Arabidopsis mutant *dnd2* exhibits increased auxin and abscisic acid content and reduced stomatal conductance

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Abstract

Arabidopsis thaliana cyclic nucleotide-gated ion channel gene 4 (*AtCNGC4*) loss-of-function mutant *dnd2* exhibits elevated accumulation of salicylic acid (SA), dwarfed morphology, reduced hypersensitive response (HR), altered disease resistance and spontaneous lesions on plant leaves. An orthologous barley mutant, *nec1*, has been reported to over-accumulate indole-3-acetic acid (IAA) and to exhibit changes in stomatal regulation in response to exogenous auxin. Here we show that the Arabidopsis *dnd2* over-accumulates both IAA and abscisic acid (ABA) and displays related phenotypic and physiological changes, such as, reduced stomatal size, higher stomatal density and stomatal index. *dnd2* showed increased salt tolerance in root growth assay and significantly reduced stomatal conductance, while maintaining near wt reaction in stomatal conductance upon external application of ABA, and probably consequently increased drought stress tolerance. Introduction of both *sid2-1* and *fmo1* into *dnd2* background resulting in removal of SA did not alter stomatal conductance. Hence, the closed stomata of *dnd2* is probably a result of increased ABA levels and not increased SA levels. The triple *dnd2sid2abi1-1* mutant exhibited intermediate stomatal conductance compared to *dnd2* and *abi1-1* (ABA insensitive, open stomata), while the response to external ABA was as in *abi1-1* suggesting that reduced stomatal conductance in *dnd2* is not due to impaired ABA signalling. In conclusion, Arabidopsis *dnd2* mutant exhibited ABA overaccumulation and stomatal phenotypes, which may contribute to the observed improvement in drought stress resistance. Thus, Arabidopsis *dnd2* mutant may serve as a model for studying crosstalk between biotic and abiotic stress and hormonal response in plants.

Keywords: Arabidopsis *dnd2*, barley *nec1*, auxin, abscisic acid, drought stress

1. Introduction

Plant lesion mimic mutants (LMM) are essential tools for understanding mechanisms of the hypersensitive response (HR) and plant disease resistance (Bruggeman et al., 2015; Lorrain et al., 2003). The link between plant disease resistance and hormonal response and abiotic stress tolerance is becoming increasingly established (Andersen et al., 2018; Kazan and Manners, 2009; Moeder et al., 2010). Arabidopsis *dnd2* and *hlm1* mutants have mutations in the *AtCNGC4* gene leading to the disruption of *cyclic nucleotide-gated ion channel 4* gene, which causes multiple phenotypes including elevated accumulation of salicylic acid (SA), defective seed development, dwarfed plant size, delayed growth and time of flowering, defective reproduction, changed leaf shape, reduced hypersensitive response, altered disease resistance, spontaneous lesions on plant leaves and defective programmed cell death (PCD) during pathogen attack, while maintaining effective gene-for-gene resistance and elevated thermal tolerance (Balague et al., 2003; Finka et al., 2012; Genger et al., 2008; Jurkowski et al., 2004; Mercier et al., 2004). While the phenotypes of the *dnd2* mutant have been well characterized, the molecular mechanisms that lead to them are not entirely clear, and, in addition to the already identified SA and reactive oxygen species (ROS) pathways and suspected Ca^{2+} signaling (Chin et al., 2013), may include other unknown signals. Different members of CNGC gene family may have different roles in Ca^{2+} signaling, e.g., *CNGC5* and *CNGC6* genes apparently encode cGMP-activated nonselective Ca^{2+}-permeable cation channels in the plasma membrane of Arabidopsis guard cells (Wang et al., 2013), while the Arabidopsis CNGC2 was demonstrated to mediate Ca^{2+} influx in mammalian HEK293T cells and in Arabidopsis leaf cells (Leng et al., 1999; Wang et al., 2017). Recently, Arabidopsis *dnd1* was reported to have reduced auxin sensitivity, impaired auxin signaling and reduced increase in Ca^{2+} after treatment with exogenous auxin,

suggesting that CNGC2 may be involved in auxin-mediated Ca^{2+} signaling (Chakraborty et al., 2018). Considering that Arabidopsis CNGC2 and CNGC4 are involved in the same signaling pathways and have been shown to form homomeric and heteromeric ion channels in plants (Chin et al., 2013), similar Ca^{2+} response in the *dnd2* mutant can be expected compared to *dnd1*. Previously, a possible auxin phenotype of *dnd2* mutant was hypothesized (Sherman and Fromm, 2009); however, no information is available on auxin content and responses in *dnd2*. *dnd2* plants have elevated plant disease resistance, but the mechanisms have not been discovered in detail. Thus, it is possible that phytohormones other than SA participate in *dnd2* disease resistance. Auxin and/or its signaling pathways may regulate plant disease resistance directly (Kazan and Manners, 2009). Auxin could also play an indirect role by inducing changes in plant development and altering plant and stomatal size and preventing plants from pathogen penetration via stomata (Melotto et al., 2008; Melotto et al., 2006).

ABA is mediating many aspects of plant growth and development (Leung and Giraudat, 1998), as well as responses to variety of abiotic stresses including salt and drought stress (Cutler et al., 2010; Finkelstein, 2013). *CNGC* genes have been shown to be induced under salinity stress in chrysanthemum leaves and roots (Cheng et al., 2018). Recently, the wheat *TaCNGC14* gene, which is an ortholog of the *AtCNGC2* gene, was found to be significantly upregulated during ABA treatment (Guo et al., 2018); however, no relation between *dnd2* mutant and ABA has been shown until now. Regulation of stomatal closure to optimize transpiration is a key step in preventing plants from water loss. Transpiration is mediated by a turgor-driven change in volume of the two surrounding guard cells (Yu et al., 2008). Many factors can influence the guard cell turgor change including light, phytohormones, potassium and calcium ions, malate, NO, and H_2O_2 (Assmann, 2003; Nilson and Assmann, 2007; Schroeder et al., 2001; Shimazaki et al.,

2007). Additionally, overaccumulation of ABA has been demonstrated in Arabidopsis lesion mimic mutant *cpr22* that contains a fusion of *CNGC11* and *CNGC12* genes (Mosher et al., 2010). ABA is also known as one of the regulators of drought stress tolerance in plants (Sauter et al., 2001). The role of ABA in pathogen defense is poorly understood, although there are some reports revealing correlation between ABA levels and resistance to pathogens (Mauch-Mani and Mauch, 2005). ABA is also one of the players in the regulation of stomatal closure, which is part of the innate immune system against bacterial infection. In view of this, it would be of interest to determinate auxin and ABA content of *dnd2* plants and measure stomatal characteristics, which could indirectly affect *dnd2* resistence to *Pseudomonas syringae*.

The orthologous mutant in barley, *nec1* (Rostoks et al., 2006), has been shown to possess essentially the same "defense–no–death" phenotype as *dnd2*/*hlm1* (Keisa et al., 2011) and provides significantly reduced disease development caused by fungal pathogens *Ramularia collo-cygni* and *Fusarium culmorum* (McGrann et al., 2015). However, *nec1* also exhibits reduced stomatal conductance and aperture, altered response to exogenous auxin and increased level of endogenous indole-3-acetic acid (Keisa et al., 2013). In this study we characterized IAA and ABA content and expression of relevant genes in Arabidopsis *dnd2* mutant, as well as changes in its physiological responses and its drought tolerance.

2. Material and methods

2.1. Arabidopsis mutants

Arabidopsis mutants used in the study are described in the Table 1. Arabidopsis accession Col-0, as well as mutants *dnd2-1* and *aba2-1* were obtained from the European Arabidopsis Stock Centre (http://arabidopsis.info/). Arabidopsis *fmo1*, *sid2* and *abi1-1* mutants were gifts

from Dr. Hans Thordal-Christensen, Dr. Jean-Pierre Métraux and Dr Julian Schroeder, respectively. Double and triple mutants were generated through crossings. Mutations were confirmed in F_2 and F_3 generations by using cleaved amplified polymorphic sequences (CAPS) primer-based PCR for *dnd2-1*, *sid2-1*, *abi1-1*, and *aba2-1*, and PCR genotyping for *fmo1*. Primer sequences for genotyping are provided in Table 2.

2.2. Measurements of IAA and ABA

Non-conjugated forms of IAA and ABA were extracted and determined by HPLC-UV-FLD as described previously (Nakurte et al., 2012). Briefly, the plant material was ground in liquid nitrogen and the samples were weighted and extracted with 100% methanol (2.5 mL g^{-1} of fresh weight (FW)). The extract was cleared by centrifugation at 4,000 g for 10 min at room temperature. The resulting supernatant was transferred to a new tube and evaporated until the volume decreased to less than one-tenth of the initial. The evaporated residue was dissolved in a 1% acetic acid solution (2.5 mL g^{-1} FW) and filtered with 0.20 μm filters (Nonpyrogenic Sterile-R, Sarstedt) to remove particulate and other suspended solid matter. The filtered samples were immediately preconcentrated by SPE using AccuBOND II ODSC18 200 mg 3 mL SPE (Agilent Technologies, Santa Clara, CA, USA). C18 SPE columns were pretreated with 2.5 mL of methanol followed by 2.5 mL of 1M acetic acid. Samples (approximately 2.5 mL) were loaded on the cartridge, the column was washed with 2.5 mL of 1M acetic acid and methanol (2.5 mL) was used to elute the analytes from the extraction column. The extract was evaporated till dryness and redissolved in 300 μL of the mobile phase (60% methanol and 40% of 1% acetic acid v/v)). Standards of indole- 3-acetic acid (>99%), and ABA (99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Chromatographic analysis was performed on a modular HPLC system, Agilent 1100 series consisting of quaternary pump, autosampler, column thermostat, UV and fluorescence detectors (Agilent Technologies, Santa Clara, CA, USA). HPLC separations were achieved by using a reverse-phase Zorbax Eclipse XDB-C8 (Agilent Technologies, Santa Clara, CA, USA) column 4.6 × 150 mm, 5 μm. Colum temperature was controlled at 30°C. Mobile phase was composed of methanol and 1% acetic acid (60:40, v/v) in isocratic mode at a flow rate of 1 mL min^{-1}. The detection of ABA was monitored using UV detection at 270 nm and the detection of IAA was monitored using FLD detection at 282 nm (Ex) 360 nm (Em). Injection volume was 50 μL. Results were evaluated by a ChemStation Plus (Agilent, Santa Clara, CA, USA).

Plants for the experiment were germinated in pots (Arasystem, Betatech bvba, Ghent, Belgium) filled with a mixture of half soil and half vermiculite. After seven days seedlings were transplanted in new pots and grown for one month. For ABA content measurements during drought stress leaf samples were collected two days after withholding water (drought stress) or from routinely watered control plants. Plants were grown in a growth chamber under long-day (16 h day, 8 h night), 22°C and medium light (ca. 150 μmol m^{-2} s^{-1}) growing conditions. Histochemical visualization of endogenous IAA level in Col-0 and *dnd2* plants was done using pIAA2-GFP-GUS reporter gene system crossed into *dnd2* background according to a published protocol (Bishopp et al., 2011).

2.3. Effects of IAA, ABA and NaCl on root elongation

Seeds were surface sterilized in 2.5% bleach solution and incubated at 4°C for three days to synchronize the germination. Seedlings were grown on Murashige and Skoog (MS basal medium) supplemented with 0.8% agar (Murashige and Skoog, 1962). Plants were grown on

Petri plates in a growth chamber under long-day (16 h day, 8 h night), 22°C and medium light (ca. 150 μmol m^{-2} s^{-1}). After growth in MS medium for seven days, plants were transferred to Petri plates with fresh medium containing different concentrations of IAA and ABA or 100 mM NaCl. During the transfer dots were marked on Petri plates showing the tip of primary root. Petri plates were incubated vertically, and the increase in length of primary roots from dots (elongation) was measured after seven days from digital images using Image J software (Schneider et al., 2012). Results are average measurements of at least 10 seedlings per treatment from three independent experiments. Results were expressed as a percentage from untreated plant root elongation.

2.4. RNA extraction and quantitative real-time PCR

Total RNA extraction from Col-0 and *dnd2* rosette leaves, cDNA synthesis, and quantitative real-time PCR were performed as described by (Keisa et al., 2011). A set of genes for quantitative real-time PCR was selected based on 1) involvement in indole-3-acetic acid biosynthesis and signaling (*YUC1*, *YUC2*, *YUC6*, *TIR1*, *TAA1*, *AMI1*, *CSN5*) and 2) ABA signaling (*ABI1*, *ABI4*). *TIR1*-encoded protein is a receptor of auxin (Dharmasiri et al.,2005; Kepinski and Leyser, 2005), as well as a member of SCF TIR1 ubiquitin-ligase complex involved in degradation of Aux/IAA proteins (Tan et al. 2007), while CSN5 has been shown to modulate auxin response in COP9 signalosome (CSN) complex with SCFTIR1 (Dohmann et al., 2008). YUCCA flavin monooxygenases encoded by different *YUC* genes are implicated in auxin biosynthesis via indole-3-pyruvic acid (IPyA) pathway (Di et al., 2016), while TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1 (TAA1) has been shown to convert L-tryptophan to IPyA and AMI1 is involved in conversion of indole-3-acetamide (IAM) to IAA

(Mano and Nemoto, 2012). *ABA INSENSITIVE1* encoding protein phosphatase 2C is a known negative regulator of ABA signaling (Gosti et al., 1999) and is itself upregulated by external ABA applications (Hoth et al., 2002). *ABI4* gene encodes an AP2 domain transcription factor (Söderman et al., 2000) which functions as a positive regulator of ABA signaling.

Primers used for qPCR analysis are listed in Table 2. Relative gene expression in *dnd2* was calculated using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and gene expression was normalized to the *AtACTIN2* gene, which is commonly used for normalization of gene expression data in Arabidopsis and its expression is not affected by external application of ABA (Hoth et al., 2002). Gene expression in *dnd2* was shown relative to the expression in Col-0 plants. Plants for the experiment were grown as described above for quantification of IAA and ABA (section 2.2).

2.5. Drought stress treatment

Plants for the drought stress treatment were germinated in pots (Arasystem, Betatech bvba, Ghent, Belgium) filled with a mixture of half soil and half vermiculite and watered three times a week, ensuring soil saturation. After seven days seedlings were individually transplanted in new pots and irrigated with 5 ml water on day one, four and seven after transplantation. On day 14 after germination drought stress was induced by withholding water for 12 days and removing baskets from plastic tray for increased water evaporation. Plants were grown in a growth chamber under long-day (16 h day, 8 h night), 22°C and medium light (ca. 150 μmol m^{-2} s^{-1}) conditions with constant ventilation. Majority of Col-0 plants showed clear symptoms of wilting that were considered as severe drought stress. The survival rates were evaluated after five days of

resuming watering (5 ml water per day). Two independent experiments were performed using in total 56 *dnd2* and 56 Col-0 plants per treatment.

2.6. Measurements of stomatal density, index and sizes

Plants for the experiment were grown as described in section 2.5. in Arasystem pots (Betatech bvba, Ghent, Belgium) filled with a mixture of soil and vermiculite (1:1) under normal watering regime. Leaves from one-month old *dnd2* and Col-0 plants were used for stomatal measurements. Stomatal sizes were determined by light microscopy. Nail polish images from silicone rubber imprints of abaxial surfaces of rosette leaves were photographed and measured according to Casson et al., 2009. Stomatal density and stomatal index were calculated as described by Royer, 2001.

2.7. Measurement of stomatal conductance

Arabidopsis seeds were planted in soil containing 2:1 (v:v) peat : vermiculite and grown as described by (Kollist et al., 2007). Briefly, the plants were grown in growth chambers (AR-66LX, Percival Scientific, IA, USA and Snijders Scientific, Drogenbos, Belgium) with 12 h photoperiod, 23 °C day, 18 °C night temperature, 150 μmol m^{-2} s^{-1} light and 70% relative humidity. Plants were 24-32 days old during gas exchange experiments.

Whole-plant stomatal conductance response to exogenous ABA was measured as described by Merilo et al. (2015) with a custom made rapid-response gas exchange measurement device (Kollist et al., 2007). Intact plants were sprayed with 5 μM ABA solution (distilled water, 0.012% Silwet L-77 (Duchefa), 0.05% ethanol). After spraying stomatal conductance was measured for 40 min. Due to the small size of rosette leaves stomatal conductance of *dnd2aba2-1*

plants was not measured (Supplemental figure 1). ANOVA with Tukey unequal N HSD *post hoc* test was used to identify significant differences among Col-0 and all the mutants at $P < 0.05$.

3. Results and discussion

Plant LMMs that exhibit spontaneous cell death, changes in the hypersensitive response and disease resistance in the absence of pathogen have proved useful to unravel the mechanisms of plant disease resistance (Lorrain et al., 2003; Moeder et al., 2011). Recently, the interplay between plant hormonal signaling and disease resistance involving salicylic acid, auxin and abscisic acid (ABA) has been established in some detail (Kazan and Manners, 2009; Moeder et al., 2010). The barley mutant *nec1*, which is an orthologue of Arabidopsis *dnd2* (Rostoks et al., 2006), was found to exhibit similar disease resistance response as *dnd2* (Keisa et al., 2011), while also containing increased level of indole-3-acetic acid (IAA) and showing altered auxin response and reduced stomatal conductance (Keisa et al., 2013). In this study, the levels of free IAA and ABA in Arabidopsis *dnd2* were studied using HPLC-UV-FLD. Approximately 2.5-fold increase of IAA in shoots and 1.4-fold increase in roots was found in *dnd2* compared to Col-0 (Fig. 1A). The 2.5-fold increase of IAA content in *dnd2* shoots was somewhat smaller than that found in barley *nec1*, which showed 4-fold increase of IAA in both shoots and roots. In terms of absolute quantity, IAA content in the Arabidopsis Col-0 shoots and roots was 49 and 30 ng g^{-1} fresh weight (FW), respectively, which was comparably higher than in wt barley shoots and roots – 14 and 2.4 ng g^{-1} FW. *dnd2* shoots and roots contained 123 and 44 ng g^{-1} FW IAA, respectively, while barley *nec1* shoots and roots contained 60 and 10 ng g^{-1} FW IAA, respectively (Keisa et al., 2013). Thus, orthologous *dnd2* and *nec1* mutants both showed

significant increase in auxin content compared to their respective wt plants, although the IAA content in wt plants from both species was different.

Under normal growth conditions *dnd2* leaves accumulated about 80-fold more ABA than Col-0 plants (98.6 vs. 1.2 ng g^{-1} FW) (Fig. 1B). Under drought stress conditions Col-0 plants exhibited approximately 27-fold ABA induction in leaves (34 ng g^{-1} FW), while *dnd2* plants showed smaller 1.4-fold induction of ABA in leaves (134 ng g^{-1} FW). Nevertheless, level of ABA in *dnd2* plants under drought stress was 4-fold higher than in Col-0 (Fig. 1B). The ABA content in barley was below limit of detection in both wt and barley *nec1* mutant (Nakurte et al., 2012); therefore no comparison with *dnd2* was possible.

The Arabidopsis line carrying the pIAA2-GUS reporter gene was crossed with *dnd2* mutant and the resulting F_2 homozygous progeny was used to assay the auxin distribution in *dnd2* plants in comparison with Col-0. The pattern of auxin-inducible GUS expression indicated that the location of auxin and auxin signaling in *dnd2* was similar to Col-0, while the somewhat stronger reporter gene signal was consistent with the observed IAA increase in *dnd2* (Fig. 2).

To study potential causes for the observed increase in IAA and ABA content, the expression of several IAA and ABA response-linked genes were studied in *dnd2* leaves (Fig. 3). Significantly increased expression was observed for *AMI1* and *CSN5* genes, which are involved in conversion of indole-3-acetamide (IAM) to IAA (Mano and Nemoto, 2012) and in regulation of auxin responses via interaction of COP9 signalosome (CSN) complex with SCF^{TIR1} (Dohmann et al., 2008), respectively. Thus, increased auxin content in *dnd2* may be caused by increased IAA biosynthesis through IAM pathway, while differences in auxin response may relate to activity of CSN complex or slightly decreased expression of auxin receptor *TIR1*. Of the three YUCCA flavin monooxygenase genes which have been implicated in auxin biosynthesis

via indole-3-pyruvic acid (IPyA) pathway (Di et al., 2016), only the *YUC2* gene showed slightly decreased expression. Increased expression of *ABI1* and *ABI4* genes, encoding protein phosphatase 2C and AP2 domain transcription factor, respectively, that are involved in ABA signal transduction may explain the observed changes in response to exogenous ABA application. *ABI1* expression was shown to be increased by exogenous ABA treatment in Arabidopsis by massively parallel signature sequencing (Hoth et al., 2002), while it has been shown that ABI1 is a negative regulator of ABA signaling (Gosti et al., 1999). Thus, increased expression of *ABI1* in *dnd2* plants may be a compensatory mechanism to reduce ABA signaling caused by higher levels of endogenous ABA. Increase of *ABI1* expression has also been observed in another lesion mimic mutant, *cpr22* with defective cyclic nucleotide-gated ion channel genes, although, unlike *dnd2*, reduced responsiveness to ABA was observed in *cpr22* (Mosher et al., 2010).

The physiological effects of altered IAA and ABA content were studied in *dnd2* using root elongation assay and under the drought stress. ABA is a mediator for several plant adaptive responses to abiotic stresses including drought and salinity stress (Fernando and Schroeder, 2016). Arabidopsis usually shows inhibited root growth, when treated with NaCl (Xu et al., 2008). Salt stress involves rapid and massive accumulation of ABA, which acts on endodermis and prevents root growth (Duan et al., 2013; Jia et al., 2002). The effects of exogenously applied IAA, ABA and NaCl were studied by measuring by how much the root growth (elongation) was reduced in treated plants (Fig. 4A-C). Increasing concentrations of exogenous IAA reduced the root growth in the *dnd2* significantly more than in Col-0 (Fig. 4A), which may be linked to already elevated level of IAA in *dnd2*. At the same time *dnd2* root growth appeared to be more insensitive to ABA and NaCl treatment than Col-0 (Fig. 4B, C). While the simplest explanation

is that increase in endogenous IAA concentration in combination with exogenous application contributes to reduced root growth in *dnd2* mutant, the crosstalk between IAA and ABA in root growth regulation may be involved (Wang et al., 2011; Xu et al., 2013). Even though different concentrations are usually used for root growth (elongation) assays for barley and Arabidopsis, the same trend in root growth was observed in both barley and Arabidopsis by applying increasing IAA concentration (Keisa, 2013). However, under relatively high IAA concentration Arabidopsis *dnd2* maintained sensitivity and showed increasing root shortening, while the barley *nec1* lost sensitivity and exhibited longer roots than wt plants (Keisa, 2013). This observation may be due to the fact that increase of IAA in Arabidopsis *dnd2* was smaller than in barley *nec1* compared to respective wt plants. In order to reach auxin homeostasis, IAA levels may need to be reduced in *nec1*; however, the process of reducing IAA levels in plants is not clearly defined. Some of the known IAA catabolism pathways involve IAA oxidation to 2-oxindole-3-acetic acid (oxIAA) and conjugation to amino acids or sugars (Normanly, 2010). Further studies on IAA conjugate content in barley *nec1* and Arabidopsis *dnd2* would be necessary to cover this issue, as increased IAA level may be due to increased biosynthesis or altered catabolism which may also be affected by abiotic stress conditions (Feng et al., 2015).

A common defense response to several abiotic stresses including drought, cold and salt stress is activation of ABA signaling (Fernando and Schroeder, 2016). Considering the elevated amount of ABA in *dnd2* and differences in response to external ABA and 100 mM NaCl between *dnd2* and Col-0 in root elongation assay (Fig. 4B, C), it was relevant to study the *dnd2* response to drought stress. The drought stress test showed that *dnd2* mutant plants survived the drought period much better than Col-0 plants (Fig. 5). Severe drought stress using water withdrawal for 12 days resulted in wilting and drying of most of the Col-0 plants (Fig. 5A), but

did not significantly affect *dnd2* plants (Fig. 5B). Once the watering was resumed, over 80% of *dnd2* plants survived, while most of the Col-0 plants did not recover (recovery rate 6.5%; Fig. 5C). The increased ABA content and reduced stomatal size (Fig. 6A) may explain the increased drought resistance of *dnd2* plants, although *dnd2* and Col-0 plants differ in development, which can also influence the response to drought stress. In addition, mutations in *CNGC2* and *CNGC4* genes have been shown to increase thermotolerance in Arabidopsis through accumutation of heat shock proteins (HSP) (Finka et al., 2012), which are often involved in responses to other abiotic stresses (dos Reis et al., 2012).

Since the level of ABA was increased in *dnd2* and the study on barley *nec1* showed reduced stomatal conductance and aperture (Keisa et al., 2013), the stomatal characteristics of *dnd2* were studied. Measurements of stomatal length and width indicated that *dnd2* has smaller stomata than Col-0 (Fig. 6A) similarly to barley *nec1* that showed reduced stomatal aperture (Keisa, 2013; Keisa et al., 2013). In addition, *dnd2* exhibited increased stomatal density per unit leaf area and increased stomatal index (Fig. 6B, C), which may represent a plant strategy to compensate for reduced stomatal size as supported by Franks and Beerling (2009). Similarly to barley *nec1*, Arabidopsis *dnd2* exhibited constitutively lower stomatal conductance (Fig. 7A), while the application of exogenous ABA resulted in even further reduction of conductance (Fig. 7B). Since the stomatal conductance was already very low in *dnd2*, the relative values were used to visualize the decrease in stomatal conductance upon ABA treatment. The ABA treatment resulted in even further decrease in stomatal conductance in *dnd2*, indicating that ABA response was not impaired in *dnd2*, although the decrease in stomatal conductance was somewhat slower than in Col-0 (Fig. 7B). To determine whether *dnd2* have more closed stomata due to increased ABA levels (Fig. 1B) or due to increased SA levels (Jurkowski et al., 2004), a genetics approach

was used to remove SA from *dnd2*. The *sid2-1* mutant is defective in the main SA biosynthesis enzyme ISOCHORISMATE SYNTHASE1 (Nawrath and Métraux, 1999) and *fmo1* encoding FLAVIN MONOOXYGENASE1 is defective in systemic acquired resistance (SAR) and exhibits lower level of SA (Hartmann et al., 2018; Mishina and Zeier, 2006). Removal of SA acid resulted in significantly increased rosette sizes in *dnd2sid2fmo1* plants compared to *dnd2* plants, although they were still smaller than *sid2fmo1* plants implying deficiency in other functions (Fig. 8, Supplemental figure 1). Introduction of both *sid2-1* and *fmo1* into *dnd2* did not alter the stomatal conductance (Fig. 7). Hence, the closed stomata of *dnd2* was likely a result of increased ABA levels and not increased SA levels. To study the interaction between ABA and *dnd2,* independent from SA, a combination mutant was constructed: *dnd2sid2-1* (to remove SA) and *abi1-1* (a strong ABA insensitive mutant). As strong ABA insensitive mutants have very high stomatal conductance (Merilo et al., 2013), this combination of mutations would allow to determine, if the low stomatal conductance of *dnd2* could suppress the high stomatal conductance of *abi1-1*. Furthermore, to exclude a role for SA, the *sid2* mutation was included. The triple *dnd2sid2-1abi1-1* mutant exhibited intermediate stomatal conductance compared to the *dnd2* and the ABA insensitive *abi1-1*, while its response to external ABA was similar to *abi1-1* (Fig. 7). Overall the phenotype of *dnd2* is likely the result of several interacting signaling pathways including responses to auxin, ABA and SA.

4. Conclusions

In conclusion, Arabidopsis *dnd2* mutant exhibited significantly increased IAA content in leaves and roots and ABA content in leaves. In addition to constitutively increased ABA content, *dnd2* showed increased tolerance to salinity, near wt-like reaction to exogenous application of

ABA, reduced stomatal conductance and probably consequently increased drought stress tolerance. Thus, Arabidopsis *dnd2* mutant may provide a useful model for studying crosstalk between biotic and abiotic stress and IAA and ABA response in plants. Further work on *dnd2* is required to substantiate the link between abiotic stress response, disease resistance and hormone signaling.

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Table 1. Description of Arabidopsis mutants used in the study.

Mutant	Genetic background	Type of mutation	Gene	Relevant phenotype	Reference
aba2-1	Col-0	EMS, G to A substitution at Ser262Asn	*ABA2*	ABA deficient 2	Leon-Kloosterziel et al., 1996
abi1-1	Col-0[a]	EMS, substitution at Gly180Asp	*ABI1*	ABA insensitive 1	Koornneef et al., 1984
dnd2-1	Col-0	Ethylmethane-sulfonate (EMS), G to A substitution at Trp89, premature stop codon	*DND2* or *HLM1* or *AtCGNC4*	Defense no death	Jurkowski et al., 2004
fmo1	Col-0	T-DNA insertion line SALK_026163	*FMO1*	Deficient in systemic acquired resistance (SAR)	Mishina and Zeier, 2006
sid2-1	Col-0	EMS, C to T substitution in exon 9 at Glu449, premature stop codon	*SID2*	Deficient in isochorismate synthase (salicylic acid (SA) biosynthesis)	Nawrath and Métraux, 1999

[a] The original *abi1-1* mutant (Koornneef et al., 1984) was in Ler background, while in this study an equivalent mutant in Col-0 background was used.

Table 2. Primers used for genotyping and qPCR.

Gene	Sequence 5'-3'	Marker or enzyme	Reference
Primer sequences for detection of mutant alleles			
aba2-1	aba2-1-left TAGCGATGACTCGCGGTACATAT	dCAPS, *Nde*I	Cui et al., 2018
	aba2-1-right GCAAAATGCATCATCTGAAGAC		
abi1-1	abi1-1 for AAGATGCTGTTTCGACTATACC	dCAPS, *Nco*I	Assmann et al., 2000
	abi1-1 rev TTTCTCCTTAGCTATCTCCTCC		
dnd2-1	dnd2-left TCCAAATGGGTTCGAGCAT	dCAPS, *Fok*I	Genger et al., 2008
	dnd2-right GCAATCTTGAACTGAATCC		
fmo1	LP CTTTTCGGTTGGACTTGGAAC	T-DNA detection	Kaurilind et al., 2015
	RP CGTAGGATACGTCCAAAGCA		
	Lba TGGTTCACGTAGTGGGCCATCG		
sid2-1	LP TGATGCTCTGCAGCTTCAAT	CAPS, *Mun*I	Kaurilind et al., 2015
	RP CGAAGAAATGAAGAGCTTGGA		
Primer sequences for quantitative real-time PCR analysis of gene expression			
ABI1	F: CGGCAAAACTGCACTTCCAT	RT qPCR	Anderson et al., 2004
	R: CACGAGCTCCATTCCACTGAA		
ABI4	F: TCAATAACTCATCCACCGCCGTTG	RT qPCR	Joseph et al., 2014
	R: AGGCCAAATGGTCGAAGATCCATC		
AMI1	F: CGCCTCCTTCTCTACAGGGTCTTAC	RT qPCR	This study
	R: GAGCTGTAGAAGTAGCTGCCGAGTG		
AtACTIN2	F: GATTCAGATGCCCAGAAGTCTTGT	RT qPCR	Alcázar et al., 2009
	R: TGGATTCCAGCAGCTTCCAT		
CSN5	F: CTGAGACAAGGGTTAATGCTCAGG	RT qPCR	This study
	R: AGGGTGAGAGTGATACCATCCAAC		
TAA1	F: CCGGTTCGACGCAGCTTTG	RT qPCR	Sakata et al., 2010
	R: CCCGACCGAACATATGTCGTC		

TIR1	F: GCCCTAAACTGCAGCGCC	RT qPCR	Sakata et al., 2010
	R: CCCCTGTTCCGTCAATGCC		
YUC1	F: ATTCCGGCATGGAAATTAGCTTAG	RT qPCR	This study
	R: AAGTATCTCCCTTGGCAACACATG		
YUC2	F: GGGATGGAAGTTTGTTTAGACCTTTGC	RT qPCR	Sakata et al., 2010
	R: CTGGAAACCACTTGAGCAGGC		
YUC6	F: GGATCTCTGCAACTTCGGTGC	RT qPCR	Sakata et al., 2010
	R: GAACATGGACAGCCCAAAAGTTGAAG		

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Figure captions

Figure 1. Changes in hormone quantity in Arabidopsis *dnd2*.

Content of IAA in Arabidopsis Col-0 and *dnd2* leaves and shoots (A) and ABA in Arabidopsis Col-0 and *dnd2* leaves under normal growth and drought stress (B). Statistical differences between Col-0 and *dnd2* plants (Student's *t*-test) are indicated (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Vertical bars indicate standard deviations (n = 12).

Figure 2. Histochemical GUS staining of Col-0 and *dnd2* plants containing pIAA2-GUS reporter gene construct. The scale bar is 1 cm.

Figure 3. Quantitative real-time PCR analysis of selected genes involved in IAA biosynthesis and in IAA and ABA signaling in *dnd2* leaves.

Relative expression rates in *dnd2* are shown compared to the expression of the same genes in Col-0 (dotted line indicates no difference to Col-0). Statistically significant differences in expression for each gene between *dnd2* and Col-0 were detected with Student's *t*-test assuming unequal variance (* $P< 0.05$; ** $P < 0.01$). Vertical bars indicate standard deviations (n = 4-6).

Figure 4. Response of Col-0 and *dnd2* plants to IAA (A), ABA (B) and NaCl (C). Results are displayed as means of 33 to 40 plants, with SDs indicated. Statistically significant differences in root elongation between Col-0 and *dnd2* were detected with Student's *t*-test (** $P < 0.01$; *** $P < 0.001$).

Figure 5. Response of Col-0 and *dnd2* plants to drought treatment.

Three weeks old Col-0 (A) and *dnd2* (B) plants after 12-day drought stress. Recovery of Arabidopsis Col-0 and *dnd2* plants after drought stress (C). Statistically significant difference between Col-0 and *dnd2* recovery after drought stress was detected with Student's *t*-test (** $P < 0.01$). Vertical bars indicate standard deviations (n = 56).

Figure 6. Comparison of stomatal length and width (A), stomatal index (B) and stomatal density (C) between Col-0 and *dnd2.* Statistical differences between Col-0 and *dnd2* plants (Student's *t*-test) are indicated (***, $P < 0.001$). Vertical bars indicate standard deviations (n = 22-64).

Figure 7. Stomatal conductance in the *dnd2* and control plants.

Stomatal conductance of Col-0, *dnd2*, *abi1-1*, *abi1-1sid2*, *fmo1sid2*, *dnd2abi1-1sid2* and *dnd2fmo1sid2* plants without ABA treatment is presented as absolute values (A) and under ABA treatment presented as relative values (B). Error bars indicate standard error of the mean (SEM, n = 10-17) values. ANOVA with Tukey unequal N HSD *post-hoc* test indicates non-significant differences in panel A with following letters: Col-0 – ab; *dnd2* – c; *abi1-1* – d; *abi1-1sid2* – d; *fmo1sid2* – a; *dnd2sid2abi1-1* – b; *dnd2sid2fmo1* – c; while the rest of comparisons are significantly different ($P < 0.05$).

Figure 8. Rosette sizes of the Col-0, *dnd2*, *fmo1sid2* and *dnd2fmo1sid2* plants. Error bars indicate standard deviations (n = 10-17), while pairwise *t*-test *P* values are indicated above (* $P< 0.05$; *** $P < 0.001$).

***Contribution**

LK – performed the experiments and drafted the manuscript

IN – performed HPLC analysis and drafted the appropriate section of the manuscript

PJ – measured stomatal conductance and drafted the appropriate section of the manuscript

LKJ – performed the experiments

MB – designed the study, performed the experiments and drafted the manuscript

NR – designed the study and wrote the manuscript

All authors have reviewed the final version of the manuscript submitted for publication.

Figure

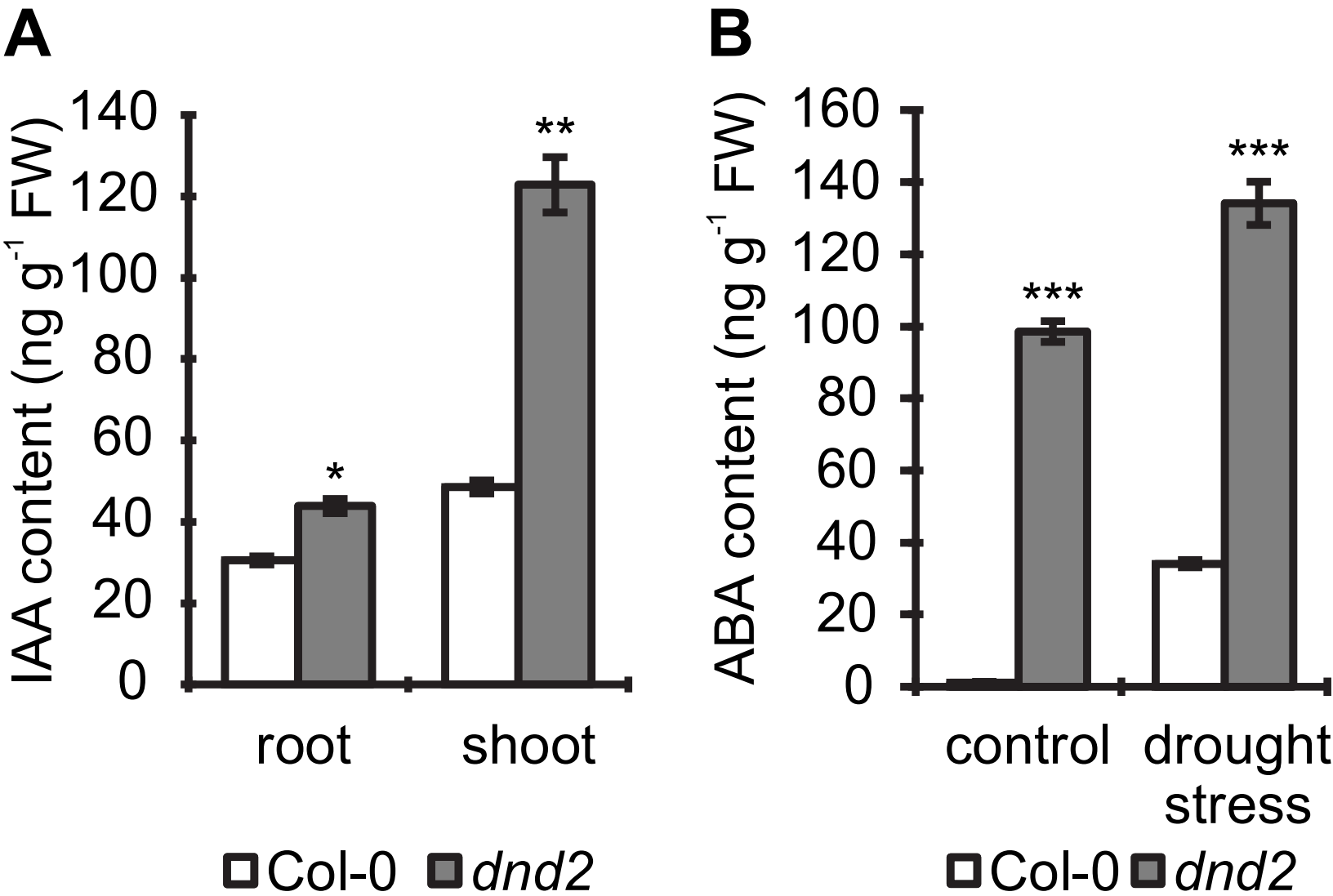
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Figure

Col-0

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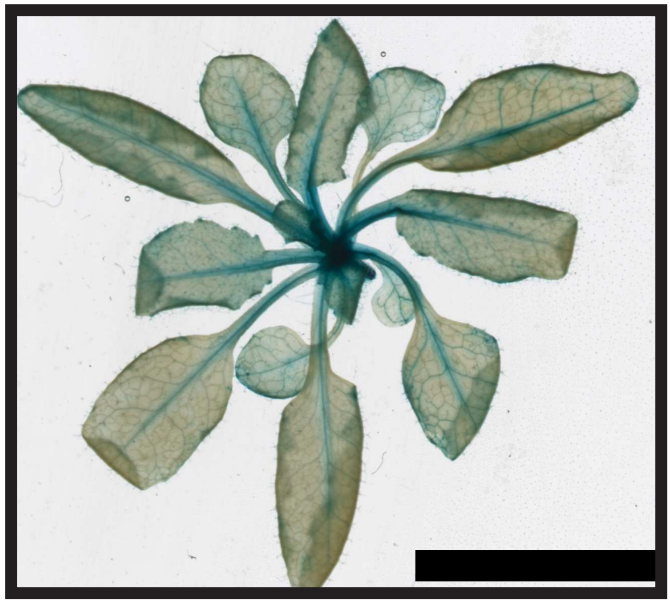

Figure

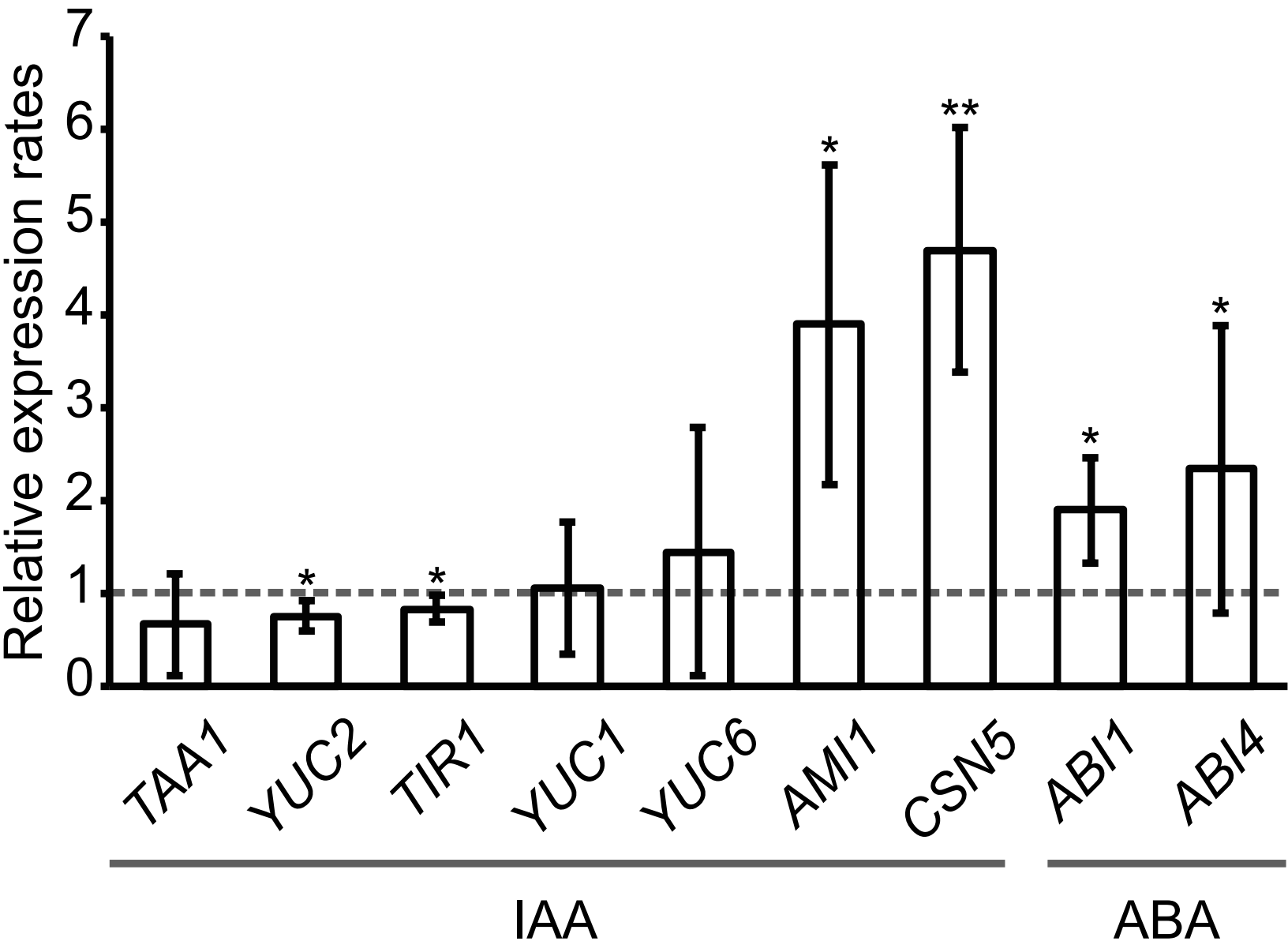
Relative expression rates
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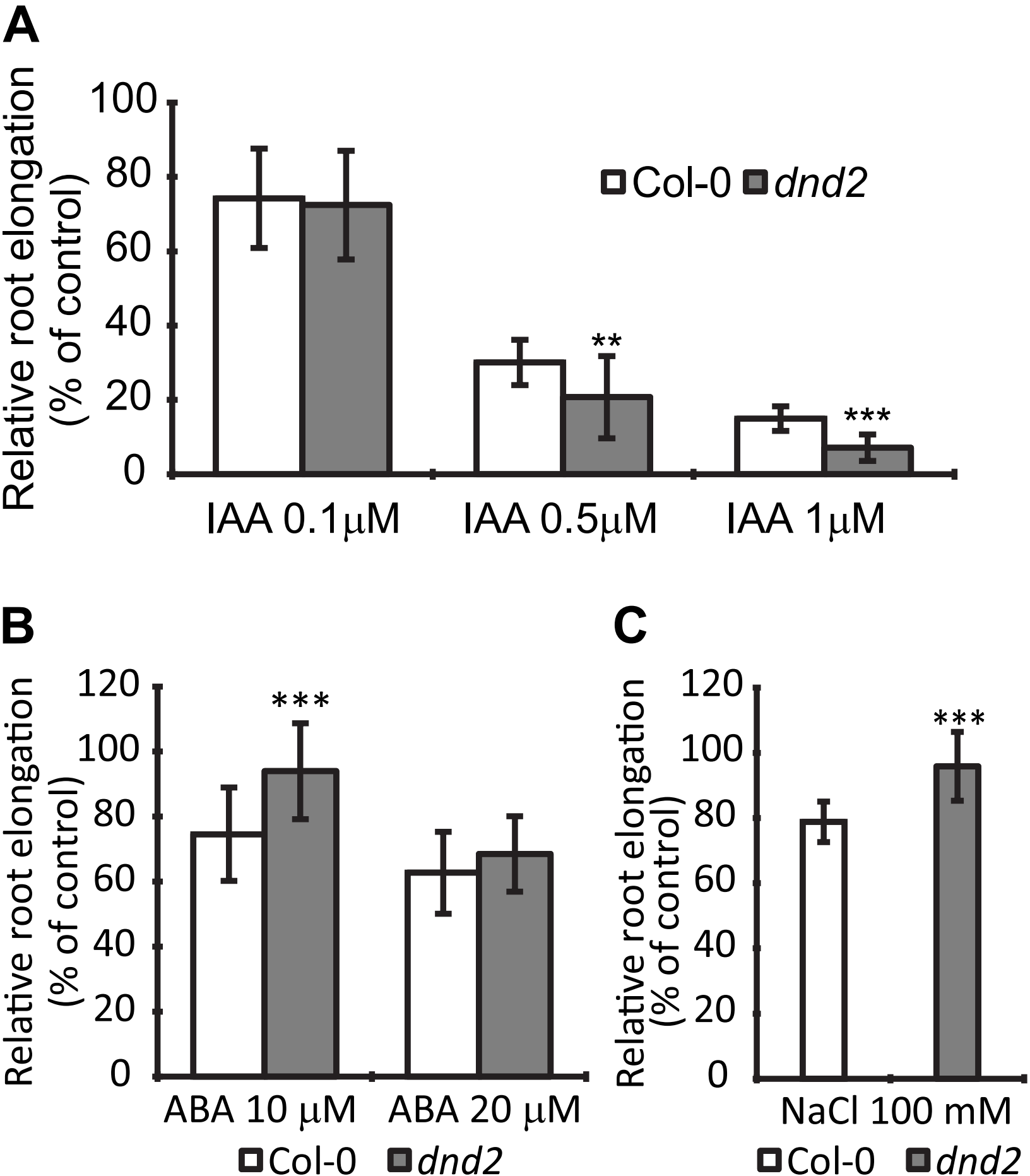
Figure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IAA 1μM
B
Relative root elongation (% of control)
120
100
80
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40
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***
ABA 10 μM
ABA 20 μM
Col-0
dnd2
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Relative root elongation (% of control)
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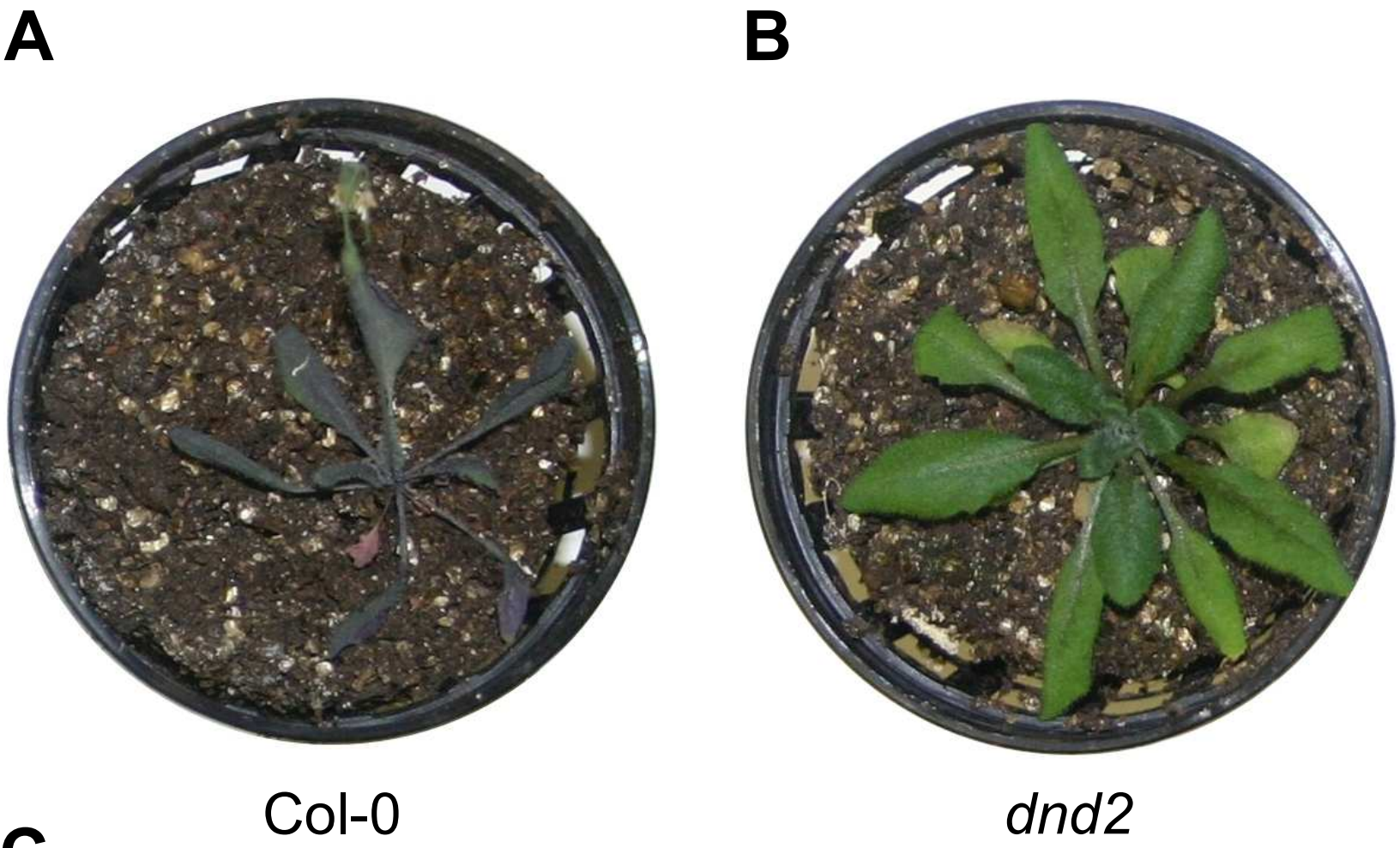
Figure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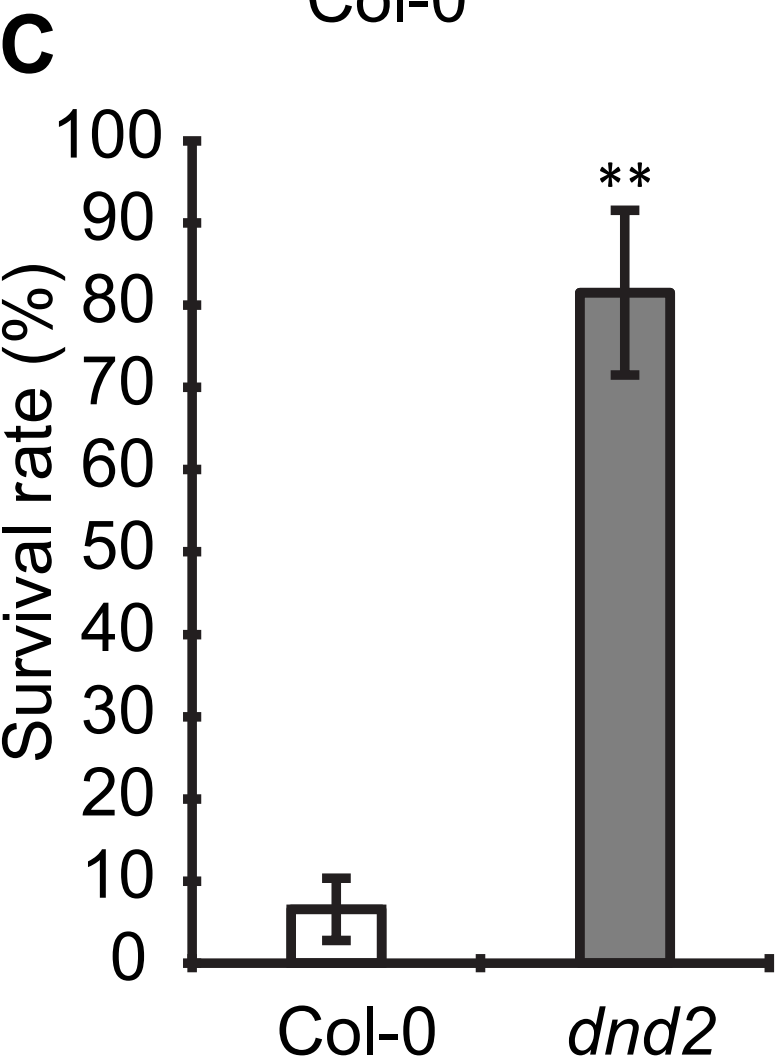
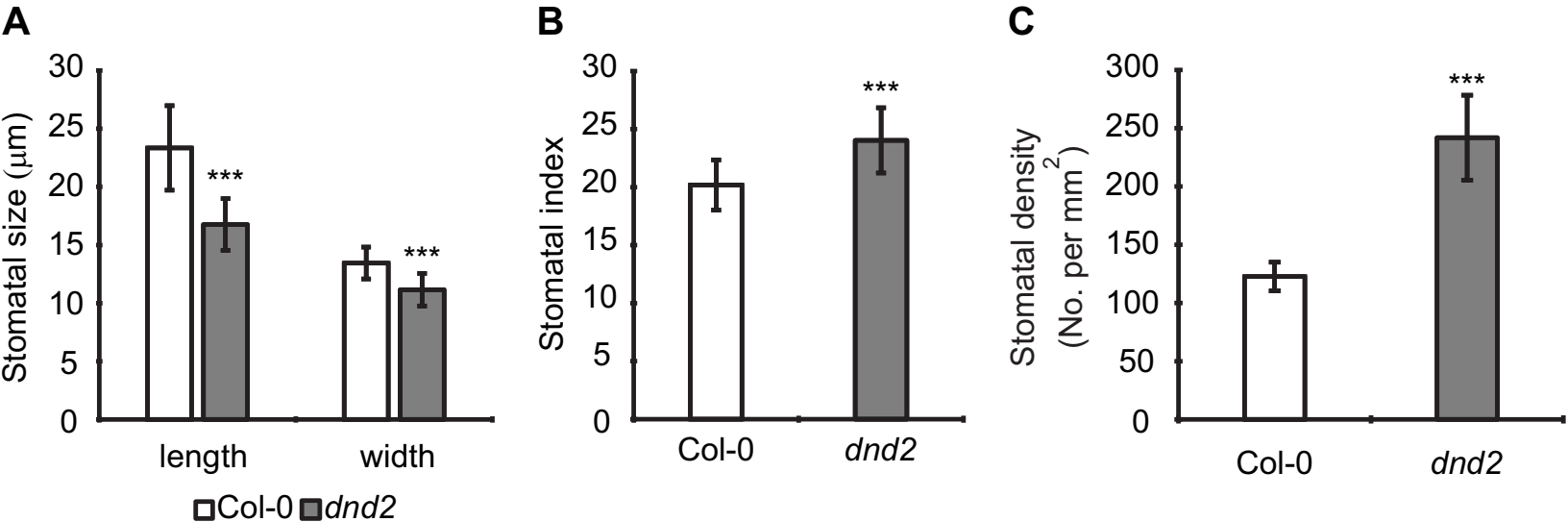
Col-0
dnd2

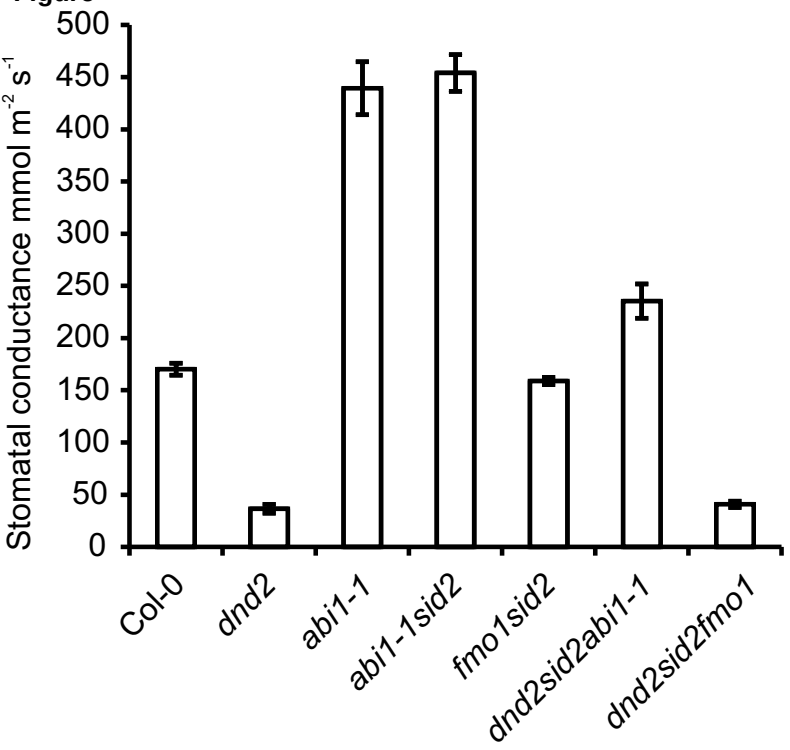

C
Survival rate (%)
100
90
80
70
60
50
40
30
20
10
0
**
Col-0
dnd2

Figure

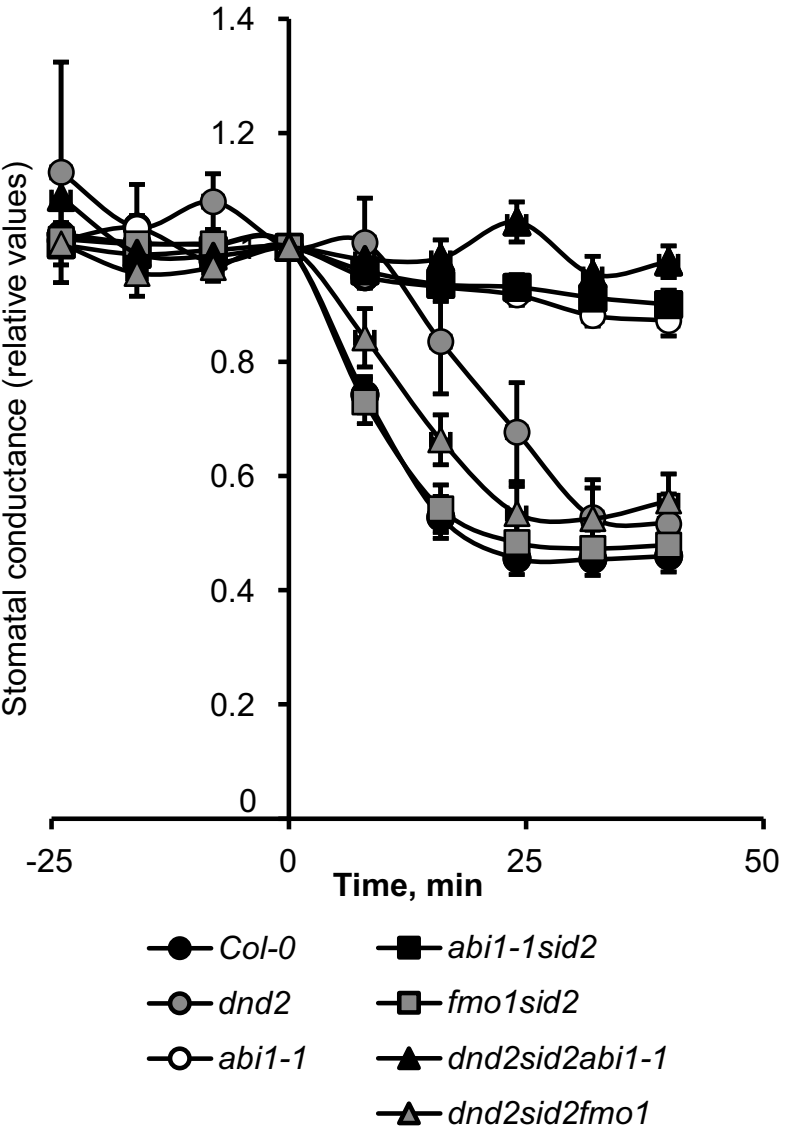

A
B
C
Stomatal size (μm)
30
25
20
15
10
5
0
***
***
length
width
Col-0
dnd2
Stomatal index
***
Col-0
dnd2
Stomatal density (No. per mm2)
300
250
200
150
100
50
0
***
Col-0
dnd2

Figure

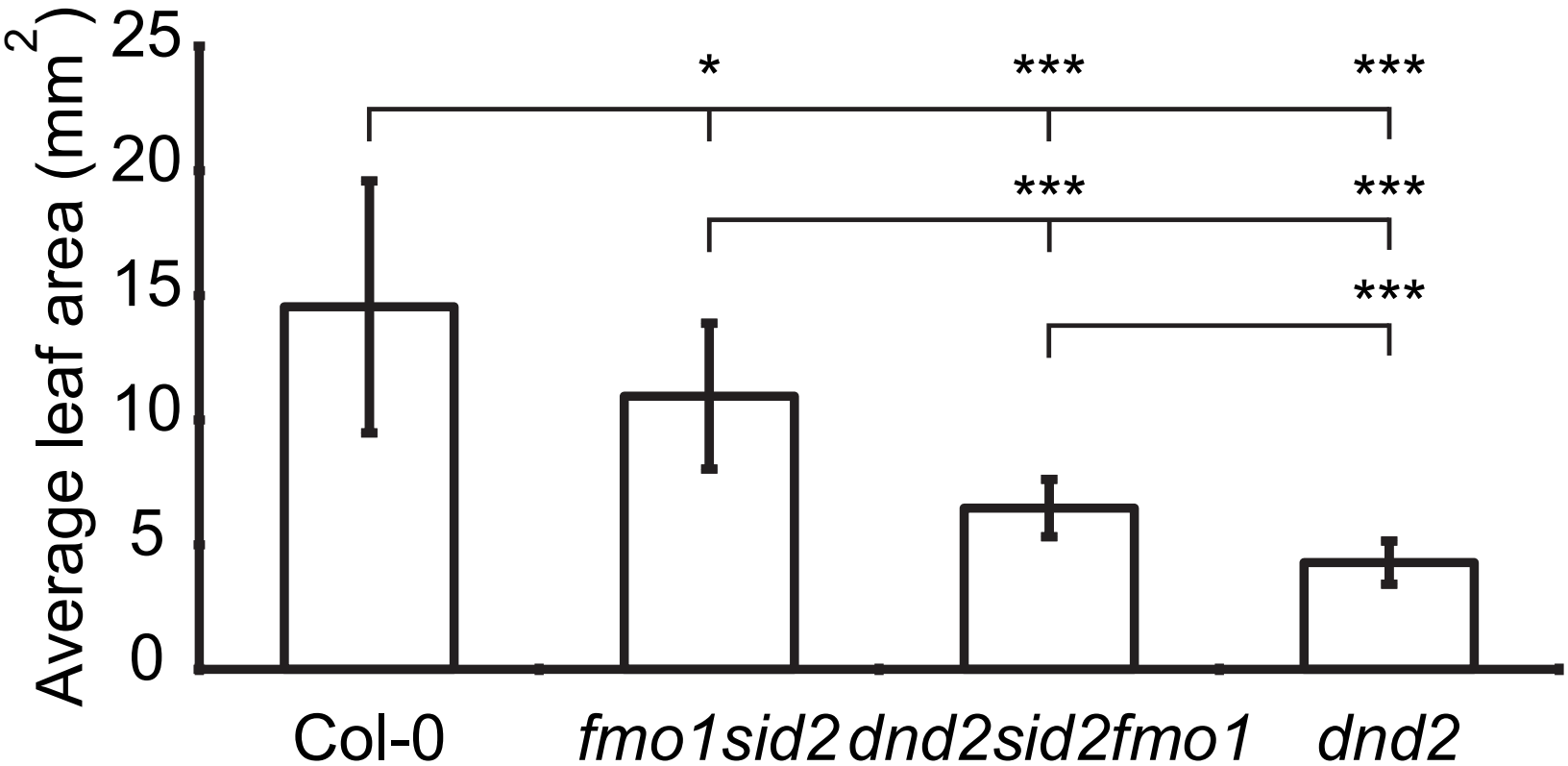
A


Stomatal conductance mmol m-2 s-1
500
450
400
350
300
250
200
150
100
50
0
Col-0
dnd2
abi1-1
abi1-1sid2
fmo1sid2
dnd2sid2abi1-1
dnd2sid2fmo1

B


Stomatal conductance (relative values)
1.4
1.2
0.8
0.6
0.4
0.2
0
-25
0
25
50
Time, min
Col-0
dnd2
abi1-1
abi1-1sid2
fmo1sid2
dnd2sid2abi1-1
dnd2sid2fmo1

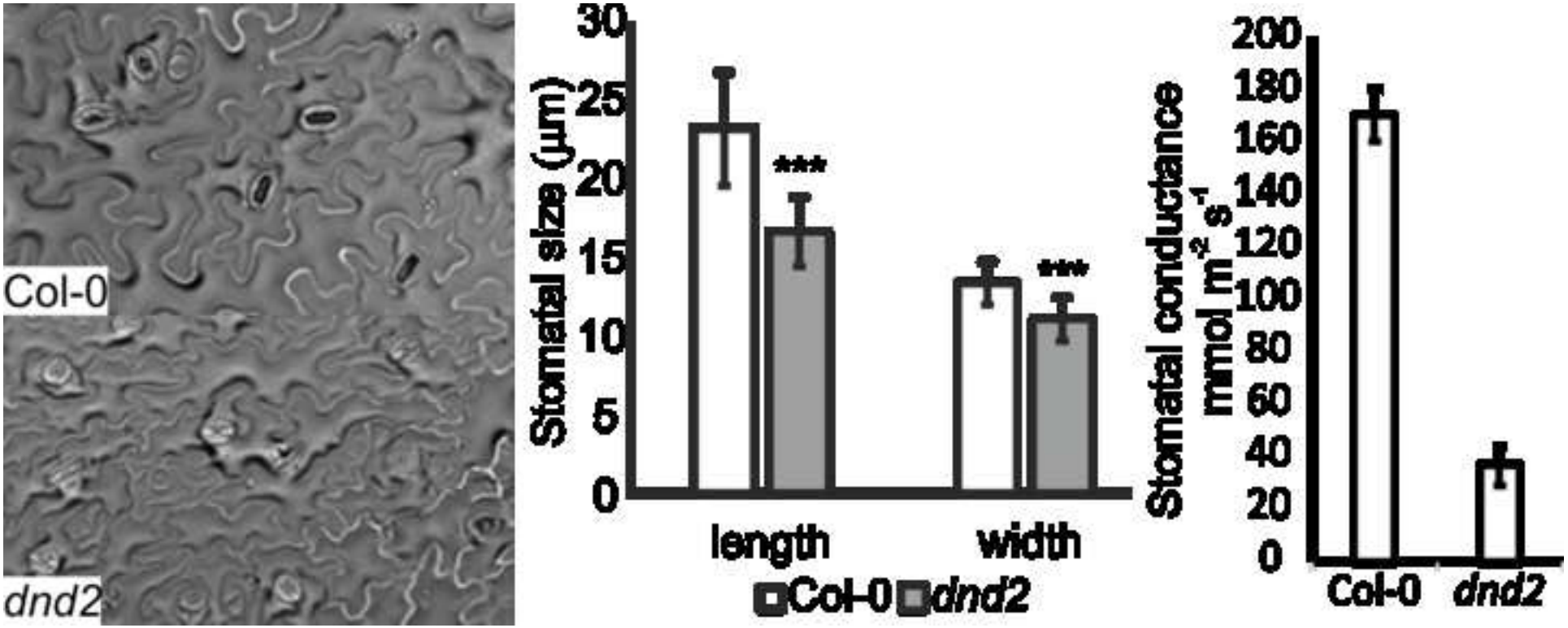
Figure


Average leaf area (mm2)
25
20
15
10
5
0
*
***
***
***
***
***
Col-0
fmo1sid2
dnd2sid2fmo1
dnd2

Supplementary figure

Click here to download Supplementary material: Kale_Supplemental_figure1.pdf

Graphical Abstract



***Highlights**

- Arabidopsis *dnd2* mutant shows increased IAA and ABA content
- Hormonal changes in *dnd2* are linked to reduced stomatal size and conductance
- Arabidopsis *dnd2* is useful to study cross-talk between abiotic and biotic stress