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NFXL2 modifies cuticle properties in Arabidopsis

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Loss of the Arabidopsis *NFX1-LIKE2* (*NFXL2*) gene (At5g05660) results in elevated ABA levels, elevated hydrogen peroxide levels, reduced stomatal aperture and enhanced drought stress tolerance. Introduction of the NFXL2-78 isoform into the *nfxl2-1* mutant is largely sufficient for complementation of the phenotype. We show here that cuticular properties are altered in the *nfxl2-1* mutant. The NFXL2-78 protein binds to the *SHINE1* (*SHN1*), *SHN2*, *SHN3* and *BODYGUARD1* (*BDG1*) promoters and mediates weaker expression of these genes. The SHN AP2 domain transcription factors influence cuticle properties. Stronger *SHN1*, *SHN2* and *SHN3* expression in the *nfxl2-1* mutant may cause altered cuticle properties including reduced stomatal density, and partly explain the enhanced drought stress tolerance. The BDG1 protein also controls cuticle development and is essential for osmotic stress regulation of ABA biosynthesis. Stronger *BDG1* expression in *nfxl2-1* plants may allow elevated ABA accumulation under drought stress. We conclude that the NFXL2-78 protein is part of a regulatory network that integrates the biosynthesis and action of ABA, ROS and cuticle components.

The human NFX1 transcription factor was identified as a protein that binds a conserved cis-acting element, the X-box, in promoters of class II MHC genes.[1] Two *NFX1*-like genes are typically present in the genomes of animals and higher plants, and the Arabidopsis genes were named *NFXL1* and *NFXL2*.[2,3] The *NFXL1* gene encodes a nuclear protein that positively affects adaptation to salt stress. In contrast, *NFXL2* may prevent unnecessary stress adaptation under favorable conditions.[3,4] The *NFXL2* gene encodes three NFXL2 isoforms, termed NFXL2-78, NFXL2-97, and NFXL2-100 according to the molecular weight of the putative proteins. The loss of *NFXL2* resulted in elevated ABA-levels, reduced stomatal aperture and enhanced survival of water stress. Introduction of the NFXL2-78 isoform largely complemented the *nfxl2-1* mutant phenotype.[4] The nuclear localization and the Cys-rich region suggest DNA-binding capacity of the NFXL2-78 protein.

Gene expression profiling experiments with Affymetrix ATH1 microarrays indicated altered transcript levels of genes involved in cuticle development in the *nfxl2-1* mutant (data not shown). The cuticle provides a protective barrier. It minimizes water loss and increases plant resistance to both biotic and abiotic stress.[5] Signals from the cuticle may influence trichome and stomatal numbers in the epidermis.[6] Cuticles consist of two types of lipophilic compounds, namely cutin polymers and monomeric very long chain fatty acids (VLCFAs, waxes).[7,8] Cutin forms the structural backbone of the cuticle. The waxes limit nonstomatal water loss and serve additional functions.[9]

The cutin polymers may be actively involved in signaling of abiotic stresses.[10] Thus, cuticle integrity is required for water stress signaling and tolerance. The molecular basis for this connection is unknown. Cutin biosynthesis could generate signaling molecules, or cuticle-associated proteins may sense stress signals, or the physical integrity of the cuticle may be required for sensing changes in the osmotic potential of the cell.[10] Surprisingly, impaired cuticle integrity can be associated with a positive effect on drought tolerance. Overexpression of the *SHN1* ERF/AP2 domain transcription factor (also termed *WIN1*) caused altered wax composition and cuticle properties.[11,12] In spite of increased cuticle permeability, *SHN1* overexpressors displayed enhanced drought tolerance and recovery.[13] This was postulated to be a consequence of reduced stomatal density.[13,14] Thus, the plant cuticle is intimately associated with stress signaling and developmental pathways. The pleiotropic phenotypic changes of mutants with defects in cuticle formation may often be a consequence of deregulated regulatory pathways rather than simple physical deficiencies.

NFXL2 Suppresses *BDG1* and *SHINE* Expression

Gene expression profiling experiments with Affymetrix ATH1 microarrays indicated elevated *BDG1* and *SHINE* transcript levels in the *nfxl2-1* mutant (data not shown). Quantitative RT-PCR analysis confirmed stronger *BDG1*, *SHN1*, *SHN2* and *SHN3* expression in the *nfxl2-1* mutant. Introduction of the *NFXL2-78* coding sequence into the *nfxl2-1* mutant background normalized *BDG1* and *SHINE* expression (**Fig. 1**).

Analysis of *BDG1*, *SHN1*, *SHN2* and *SHN3* promoters revealed sequences with similarity to formerly identified elements

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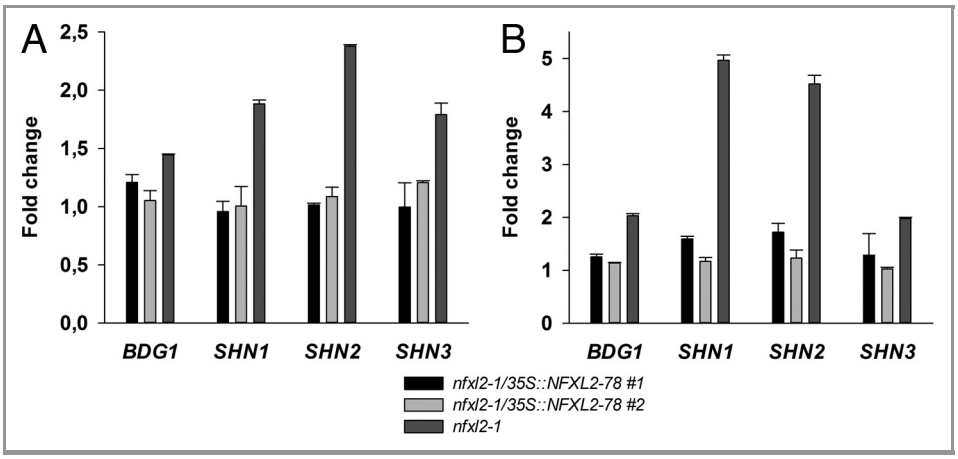

Figure 1. Quantitative RT-PCR analysis of *BDG1*, *SHN1*, *SHN2* and *SHN3* expression in leaves. The mean of the C_T (cycle threshold) values of the reference gene (*eIF1α*) was subtracted from the respective C_T value of the gene of interest. Subsequently, differences were subtracted from the wild-type value. Numbers give fold changes in comparison to the wild type. Error: SE of gene of interest in three technical replicates. (A) Relative transcript levels in 19-d-old plants grown in half-concentrated MS medium. (B) Relative transcript levels in 4-week-old soil-grown plants.

with demonstrated binding of the human NFX1 protein (**Fig. 2**).[1,15] ChIP experiments were performed with Arabidopsis wild-type plants and two independent transgenic lines expressing a green fluorescent protein (GFP)-tagged NFXL2-78 protein (35S::GFP-NFXL2-78). Both lines accumulate *GFP-NFXL2-78* mRNA (**Fig. 3B**), and the GFP-NFXL2-78 protein was detected in nuclei by means of confocal laser scanning fluorescence microscopy.[4] ChIP experiments were performed with and without the anti-GFP antibody. *BDG1*, *SHN1*, *SHN2* and *SHN3* promoter fragments were enriched in DNA samples of both 35S::GFP-NFXL2-78 lines probed with the anti-GFP antibody in comparison to the control experiments in which the antibody was omitted (**Fig. 3A**). No enrichment was observed in wild-type samples (data not shown). Thus, the *BDG1* and the *SHINE* genes represent direct targets of the NFXL2-78 transcription factor. However, additional experimentation is required to demonstrate binding of the NFXL2-78 protein to X-box-like promoter elements.

MHC class II X-box		CCT AGCAA CAGA TG
HTERT	-225	TGCGCACGTG GGAA GCCCTG
BDG1	-657	**GT ACGT AA** TCACATT**TG**
SHN1	-465	**GT ACGT** ACG **AA** GC **TG**
SHN2	-341	**GT ACGT**TGTAGGGT**AA**TTATTAACA **TTG**
SHN3	-764	**GT**CT**ACGT**AC ACCC**AA** AG TT**TG**
SHN3	-302	**GT** T**ACGT**CCCTCACT**AA** GGC T**TG**
		GTX(0-2) **ACGT** X(0-8) **AA** X(0-7)SX(1-3)**TG**

Figure 2. Putative X-box-like sequences in *BDG1* and *SHINE* promoters. The X-box sequence in MHC class II promoters and the X-box-like sequence preceded by the overlapping E-box within the *hTERT* promoter are shown in comparison to sequences identified in the *BDG1*, *SHN1*, *SHN2* and *SHN3* promoters. A consensus sequence matching the depicted Arabidopsis sequences is given at the bottom. Highly conserved residues include the ACGT sequence. This sequence is part of the human E-box and resembles the core sequence of the ABA response element (ABRE).[20] The conserved AA and TG nucleotides were among the five residues that were shown to cause diminished NFX1-91 binding to the *hTERT* promoter when being mutated.[15] X: any nucleotide. S: C or G.

Alterations of Epidermis Properties in the *nfxl2-1* Mutant

SHN1, *SHN2* and *SHN3* overexpression caused increased cuticle permeability.[13] The most widely used indicators of cuticle permeability are the elution rate of chlorophyll from leaves and staining with toluidine-blue.[16-19] The release of chlorophyll from submerged wild-type and *nfxl2-1* leaves in 80% ethanol was measured every 10 min during a period of 140 min. The chlorophyll concentration in the solution was determined. Chlorophyll loss was faster from *nfxl2-1* leaves in comparison to the wild type (**Fig. 4A**). Expression of the NFXL2-78 isoform in the *nfxl2-1* mutant reduced chlorophyll loss (**Fig. 4A**). The initial chlorophyll concentration in wild-type, *nfxl2-1*, and *nfxl2-1*/35S::NFXL-78 plants was identical (data not shown).

Leaf cuticle defects are rapidly visualized by toluidine-blue staining.[19] Leaves of the *nfxl2-1* mutant showed stronger staining in comparison to wild-type plants. The complemented mutant (*nfxl2-1*/35S::NFXL2-78) was at a similar level to the wild type (**Fig. 4B**).

Despite an increase in cuticle permeability, *SHN1* overexpression enhanced drought tolerance and recovery. This was attributed to a reduced stomatal density.[13,14] In line with the elevated *SHN1* transcript levels (**Fig. 1**), stomatal density of *nfxl2-1* leaves was reduced (**Fig. 5**). Thus, stronger *SHN1* expression may contribute to the enhanced drought resistance of the *nfxl2-1* mutant via a reduction of stomatal density.

SHN1 overexpression confers additional changes in the epidermal cell differentiation. 35S::SHN1 plants hardly formed trichomes on the surface of the first and second pairs of true leaves.[13] In agreement with stronger *SHINE* expression, the trichome number was significantly reduced in leaves of *nfxl2-1* plants grown under standard greenhouse conditions (data not shown).

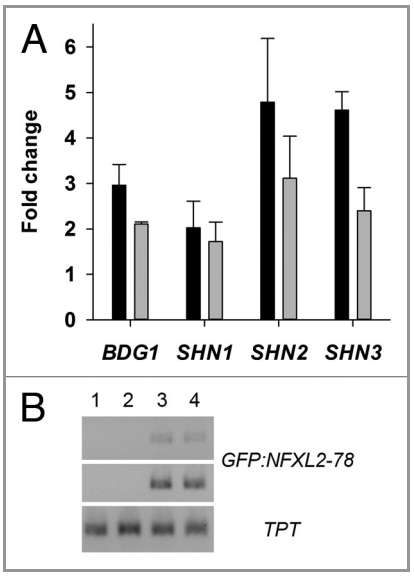

Figure 3. In vivo binding of GFP-NFXL2-78 to *BDG1*, *SHN1*, *SHN2* and *SHN3* promoters and analysis of *GFP-NFXL2-78* transcript levels. (A) PCR amplification of promoter fragments after ChIP with an anti-GFP antibody in leaf extracts of 4-week-old soil-grown plants. Numbers give fold changes ± SE in three technical replicates (two *nfxl2-1*/35S::GFP-NFXL2-78 lines plus vs. minus anti-GFP antibody). (B) Semiquantitative RT-PCR was used to determine *GFP-NFXL2-78* mRNA levels in 4-week-old soil-grown plants. As an internal control, the same cDNAs were used to quantify *TPT* transcript levels. The *GFP-NFXL2-78* and *TPT* transcripts were amplified by PCR for 24/27 and 24 cycles, respectively. The PCR products were separated on a 1.5% agarose gel. 1, wild type; 2, *nfxl2-1*; 3, 35S::GFP-NFXL2-78 #1; 4, 35S::GFP-NFXL2-78 #2.

Thus, 35S::SHN1 and *nfxl2-1* plants have several phenotypic changes in common, including increased cuticle permeability, lower stomatal density, enhanced drought tolerance and lower trichome number. In contrast to 35S::SHN1 plants and the *shn* gain-of-function mutant,[11,13,14] the *nfxl2-1* mutant was neither characterized by deep shiny green appearance, nor curled leaves, nor stunted growth.[4] The comparatively mild phenotypic changes of the *nfxl2-1* mutant presumably are due to the moderate increase of *SHINE* transcript levels (**Fig. 1**).

BDG1 (also termed CED1) and further proteins involved in cutin biosynthesis are essential for osmotic stress induction of ABA biosynthesis and osmotic stress tolerance.[10] Binding of the NFXL2-78 protein to the *BDG1* promoter may repress *BDG1* expression (**Figs. 1 and 3A**). Stronger *BDG1* expression in the *nfxl2-1* mutant may allow ABA accumulation and enhanced osmotic stress tolerance.[10]

NFXL2 is Part of a Regulatory Network with Manifold Input Signals

NFXL2-78 suppresses ABA and hydrogen peroxide accumulation, controls stomatal aperture, and mediates weaker expression of stress-related genes.[4] Here we have shown altered cuticle properties and elevated *BDG1* and *SHINE* transcript levels in the *nfxl2-1* mutant. Stronger *SHN1*, *SHN2* and *SHN3* expression in the *nfxl2-1* mutant (**Fig. 1**) presumably causes altered cuticle properties and associated effects (**Figs. 4 and 5**), and the reduced stomatal density may partly account for the reduced transpiration rate of the *nfxl2-1* mutant.[4]

The multifaceted phenotypic changes of the *nfxl2-1* mutant suggest that *NFXL2* is not only involved in a single regulatory pathway. NFXL2 may represent a component in a network of interacting processes, in which global changes can emanate from multiple sites. Input signals include, but may not be limited to, ABA, ROS, and cuticle components. The molecular basis of their interplay is poorly understood. Analysis of the mode of action of *NFXL2* may provide new insights into the complex regulatory network of drought resistance.

Materials and Methods

Growth conditions. Plants were established in soil. Seeds were allowed to germinate and grew for two weeks in controlled growth

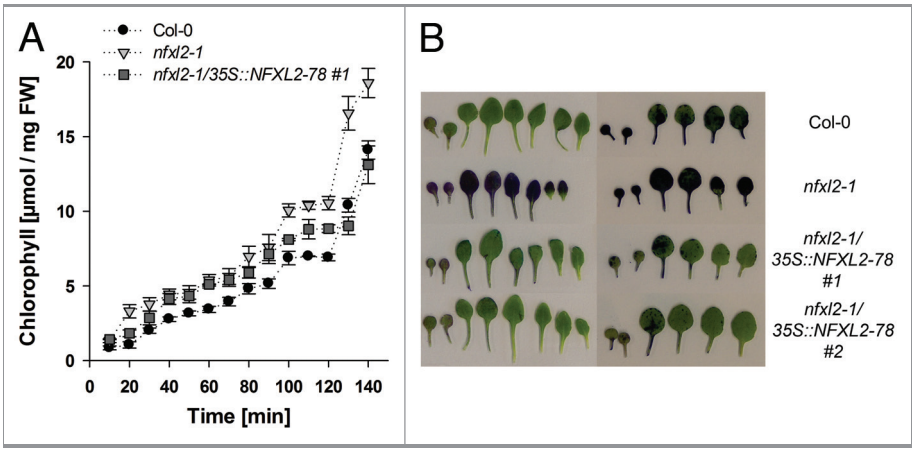

Figure 4. Analysis of cuticle permeability. (A) Chlorophyll leaching assays with mature rosette leaves. Rosette leaves of 4-week-old soil-grown plants were immersed in 80% ethanol for different time periods. Results are given as mean ± SE (B) Toluidine-blue staining of rosette leaves. Representative rosette leaves are shown after staining with an aqueous solution (0.05%) of toluidine blue. Left: plants grown under aseptic conditions. Right: plants grown in soil.

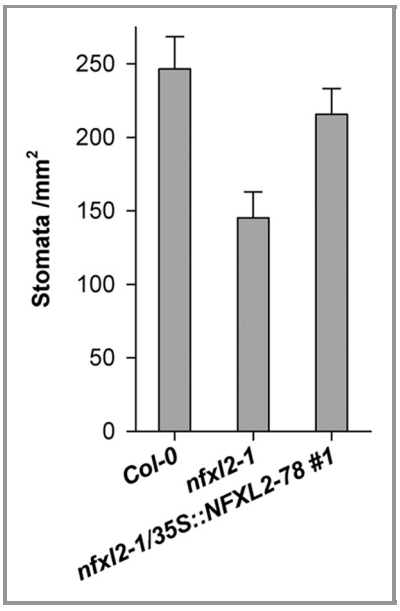

Figure 5. Stomatal density of mature abaxial leaf blades. Stomatal density was determined in mature rosette leaves of 4-week-old soil-grown plants. Results are given as mean ± SE. Stomatal density of *nfxl2-1* plants is significantly different from the wild type (t test, $p < 0.01$).

chambers (7 d: 16 h light, 140 µmol m^{-2} sec^{-1}, 20°C, 75% relative humidity; 8 h night, 6°C, 75% relative humidity; thereafter 7 d: 8 h light, 140 µmol m^{-2} sec^{-1}, 20°C, 60% relative humidity; 16 h night, 16°C, 75% relative humidity). Subsequently, plants used for chlorophyll leaching, toluidine-blue staining, and quantification of stomata number were transferred to long day conditions in a greenhouse with artificial light (16 h light, 21°C, 50% relative humidity; 8 h night, 19°C, 50% relative humidity). Plants used for ChIP and gene expression experiments were transferred to long day conditions in a phytotron with artificial light (16 h light, 20°C, 60% relative humidity; 8 h night, 16°C, 75% relative humidity). All genotypes were grown side by side in a randomized manner. Alternatively, plants were grown under aseptic conditions as described before.[4]

Gene expression analysis and ChIP. Sequences of primers used for real-time RT-PCR analysis were as follows: BDG1_fw AGC TAC GGC GTC AAG AGG AA, BDG1_rev TGG CAA CCA CAA AAG AAA CA (At1g64670), SHN1_fw GTC ATG ACG GTG GAG CTA GG, SHN1_rev TTC TTC TCT GCT GCC ACC AA (At1g15360), SHN2_fw GGC TAG GAA CAT TCG ACA CG, SHN2_rev CAT CTG CAT CGC CAT TTG AT (At5g11190), SHN3_fw AGT GTG GCT TGG AAC TTT CG, and SHN3_rev TTT GGT GAC ATC AAC GGA GA (At5g25390). The *eIF1α* gene was used to normalize the expression levels.[4]

Sequences of primers used for semiquantitative RT-PCR were as follows: EGFP_fw ACA TGG TCC TGC TGG AGT TC, NFXL2_rev CGC GCT CCC TGC AAG GTG GAC, TPT_fw GCT GCT TCT CAA TTC ATT ATG GGA C, and TPT_rev TGT CTG TGT CGA TAT CTT GTT TCC G.

Primers used for ChIP were as follows: BDG1_SN CCT AAG GAA GGA ACC GCA CA, BDG1_AS TCC GGT GAT GAT CCA AAA TG, SHN1_SN CCA CTG AAC AAA GTC CCA AC, SHN1_AS TGT CAA TCC AAT CCC CAA GA, SHN2_SN CCC CAT GAC ACG GAA GAG AT, SHN2_AS CTG CTT TTC CCA CGT CCT CT, SHN3_SN CGA CCC ACC GAC ATT TCT TT, and SHN3_AS AAG GCC ACC TCG TAA GTT CG. ChIP was performed using the EpiTect ChIP One-Day Kit (SABiosciences/Qiagen) and an anti-GFP antibody (Roche Applied Science) according to the manufacturer's instruction.

Chlorophyll leaching and toluidine-blue staining. Chlorophyll leaching experiments were performed as described in Aharoni et al.[13] Toluidine-blue staining was performed for 5 min (plants grown under aseptic conditions) or 8 h (plants grown in a greenhouse) as described in Tanaka et al.[19]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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