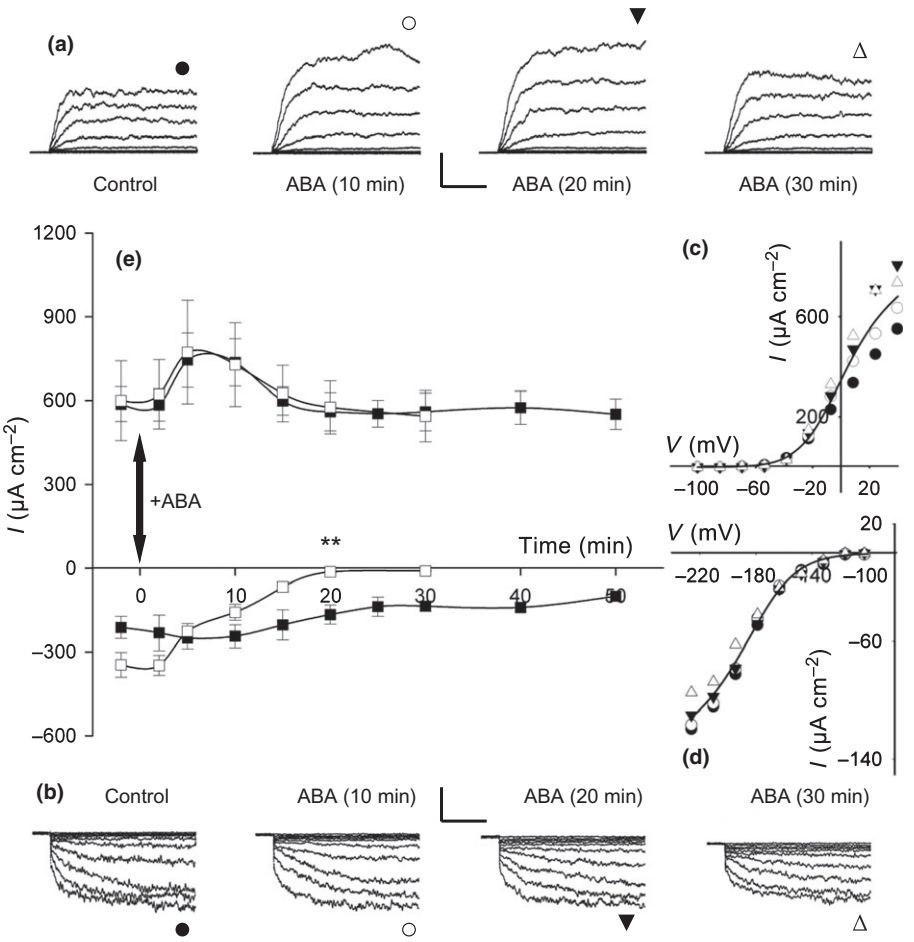


Fig. 6 Abscisic acid regulates K^+_{out} and K^+_{in} channels in guard cells of Columbia-0 (Col-0) and *nia1nia2* of *Arabidopsis thaliana*. (a, b) Representative traces of $I_{K,out}$ (a) and $I_{K,in}$ (b) were from one guard cell of *nia1nia2* before (closed circles), and 10 (open circles), 20 (close triangles) and 30 (open triangles) min after ABA treatment. Scales: vertical, (a) 300 and (b) 50 μA cm^{-2}; horizontal, 2 s. The voltage protocols were designed to hold at −100 mV for 2 s and stepped to + 40 mV (10 steps for $I_{K,out}$) and to −230 mV (nine steps for $I_{K,in}$) for 4 s. (c, d) *I*/*V* curves were $I_{K,out}$ (c) and $I_{K,in}$ (d) from the same guard cell of *nia1nia2* and symbols were cross-referenced. Owing to the lack of changes in the gating properties of K^+_{in} and K^+_{out} channels, all data points were fitted globally with a Boltzmann function, yielding conductance maxima, g_{max}, of 0.52 ($I_{K,in}$) and 3.72 ($I_{K,out}$), half-maximal voltages for gating, $V_{1/2}$, of −178 ($I_{K,in}$) and −7 ($I_{K,out}$) mV, and gating charges, δ, of 1.5 ($I_{K,in}$) and 1.8 ($I_{K,out}$). (e) Average $I_{K,out}$ at + 40 mV and $I_{K,in}$ at −200 mV from guard cells of Col-0 (open squares) and *nia1nia2* (closed squares) responded to ABA over time ($n = 6$). Data are means ± SE. **, $P < 0.01$ for $I_{K,in}$ at 20 min.

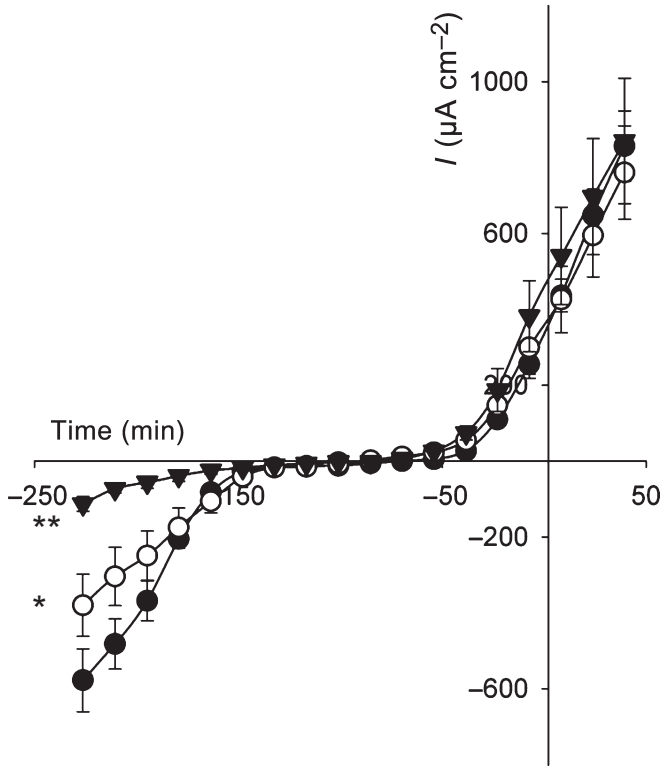


Fig. 7 Nitric oxide (NO) scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethyllimidazoline-1-oxyl-3-oxide (c-PTIO) alleviates ABA-induced inhibition of $I_{K,in}$ in Columbia-0 (Col-0) of *Arabidopsis thaliana*. Average *I/V* curves for $I_{K,out}$ and $I_{K,in}$ from guard cells in control (closed circles), c-PTIO pretreatment + ABA (open circles) and ABA (triangle) for 20 min in Col-0. The voltage protocols were designed to hold at −100 mV for 2 s and stepped to + 40 mV (10 steps for $I_{K,out}$) and to −230 mV (nine steps for $I_{K,in}$) for 4 s. The average data of $I_{K,out}$ and $I_{K,in}$ were fitted individually to a Boltzmann function for Col-0 in the control and treatments ($n = 12$–18). Data are means ± SE. *, $P < 0.05$; **, $P < 0.01$ (as compared with the control).

Therefore, we measured the photosynthetic parameters of Col-0 and *nia1nia2* plants under the normal growth conditions (100 μmol m^{-2} s^{-1} PAR) and saturating light. The light response of net CO_2 assimilation (P_n) of *nia1nia2* was significantly impaired across the entire range of PAR intensities, resulting in an average of 40% of P_n in Col-0 (Fig. 3a). The *nia1nia2* plants also showed significantly lower stomatal conductance (g_s) and transpiration rates (E) in both light conditions (Table S2). To test whether smaller aperture and lower g_s might limit P_n or whether the mutant phenotype might be affected by its capacity of PSII, we measured the photochemical capacity of PSII (F_V/F_M) in the leaves. The F_V/F_M ratio of both lines was above the lower limit of natural variation of healthy leaves (> 0.7) (Maxwell & Johnson, 2000) and there was no significant genotypic difference (Fig. 3b). In addition, no differences in stomatal density were observed between Col-0 and *nia1nia2* (Fig. S2).

We examined the leaf sap K^+ concentration and total leaf K^+ content of the two lines under normal growth conditions‘ to assess whether the effects on aperture, photosynthesis and growth might be correlated with K^+ nutrition. In each case, *nia1nia2* showed up to 60% of K^+ reduction compared with Col-0 (Fig. 4a,b). Leaf water content of Col-0 and *nia1nia2* ranged from 92 to 94% (data not shown), excluding a possible effect of water stress-altered leaf sap K^+ concentration. There was a significant positive correlation ($r^2 = 0.58$, $P < 0.01$) between leaf sap K^+ concentration and P_n of individual plants (Fig. 4c). These results indicate strong links between stomatal aperture (Fig. 1), P_n (Fig. 3a; Table S2) and leaf K^+ nutrition (Fig. 4).

Impaired stomatal regulation in *nia1nia2* is related to reduced $I_{K,in}$ and I_{anion}

Stomatal opening and closure are regulated by the activity of K^+_{in} and slow anion channels (Kim *et al.*, 2010; Chen *et al.*, 2012a,b; Hills *et al.*, 2012). We carried out two-electrode voltage clamp measurements on intact guard cells using an improved method (Chen *et al.*, 2012a; Wang *et al.*, 2013). The basic characteristics of K^+_{in} and K^+_{out} channels, including current activation half times ($t_{1/2}$), deactivation time constants (τ_b), the maximum conductance (g_{max}), the half-maximal conductance voltage ($V_{1/2}$), and the gating charge (δ) were similar in Col-0 and *nia1nia2* (Figs 5 and S3 legends), and differed little from data obtained in measurements earlier from *V. faba* (Blatt, 1992; Blatt & Armstrong, 1993), *Nicotiana benthamiana* (Armstrong *et al.*, 1995) and *Arabidopsis* (Roelfsema & Prins, 1995; Wang *et al.*, 2012, 2013). When measured within the first 10–15 min after stripping and mounting, guard cells of *nia1nia2* showed little $I_{K,in}$, although $I_{K,out}$ appeared normal (Fig. S3). After first superfusing the guard cells with an opening buffer for 2 h, the mean $I_{K,in}$ of *nia1nia2* increased significantly, but remained substantially lower than Col-0 at −200 mV, giving 36% of the wild-type current (Fig. 5; Table S3). In addition, steady-state I_{anion} was significantly impaired in guard cells of *nia1nia2*, which was only 64% of that in Col-0 at −160 mV (Table S3). To verify whether this low $I_{K,in}$ is related to a high baseline of $[Ca^{2+}]_{cyt}$ in guard cells of *nia1nia2*, we added 50 mM of the Ca^{2+} chelator BAPTA in the electrodes. There was a significant recovery of $I_{K,in}$ (five out of 11 cells) with an overall shift of activation voltage to more positive when the $[Ca^{2+}]_{cyt}$ was buffered to lower nanomolar concentrations by BAPTA (Fig. S4a), indicating that $[Ca^{2+}]_{cyt}$ may be one of the factors reducing the stomatal aperture of *nia1nai2*.

NO inhibits $I_{K,in}$ and activates I_{anion} in guard cells of *nia1nia2*

Abscisic acid-mediated regulation of stomatal closure is dominated by changes in the activity and kinetic characteristics of major K^+ and anion channels (Blatt, 2000; Ward *et al.*, 2009; Kim *et al.*, 2010). In both lines, guard cell $I_{K,out}$ was slightly enhanced by ABA over the first 15 min, and then maintained a high activity in ABA over 20–50 min (Figs 6a,e, S5; Table S3). $I_{K,in}$ was completely inhibited by ABA after 20 min in guard cells of Col-0; however, $I_{K,in}$ of *nia1nia2* was marginally reduced by 16% at −200 mV after 40 min of ABA treatment (Fig. 6b,e). Analysis of I_{anion}, recorded separately in the presence of TEA-Cl and CsCl, yielded an increase of I_{anion} in guard cells of Col-0 of 234% at

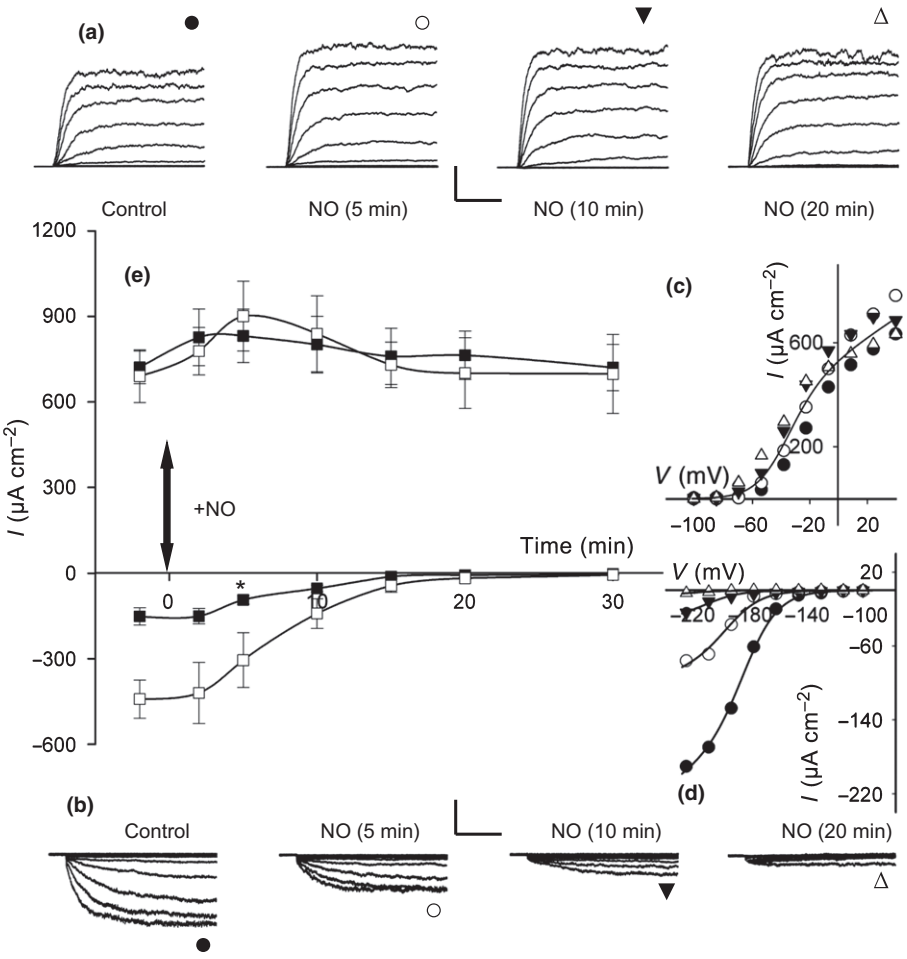


Fig. 8 Nitric oxide (NO) affects K^+_{out} and K^+_{in} channels in guard cells of Columbia-0 (Col-0) and *nia1nia2* of *Arabidopsis thaliana*. (a, b) Representative traces of $I_{K,out}$ (a) and $I_{K,in}$ (b) were from one guard cell of *nia1nia2* before (closed circles), and 5 (open circles), 10 (closed triangles), and 20 (open triangles) min after NO treatment. Scale: vertical, 200 μA cm^{-2} (a) and 50 μA cm^{-2} (b); horizontal, 2 s. (c, d) *I/V* curves of $I_{K,out}$ (c) and $I_{K,in}$ (d) were from the same guard cell of *nia1nia2* and symbols were cross-referenced. The voltage protocols were designed to hold at −100 mV for 2 s and stepped to + 40 mV (10 steps for $I_{K,out}$) and to −230 mV (nine steps for $I_{K,in}$) for 4 s. Curves were the result of fitting the data individually to a Boltzmann function with a fixed $\delta = 1.6$ for $I_{K,in}$. The analysis yielded parameters for the control, and at 5, 10, and 20 min after NO treatment of 0.89, 0.39, 0.13 and 0.02 for g_{max}, respectively, and −184, −196, −211 and −224 mV for $V_{1/2}$, respectively. Owing to the lack of changes in the gating property of $I_{K,out}$, all data points were fitted globally with a Boltzmann function, yielding g_{max} of 3.7, $V_{1/2}$ of −17 mV, and δ of 2.1. (e) Average $I_{K,out}$ at + 40 mV and $I_{K,in}$ at −200 mV from guard cells of Col-0 (open squares) and *nia1nia2* (closed squares) responding to NO over time ($n = 7$). Data are means ± SE. *, $P < 0.05$ after 10 min in NO.

−160 mV in ABA treatment, significantly higher than that of *nia1nia2* (Table S3). To test whether the lack of response of $I_{K,in}$ and I_{anion} to ABA could be attributed to the reduction of NO production, we carried out experiments challenging the guard cells in the presence of the NO scavenger c-PTIO (Fig. 7) or the NO donor SNP (Fig. 8). After 20 min of ABA treatment, the guard cells of Col-0 pretreated with c-PTIO showed a 37% reduction in $I_{K,in}$ at −200 mV, compared with an 85% decrease in guard cells treated with ABA only (Fig. 7). Exogenous addition of NO for 15 min led to an average 92% reduction in $I_{K,in}$ at −200 mV in *nia1nia2* (Fig. 8). Furthermore, there was a significant activation of steady-state I_{anion} in guard cells of *nia1nia2* subjected to NO treatment (138%), which was similar to the typical I_{anion} response in Col-0 for stomatal closure (Fig. 9; Table 1).

Key genes encoding K^+ and anion transport, nitrogen metabolism and ABA-responsive TFs are differentially regulated by *NIA1* and *NIA2* mutation

The unique stomatal and ion channel phenotype of *nia1nia2* led us to further investigate the response of key guard cell genes encoding the $I_{K,in}$, $I_{K,out}$ and I_{anion} channels, nitrate transporters, nitrogen metabolism, and ABA-responsive protein phosphatase 2C (PP2Cs) and TFs in ABA and NO treatments (Fig. 10; Table 1). In comparison to Col-0, *nia1nia2* showed a dramatic increase in *KAT1* and *AKT1* transcripts but a significant decrease in *KAT2*, *AKT2* transcripts and that of the guard cell outwardly rectifying K^+ channel (*GORK*) in the control, ABA and NO treatments (Fig. 10a–d,f). There was no genotypic and treatment difference for the expression levels of *SLAC1*, but surprisingly, *SLAH3* was significantly induced, by seven- and 10-fold in *nia1nia2* by ABA and NO, respectively (Fig. 10g,h). In *nia1nia2*, NO significantly enhanced guard cell *NRT1.1*, but *NRT2.5* was largely suppressed in the control and both treatments (Fig. 10i,j), which is consistent with results in Wang *et al.* (2004). Very little *NIA1* and *NIA2* transcription was detected by qPCR in *nia1nia2*, confirming the insertion in *NIA1* and deletion of *NIA2* (Table 1). There was little difference in the expression of NO synthase associated 1 (*NOA1*) between the genotypes and treatments. Glutamate synthase 1 (*GLU1*) and glutamine synthetase 1 (*GLN1*) genes for ammonium metabolism were significantly up-regulated, 21- and 790-fold in *nia1nia2*, respectively, in comparison to Col-0 in the control. ABA and NO slightly reduced the *GLU1* and *GLN1* transcripts in *nia1nia2* but showed little effects on those in Col-0 (Table 1). We also identified, in both Col-0 and *nia1nia2*, significant ABA-induced up-regulation of PP2Cs *ABI1* and *ABI2*, which were up to threefold

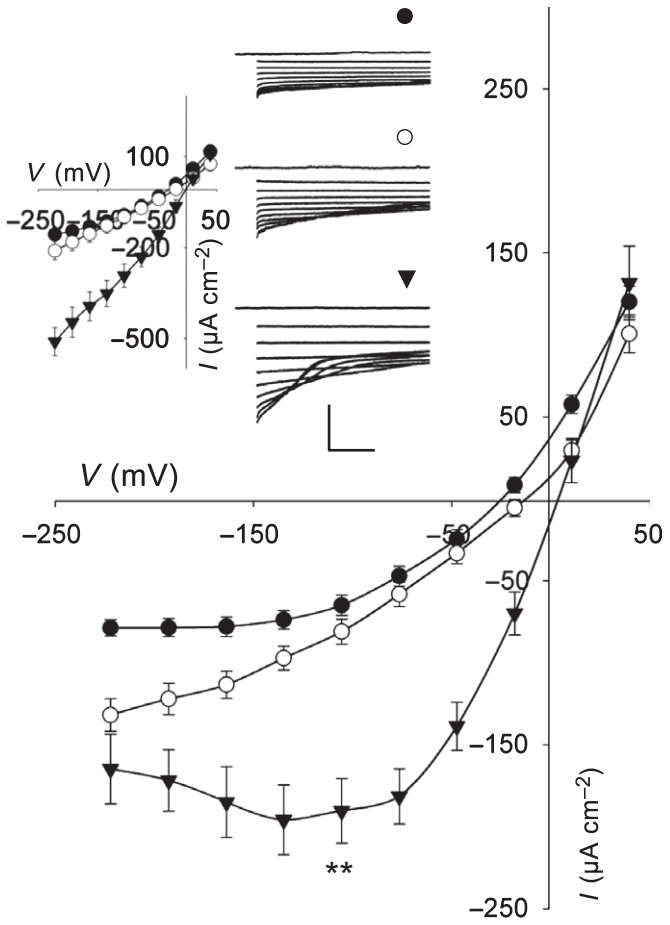


Fig. 9 Abscisic acid and nitric oxide (NO) modulate the anion channel in guard cells of *nia1nia2* of *Arabidopsis thaliana*. Mean steady-state *I*/*V* curves were measured in the control (closed circles), followed by 20 min in ABA (open circles) and NO (triangles) treatments. The inset on the upper left is the average instantaneous I_{anion} ($n = 5$–24). Data are means ± SE. **, $P < 0.01$ for I_{anion} at −130 mV in the NO treatment as compared with the control. The insets on the upper right are representative anion current traces in the control (top inset), followed by 20 min in ABA (middle inset) and NO (bottom inset) treatments, and symbols were cross-referenced in the *I*/*V* curves. A voltage protocol was used with holding voltage at −100 mV for 2 s, followed by an activation voltage of + 40 mV for 5 s. Current was then measured at 10 testing voltages from + 40 to −220 mV for 8 s. Scale: vertical, 200 μA cm^{-2}; horizontal, 2 s.

higher in *nia1nia2* (Table 1). In the control, with the exception of *AKS1* and the MYB domain protein 61 (*MYB61*), expression of all TFs was larger in *nia1nia2* than that in Col-0. Applying ABA and NO resulted in differential expression of those genes between Col-0 and *nia1nia2* with no exception (Table 1).

Discussion

Impaired plant growth, photosynthesis and K^+ nutrition in *nia1nia2* plants is associated with a disrupted K^+-uptake system

Potassium nutrition is so crucial that land plants have evolved to have a large number of K^+-selective transporters for their survival in adverse environmental conditions (Armengaud *et al.*, 2004; Amtmann & Blatt, 2009; Ward *et al.*, 2009; Marschner, 2012). Regulatory proteins, such as kinases, phosphatases, syntaxins and farnesyl transferase, add more complexity to the large number of K^+ transporters and their regulation (Blatt, 2000; Véry & Sentenac, 2003; Ward *et al.*, 2009). A robust K^+-selective inward channel activity at guard cell plasma membrane has been proposed to be a major actor in plant adaptation to atmospheric and soil fluctuating and challenging conditions (Lebaudy *et al.*, 2008). In this study, the *nia1nia2* mutant showed largely reduced growth, leaf K^+ content and photosynthesis (Figs S1, 3, 4), so we proposed that disruption in nitrate reductase may also affect the K^+-uptake system in *Arabidopsis*. Indeed, three major K^+_{in} channel genes, *KAT2*, *AKT2* and *KC1*, were significantly more suppressed in *nia1nia2* than those in Col-0. To compensate, in the *nia1nia2* mutant, the expression of the channels *KAT1* and *AKT1* was substantially enhanced for K^+ uptake (Fig. 10). Even so, we found $I_{K,in}$ from guard cells of *nia1nia2* to be significantly reduced under both normal growth conditions and after the opening buffer treatment (Figs 5, S3). In addition, the significant reduction of *GORK* transcripts in *nia1nia2* (Fig. 10f) indicates that the mutant may reduce K^+ efflux through this pathway to compensate its impaired K^+-uptake capacity, although no difference was found for $I_{K,out}$ activities in guard cells between Col-0 and *nia1nia2* (Figs 5, S3; Table S3). Overall, such a largely disrupted K^+ transport system renders *nia1nia2* unable to grow like a normal wild-type plant.

Reduced channel-mediated K^+ uptake and membrane hyperpolarization affect stomatal opening in *nia1nia2*

In guard cells, five Shaker genes, *KAT1*, *AKT1*, *KAT2*, *AKT2* and *KC1*, encode K^+_{in} channels, but only *GORK* is found for K^+_{out} channels (Szyroki *et al.*, 2001; Hosy *et al.*, 2003). A rapid K^+ uptake upon hyperpolarization of plasma membrane may be more complex to achieve than K^+ loss upon depolarization (Lebaudy *et al.*, 2008). This is supported by the fact that absence of $I_{K,in}$ in guard cells leads to a sixfold (20 vs 120 min) increase in the time constant of stomatal opening, whereas the time constant of stomatal closure induced by disrupting $I_{K,out}$ is extended only twofold (10 vs 20 min) (Szyroki *et al.*, 2001; Hosy *et al.*, 2003; Lebaudy *et al.*, 2008). We also found that stomatal opening is significantly affected in the *nia1nia2* in normal growth condition throughout the day (Fig. 1). Moreover, *nia1nia2* was unable to fully open its stomata even under high external K^+ (Fig. 1), suggesting the *NIA1* 'knockout' and *NIA2* deletion (Table 1) may affect guard cell K^+ transport, as K^+ is the main solute for stomatal opening (Blatt, 2000; Hills *et al.*, 2012). Indeed, an inhibited guard cell $I_{K,in}$ and down-regulation of *KAT2*, *AKT2*, and *KC1* are both likely to contribute significantly to the reduced stomatal opening in *nia1nia2* (Figs 5, 10, S3).

Apart from the K^+ transport, stomatal opening is inducible by light via the regulation of plasma membrane H^+-ATPase and membrane hyperpolarization (Shimazaki *et al.*, 2007). This has been recently incorporated in the OnGuard modelling platform (Chen *et al.*, 2012b; Hills *et al.*, 2012; Wang *et al.*, 2012, 2014).

Table 1 Effect of ABA and nitric oxide (NO) on genes involved in nitrogen metabolism, ABA-responsive protein phosphatase 2Cs (PP2Cs) and transcription factors in *Arabidopsis thaliana* Col-0 and *nia1nia2* leaves

	Control		ABA		NO	
Gene	Col-0	*nia1nia2*	Col-0	*nia1nia2*	Col-0	*nia1nia2*
Nitrogen metabolism						
NIA1	2.62 ± 0.43	0.18 ± 0.05**	1.93 ± 0.15	0.30 ± 0.04**	3.92 ± 0.37	0.38 ± 0.07**
NIA2	1.18 ± 0.14	0.03 ± 0.006**	0.32 ± 0.09	0.01 ± 0.004**	0.90 ± 0.06	0.02 ± 0.004**
NOA1	0.83 ± 0.08	0.52 ± 0.10*	0.87 ± 0.06	0.85 ± 0.33	0.67 ± 0.03	0.49 ± 0.071*
GLU1	0.27 ± 0.06	5.68 ± 2.11**	0.05 ± 0.02	1.40 ± 0.54*	0.31 ± 0.08	3.87 ± 1.56**
GLN1	0.76 ± 0.09	601 ± 77**	0.89 ± 0.11	385 ± 132**	1.87 ± 0.11	502 ± 18**
ABA-responsive PP2Cs						
ABI1	0.35 ± 0.02	1.22 ± 0.13**	1.78 ± 0.23	5.70 ± 0.94**	1.01 ± 0.14	1.57 ± 0.14**
ABI2	0.46 ± 0.05	1.04 ± 0.12**	2.10 ± 0.25	5.27 ± 1.19*	0.63 ± 0.12	1.63 ± 0.34**
ABA-responsive transcription factors						
AKS1	1.09 ± 0.15	0.66 ± 0.09*	1.44 ± 0.23	3.54 ± 0.29**	0.30 ± 0.02	1.43 ± 0.21**
AKS2	0.89 ± 0.11	1.81 ± 0.16**	1.24 ± 0.09	0.69 ± 0.07**	1.65 ± 0.23	0.75 ± 0.06**
AKS3	0.61 ± 0.04	0.51 ± 0.03*	0.92 ± 0.12	0.26 ± 0.04**	0.97 ± 0.08	0.38 ± 0.02**
MYB15	0.36 ± 0.09	0.39 ± 0.06	7.85 ± 1.29	3.94 ± 0.63*	9.82 ± 0.51	5.13 ± 1.09**
MYB44	0.67 ± 0.15	2.00 ± 0.24**	1.73 ± 0.31	9.12 ± 2.00**	1.01 ± 0.17	10.81 ± 3.04**
MYB60	0.96 ± 0.14	1.38 ± 0.15	0.90 ± 0.21	0.24 ± 0.02*	1.11 ± 0.10	0.64 ± 0.02**
MYB61	1.01 ± 0.27	0.45 ± 0.10*	1.05 ± 0.36	0.36 ± 0.17	1.43 ± 0.63	0.16 ± 0.05*
ABF1	0.52 ± 0.06	0.74 ± 0.03**	0.69 ± 0.11	1.15 ± 0.13*	0.38 ± 0.03	0.57 ± 0.07*

Data are relative expressions of genes over the reference gene *RPB1*. Data are means ± SE ($n = 6$–10).
*, $P < 0.05$; **, $P < 0.01$ (as compared with Col-0).

Light-induced stomatal opening can be inhibited by ABA, NO and H_2O_2 through the inhibition of H^+ pumping and phosphorylation of plasma membrane H^+-ATPase (Garcia-Mata & Lamattina, 2007; Zhang *et al.*, 2007). In this study, white light at high PARs was unable to increase stomatal opening, P_n and g_s of *nia1nia2* to the same extent as Col-0, despite no depression of the photosynthetic apparatus in *nia1nia2* (Fig. 3; Table S2). In our previous study, *nia1nia2* showed a 14% lower membrane hyperpolarization in guard cells than that of Col-0 (Chen *et al.*, 2012a). Notably, a significant lower expression of bHLH TF *AKS1* in *nia1nia2* in the control (Table 1) could potentially be linked to a reduced binding to 14-3-3 proteins for phosphorylation of plasma membrane H^+-ATPase (Takahashi *et al.*, 2013), and consequently a less negative guard cell membrane potential (Chen *et al.*, 2012a). Therefore, less negative membrane potential and reduced expression of K^+_{in} channel genes (Fig. 10) are likely to result in a decrease of K^+ influx for stomatal opening (Blatt, 2000; Shimazaki *et al.*, 2007; Figs 5, S3). Future investigation should be focused on the light-induced H^+ pumping and regulation of plasma membrane H^+-ATPase in the guard cells of *nia1nia2*.

NO is required for the ABA regulation of ion channels in *Arabidopsis* guard cells

Nitric oxide has been a vital secondary messenger in various signalling pathways for plant stress response over the past two decades (Neill *et al.*, 2003; Garcia-Mata & Lamattina, 2007, 2013). Taking advantage of the improved experimental techniques (Chen *et al.*, 2012a; Wang *et al.*, 2013), we found that exogenous NO rapidly inhibits $I_{K,in}$ and activates I_{anion} in *Arabidopsis* guard cells (Figs 8, 9, S5; Table S3). However, ABA has little effect on the inhibition of $I_{K,in}$ and activation of I_{anion} in guard cells of *nia1nia2* and in guard cells of Col-0 preincubated with NO scavenger c-PTIO (Figs 6, 7). In ABA and NO treatments, the H_2O_2 production was not affected in guard cells of *nia1nia2* or Col-0 (Fig. 2). These results demonstrate unambiguously that NO production in guard cells modulates $I_{K,in}$ and I_{anion} and is required for ABA-induced stomatal closure in *Arabidopsis*.

We further explored the underlying mechanisms for the largely attenuated stomatal response to ABA in *nia1nia2* by investigating the core ABA signalling pathway. Under normal conditions, two PP2Cs, ABI1 and ABI2, suppress the activation of the SNF1-related protein kinase 2 (SnRK2) and its downstream signalling in guard cells (Geiger *et al.*, 2009; Sato *et al.*, 2009). ABA is known to bind PYR/PYL/RCAR receptor proteins and PP2Cs (Park *et al.*, 2009), and as a consequence SnRK2 is released for direct phosphorylation of both anion and K^+ channels for stomatal closure. Interestingly, we found that the expression of *ABI1* and *ABI2* in the *nia1nia2* was consistently higher than that in Col-0 in the control, ABA and NO treatments (Table 1). These observations could imply that an elevation in ABI1 and ABI2 in *nia1nia2* could reduce SnRK2 activity, therefore blocking the effective phosphorylation of KATs and SLAC/SLAHs. It is possible, too, that changes in the transcription and translation of the *KAT1* and *AKT1* genes in *nia1nia2* could affect stomatal function (Fig. 10). These ion channel genes are regulated at transcriptional and post-translational levels (Eisenach *et al.*, 2012; Takahashi *et al.*, 2013). For instance, *AKS1* binds the promoter region of *KAT1* through an E-box motif to stimulate *KAT1* transcription (Takahashi *et al.*, 2013). Here, ABA-induced up-

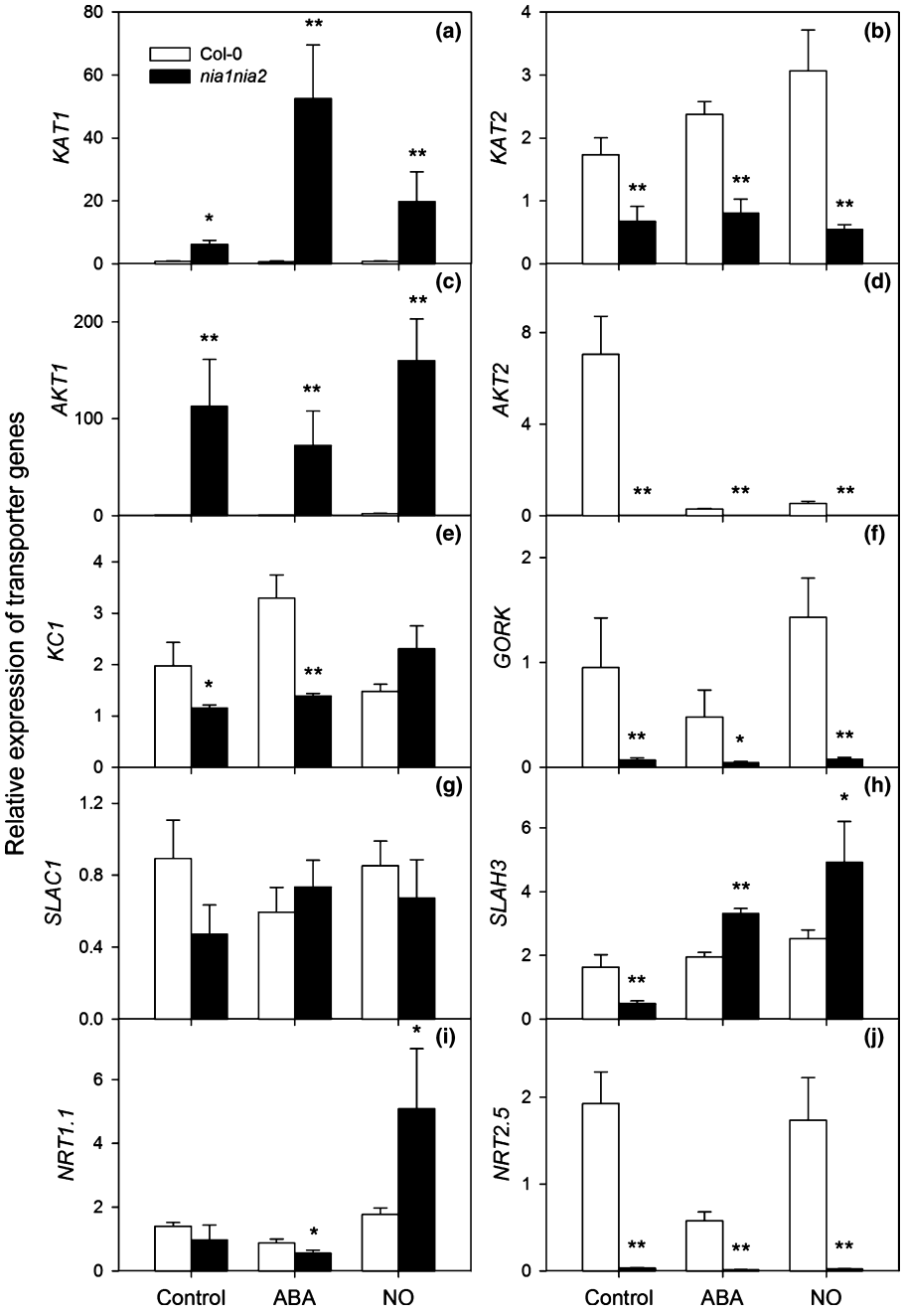


Fig. 10 Abscisic acid- and nitric oxide (NO)-induced relative expression of genes encoding major potassium (K^+) and anion channels and nitrate transporters (NRTs) in leaves of Columbia-0 (Col-0) and *nia1nia2* of *Arabidopsis thaliana*. Relative transcripts of inwardly rectifying K^+ channels *KAT1*, *KAT2*, *AKT1*, *AKT2* and *KC1* (a–e), guard cell outwardly rectifying K^+ channel *GORK* (f), slow anion channels *SLAC1* and *SLAH3* (g, h) and NRTs *NRT1.1* and *NRT2.5* (i, j) over RNA polymerase II subunit (RPB1) in leaves of Col-0 (open bars) and *nia1nia2* (closed bars). Data are means ± SE ($n = 6$–10). *, $P < 0.05$; **, $P < 0.01$ (as compared with Col-0).

regulation of *AKS1* (Table 1) was accompanied by an 8.5-fold increase of *KAT1* in *nia1nia2* in the ABA treatment (Fig. 10a). *KAT1* transcripts were 72-fold higher in ABA-treated *nia1nia2* than in Col-0, in which *KAT1* showed little change as a result of ABA (Fig. 10a). Moreover, it was reported that ABA increases 14-3-3 protein binding to guard cell AKS1 and AKS3 in a phosphorylation-dependent manner to enhance stomatal opening (Takahashi *et al.*, 2013). Similarly, ABA induced a fivefold increase of *AKS1* in *nia1nia2* compared with Col-0 (Table 1), which may imply that AKS1 regulates H^+-ATPase by binding 14-3-3 protein to counteract the ABA-induced stomatal closure in *nia1nia2*, although direct evidence is required in future studies. By contrast, ABA imposed only small changes in *SLAC1* transcript abundances and I_{anion} activity in the *nia1nia2* mutant (Figs 9, 10g; Table S3), despite these channels being known as a 'master switch' for stomatal closure (Roelfsema *et al.*, 2012). In summary, all this evidence might account for *nia1nia2*'s impaired ABA-induced stomatal closure.

Interactions of nitrogen and potassium nutrition in *nia1nia2*

Under normal growth conditions, K^+ and NO_3^- are the major forms of macronutrients that are usually transported and utilized efficiently by plants (Leigh & Wyn Jones, 1984; Schachtman & Shin, 2007; Amtmann & Blatt, 2009; Marschner, 2012). Plants

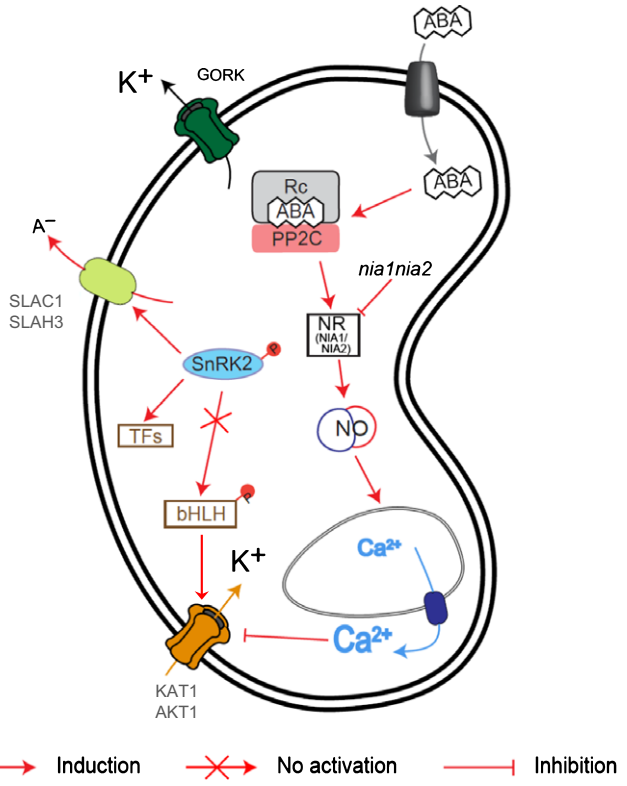


Fig. 11 Schematic diagram of ABA and nitric oxide (NO) interaction in the guard cells of *nia1nia2* of *Arabidopsis thaliana*. Based on the well established ABA signalling pathway, we hypothesize that, in the absence of NO production of *nia1nia2*, ABA is still transported to guard cells and sensed by the PYR/PYL/RCAR receptor complex (Rc). However, ABA-induced up-regulation of protein phosphatase 2C (PP2C; *ABI1* and *ABI2*) genes in *nia1nia2* blocks the downstream SnRK2.6/OST1 that inactivates anion channels (A−). Further, low endogenous NO is unable to inhibit $I_{K,in}$ in a Ca^{2+}-dependent manner. Basic helix–loop–helix (bHLH) transcription factor AKS1 enhances both the K^+_{in} channel gene *KAT1* at the transcriptional level and 14-3-3 protein for phosphorylation of H^+-ATPase at the post-translational level. K^+_{out} channel GORK is still responsive to ABA via protein phosphorylation. Also, other transcription factors (TFs; e.g. *MYBs*, *ABF1*) are involved in this impaired stomatal closure as positive or negative regulators (see Fig. 10 and Table 1). Red arrows, signal induction; red arrows with cross, no signal activation; red stop arrows, signal inhibition.

normally balance the nutritional status of these two inorganic elements. K^+ starvation affected nitrogen nutrition through the down-regulation of high-affinity *NRT2.1*, *NRT2.3* and *NRT2.6*, and up-regulation of *NIA1* and *NIA2* and nitrate reductase activity in *Arabidopsis* (Armengaud *et al.*, 2004, 2009). We found that eliminating *NIA1* and *NIA2* not only disrupted six major K^+ channels but also down-regulated high-affinity *NRT2.5* and up-regulated ammonium transporters *GLU1* and *GLN1* (Fig. 10; Table 1). Disruption of nitrogen metabolism in *nia1nia2* has been interpreted as the reason for the failure of ABA to induce NO production in guard cells (Ribeiro *et al.*, 2009). Stomatal closure was induced by NO production during the transition from light to dark in both wild-type and *nia2* mutants, but stomata of *nia1* failed to close under the same conditions (Wilson *et al.*, 2008). This difference highlights a unique function of *NIA1* in NO production and implies a role for *NIA2* primarily in nitrogen assimilation (Wilkinson & Crawford, 1993; Bright *et al.*, 2006). There is growing evidence to show that the comodulation of AKT1 and NRT1.1 by the CBL1-CIPK23 complex ensures a concerted transport and sensing of potassium and nitrogen nutrition in *Arabidopsis* (Xu *et al.*, 2006; Léran *et al.*, 2015). In our experiment, *AKT1* was constitutively up-regulated in the *nia1nia2* mutant regardless of ABA and NO treatment (Fig. 10c). It will be interesting in the future to see whether a connection might be made to CBL-CIPK regulation of NRT1.1. Thus, these findings demonstrate the involvement of multiple regulatory mechanisms such as transcriptional, post-transcriptional and post-translational regulation of K^+ and nitrogen transporters for the interactions of nitrogen and potassium nutrition in *nia1nia2*.

A hypothetic model for ABA-induced stomatal response in *nia1nia2*

Based on the well-established ABA signalling pathway (Kim *et al.*, 2010; Boursiac *et al.*, 2013; Takahashi *et al.*, 2013; Osakabe *et al.*, 2014) and our current results, we proposed a hypothetic model for attenuated ABA-induced stomatal response in *nia1nia2* (Fig. 11). We hypothesize that, in the absence of NO production of *nia1nia2*, ABA is still transported to guard cells and sensed by the PYR/PYL/RCAR receptor complex (RC). However, ABA-induced up-regulation of PP2C (*ABI1* and *ABI2*) genes in *nia1nia2* blocks the downstream SnRK2.6/OST1 that inactivates anion channels (A^-) for stomatal closure. Also, low endogenous NO is unable to inhibit $I_{K,in}$ in a Ca^{2+}-dependent manner. bHLH TF *AKS1* also enhances both K^+_{in} channel *KAT1* at the transcriptional level and 14-3-3 proteins for phosphorylation of H^+-ATPase at the post-translational level to counteract ABA-induced stomatal closure. The K^+_{out} channel in guard cells of *nia1nia2* is still ABA-responsive, potentially via protein phosphorylation and changes in cytosolic pH (Blatt & Armstrong, 1993; Grabov & Blatt, 1997). However, future work is needed to consider a wider range of signal transductions, including ABA, Ca^{2+}, pH, H_2S, H_2O_2 and CO (Garcia-Mata & Lamattina, 2013) and signalling mutants (e.g. *abi1*, *ost1*), which may regulate NO accumulation and NO-mediated downstream events (Fig. 11).

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Author contributions

Z-H.C., Y.W., J-W.W., A.A. and M.R.B. planned and designed the research. Z-H.C., Y.W., J-W.W., M.B., C.Z., E.S., C.D. and M.M. performed experiments. Z-H.C., Y.W., M.B., C.Z., E.S., C.D., M.M. and A.H. analysed the data. Z-H.C., Y.W., C.G-M. and M.R.B. prepared the figures and tables. Z-H.C. and M.R.B. wrote the manuscript.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Shoot dry weight of Col-0 and *nia1nia2* plants.

Fig. S2 Stomatal density of Col-0 and *nia1nia2* plants.

Fig. S3 Characteristics of K^+_{out} and K^+_{in} channels in guard cells of Col-0 and *nia1nia2*.

Fig. S4 Effects of Ca^{2+} chelator BAPTA on K^+_{in} channels in guard cells and the comparison of Ca^{2+} content in leaves of Col-0 and *nia1nia2* plants.

Fig. S5 ABA and NO modulate K^+_{out} and K^+_{in} channels in guard cells of Col-0 and *nia1nia2*.

Table S1 Primer and gene information of the quantitative real-time PCR experiment

Table S2 Comparison of photosynthetic characteristics in Col-0 and *nia1nia2* plants

Table S3 Comparison of K^+ and anion channel current in the control, ABA and NO treatments in guard cells of Col-0 and *nia1nia2* plants

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