NRPB3, the third largest subunit of RNA polymerase II, is essential for stomatal patterning and differentiation in Arabidopsis

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ABSTRACT

Stomata are highly specialized epidermal structures that control transpiration and gas exchange between plants and the environment. Signal networks underlying stomatal development have been previously uncovered but much less is known about how signals involved in stomatal development are transmitted to RNA polymerase II (Pol II or RPB), which plays a central role in the transcription of mRNA coding genes. Here, we identify a partial loss-of-function mutation of the third largest subunit of nuclear DNA-dependent Pol II (NRPB3) that exhibits an increased number of stomatal lineage cells and paired stomata. Phenotypic and genetic analyses indicated that NRPB3 is not only required for correct stomatal patterning, but is also essential for stomatal differentiation. Protein-protein interaction assays showed that NRPB3 directly interacts with two basic helix-loop-helix (bHLH) transcription factors, FAMA and INDUCER OF CBF EXPRESSION1 (ICE1), indicating that NRPB3 serves as an acceptor for signals from transcription factors involved in stomatal development. Our findings highlight the surprisingly conserved activating mechanisms mediated by the third largest subunit of Pol II in eukaryotes.

KEY WORDS: Stomata, RNA polymerase II, Patterning, Differentiation, Arabidopsis

INTRODUCTION

Stomata, which consist of paired guard cells, are known to have played crucial roles in the colonization of land by plants. Turgor-driven stomatal movement requires ion and water exchange with neighboring cells and controls transpiration and gas exchange between plants and the environment. To function efficiently, the development of stomata complies with the one-cell-spacing rule, that is, two stomata are separated by at least one non-stomatal cell. In Arabidopsis, the stomatal lineage begins with an asymmetric entry division, which takes place in a fraction of protodermal cells known as meristemoid mother cells (MMCs). The division gives rise to two daughter cells with distinct morphologies: a large sister cell known as the stomatal lineage ground cell (SLGC) and a small triangular meristemoid. The meristemoid undergoes asymmetric amplifying division and regenerates an SLGC and a meristemoid that ultimately converts into a guard mother cell (GMC). The GMC divides symmetrically once to form a pair of guard cells (GCs) (Nadeau and Sack, 2002a; Bergmann and Sack, 2007). The SLGCs produced by asymmetric entry and amplifying divisions can either initiate stomatal development by undergoing oriented asymmetric spacing division or terminally differentiate into pavement cells (Geisler et al., 2000).

Stomata are highly specialized epidermal structures that control transpiration and gas exchange between plants and the environment. Signal networks underlying stomatal development have been previously uncovered but much less is known about how signals involved in stomatal development are transmitted to RNA polymerase II (Pol II or RPB), which plays a central role in the transcription of mRNA coding genes. Here, we identify a partial loss-of-function mutation of the third largest subunit of nuclear DNA-dependent Pol II (NRPB3) that exhibits an increased number of stomatal lineage cells and paired stomata. Phenotypic and genetic analyses indicated that NRPB3 is not only required for correct stomatal patterning, but is also essential for stomatal differentiation. Protein-protein interaction assays showed that NRPB3 directly interacts with two basic helix-loop-helix (bHLH) transcription factors, FAMA and INDUCER OF CBF EXPRESSION1 (ICE1), indicating that NRPB3 serves as an acceptor for signals from transcription factors involved in stomatal development. Our findings highlight the surprisingly conserved activating mechanisms mediated by the third largest subunit of Pol II in eukaryotes.

Several key genes and regulatory networks underlying stomatal development have been uncovered by molecular genetic analyses. Three ERECTA family (ERI) leucine-rich repeat receptor-like kinases [LRR-RLKs; ER, ERECTA-LIKE1 (ERL1) and ERL2], four SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) LRR-RLKs (SERK3/BAK1, SERK2, SERK1 and SERK4) and a leucine-rich repeat receptor-like protein (LRR-RLP) TOO MANY MOUTHS (TMM) have been identified as stomatal development receptors (Nadeau and Sack, 2002b; Shpak et al., 2005; Meng et al., 2015). Regarding their ligands, several small secreted, putative peptides belonging to the EPIDERMAL PATTERNING FACTOR-LIKE (EPFL) family have been discovered. Among these peptides, EPF1, EPF2 and CHALLAH family ligands (EPFL4-EPFL6) are negative regulators of stomatal density (Hara et al., 2007, 2009; Hunt and Gray, 2009; Abrash and Bergmann, 2010; Abrash et al., 2011; Lee et al., 2012; Niwa et al., 2013). By contrast, EPFL9/STOMAGEN positively regulates stomatal density (Hunt et al., 2010; Kondo et al., 2010; Sugano et al., 2010; Lee et al., 2015). A mitogen-activated protein kinase (MAPK) cascade, which consists of a MAPKKK (YODA), four MAPKs (MKK4/5/7/9) and two MAPKs (MPK3/6), regulates stomatal development downstream of the receptors (Bergmann et al., 2004; Wang et al., 2007; Lampard et al., 2008, 2009). In addition, STOMATAL DENSITY AND DISTRIBUTION1 (SDD1), a putative subtilisin acting upstream of TMM, is also a negative regulator of stomatal density (Berger and Altmann, 2000; von Groll, 2002). All of these genes are stomatal patterning genes, which regulate stomatal development with the correct pattern and proper density (Pillitteri and Torii, 2012).

As intrinsic positive regulators of stomatal differentiation, the closely related basic helix-loop-helix (bHLH) transcription factors, SPEECHLESS (SPCH), MUTE and FAMA control the consecutive cell fate transitions, MMC to meristemoid, meristemoid to GMC and GMC to GCs, respectively (Ohashi-Ito and Bergmann, 2006; MacAlister et al., 2007; Pillitteri et al., 2007). To specify each cell state transition, SPCH, MUTE and FAMA can also form heterodimers with two paralogous bHLH-leucine zipper (bHLH-LZ) transcription factors, INDUCER OF CBF EXPRESSION1 (ICE1) and SCREAM2 (SCRM2) (Kanaoka et al., 2008). In addition, two partially redundant R2R3 MYB transcription factors, FOUR LIPS (FLP) and MYB88, which are independent of FAMA, control stomatal terminal differentiation (GMC to GCs) (Lai et al., 2005; Ohashi-Ito and Bergmann, 2006).

Programs of gene expression, which are induced by developmental signals, lead to the differentiation of a variety of...
RESULTS

Phenotypic analysis and cloning of nrbp3-1

To identify new genes involved in stomatal development, we isolated a mutant with increased stomatal density and paired stomata in an ethyl methanesulfonate mutagenesis screen. The mutant displayed deficient developmental phenotypes, such as etiolation, late flowering and dwarfness (Fig. 1A,B). Its fully expanded rosette leaves were smaller than those of the wild type (see Fig. S1), suggesting that the mutation resulted in a defect in leaf expansion. Epidermal cell density in the abaxial epidermis of the mutant leaves was increased and further statistical analysis indicated that Pol II plays essential roles in stomatal development. Genetic analysis indicated that NRPB3 synergistically interacts with stomatal patterning and differentiation regulators. We also found physical associations of NRPB3 with two bHLH transcription factors, FAMA and ICE1. Our study reinforces the idea that mechanisms needed for the differentiation of skeletal muscle cell in animals are also required for stomatal development in plants.

To characterize the clustered meristemoid-like cells in leaves of GVG-NRPB3RNAi transgenic plants, we investigated the expression patterns of the stomatal cell-specific markers TMM, which marks stomatal lineage cells (Nadeau and Sack, 2002b) and MUTE, which marks late meristemoids, GMCs and immature GCs (Pillitteri et al., 2007). In GVG-NRPB3RNAi plants transformed with TMMpro::nucGFP, clusters of small, highly divided meristemoid-like cells exhibited strong GFP signals (Fig. 2D-H).
suggesting that downregulation of NR PB3 leads to a large increase in disorganized stomatal lineage divisions. In GVG-NRPB3RNAi plants transformed with MUTE\textit{pro}::GFP, fluorescence could be detected in clusters of immature stomata and multiple adjacent cells, which likely eventually formed stomatal clusters (Fig. 2I-K). Importantly, caterpillar-like structures similar to those of \textit{fama} also expressed MUTE\textit{pro}::GFP (Fig. 2K-M). These findings suggested that NR PB3 is required for limiting stomatal lineage cell divisions.

**Expression pattern and subcellular localization of NR PB3**

Histochemical expression pattern analysis showed that NR PB3 was expressed in almost all tissues. In seedlings, strong NR PB3 expression was observed in both the shoot and root, and high GUS activity was detected in the shoot apex, root tip, stele, lateral root primordium and newly formed lateral root (Fig. 3A-G). In developing inflorescences, strong staining was present in immature axillaries, the inflorescent apex, and the siliques apex and base (Fig. 3H-K).

Fig. 1. Isolation of an \textit{nrpb3} mutant. (A) Two-week-old seedlings of wild type, \textit{nrpb3-1} and NR PB3/NRP B3-1. (B) Five-week-old plants of wild-type, \textit{nrpb3-1} and NR PB3/NRP B3-1. (C-F) SEM images of the abaxial epidermis of the seventh fully expanded rosette leaf of wild type (C), \textit{nrpb3-1} (D,E) and NR PB3/NRP B3-1 (F). Arrow, parallel-aligned stomata; arrowhead, none parallel-aligned stomata; asterisk, GMC. (G) Densities of epidermal cells, stomatal cells (meristemoids, GMCs and stomata) and non-stomatal cells on the abaxial epidermis of the seventh mature leaves. (H) The proportion of stomata and stomatal precursors (meristemoids and GMCs) on the abaxial epidermis of the seventh mature leaves. (I) Densities of paired stomata on the abaxial epidermis of the seventh mature leaves. (J) The \textit{Arabidopsis} NRP B3 locus. Boxes, exons; lines, introns. The \textit{nrpb3-1} missense allele is indicated by a vertical line and the \textit{nrpb3-2} insertion allele is indicated by a triangle. (K) Partial amino acid sequence of RPB3 in various species. At, \textit{Arabidopsis thaliana}; Os, \textit{Oryza sativa}; Hs, \textit{Homo sapiens}; Ce, \textit{Caenorhabditis elegans}; Sc, \textit{Saccharomyces cerevisiae}; asterisk, mutation site in \textit{nrpb3-1}. Error bars indicate s.e.m.; NS, not significant; **\textit{P}<0.01 by Student’s \textit{t}-test. \textit{n}=30 per genotype. Scale bars: 1 cm in A,B; 50 µm in C,D,F; 20 µm in E.
At the cellular level, NRBP3 was broadly expressed in the leaf epidermal cells (Fig. 4A-C). In the cells of the root elongation zone, we observed NRBP3-GFP in the nucleus (Fig. 4D-F). Transient expression of NRBP3-GFP in Arabidopsis protoplasts indicated that it localized to the cytoplasm as well as the nucleus (Fig. 4G-L).

NRBP3 is essential for the proper expression of stomatal development genes

The fact that NRBP3 was a key subunit of Pol II led us to investigate the expression levels of genes for stomatal development in nrpb3-1. Except for EPF2, negative stomatal patterning regulators TMM, ER, EPF1, YODA and SDD1 were significantly downregulated (see Fig. S7B), consistent with the deficient stomatal phenotypes observed in nrpb3-1. Regarding the stomatal-promoting genes, SPCH and MUTE transcripts were abundant in the mutants (see Fig. S7B), consistent with the increased number of stomatal lineage cells in nrpb3-1. To confirm the RT-PCR results, TMM<sub>pro::</sub>TMG-GFP and SPCH<sub>pro::</sub>nucGFP were crossed to nrpb3-1, and the fluorescence intensity at the base of the fifth rosette leaf was compared between the wild type and nrpb3-1 under the same conditions. Weaker TMM expression and stronger SPCH expression were observed in nrpb3-1 (see Fig. S7C-F). The relative expression of these genes was also detected in GVG-NRPB3RNAi and amiR-NRPB3-1 transgenic plants and the results were similar to those of nrpb3-1 (see Fig. S7G,H). These results indicated that NRBP3 is essential for the proper expression of stomatal development genes.

NRBP3 interacts synergistically with stomatal patterning genes

To investigate the genetic interactions of NRBP3 with regulators of stomatal patterning, double, triple or quadruple mutants were produced between nrpb3-1 and tmm-1, er105 erl1 erl2, er105 erl2, er105 erl2, er105 epf2 and sdd1-1 (Fig. 5 and see Figs S8,S9). The nrpb3-1 tmm-1 double mutants exhibited dramatically exaggerated tmm-1 phenotypes. The stomatal density of nrpb3-1 tmm-1 was significantly higher than that of either the nrpb3-1 or the tmm-1 single mutants. Compared with tmm-1 individual mutants, nrpb3-1 tmm-1 double mutants not only exhibited larger clusters, but also had a larger number of stomatal clusters of all sizes (Fig. 5B,D,M,N). Similar results were also obtained for amiR-NRPB3-1 tmm-1 (see Fig. S8). The expression of nrpb3-1 er105 erl1 erl2 quadruple mutants exhibited much higher stomatal density, larger stomatal clusters and an increased number of clustered stomata compared with er105 erl1 erl2 triple mutants, thus greatly enhancing the er105 erl1 erl2 phenotypes (Fig. 5E,F,O,P). Furthermore, nrpb3-1 also exaggerated the stomatal phenotypes of erl1 erl2, er105 erl2 and er105 (Fig. 5G-L,Q-S). Compared with nrpb3-1 or epf2, many more stomata, paired stomata and meristemoid-like cells were found in nrpb3-1 epf2 (see Fig. S9C,D,G-I). In addition, surges in both the stomatal density and the number of clustered stomata were observed in nrpb3-1 sdd1-1 (see Fig. S9E,F,J,K). Overall, NRBP3 interacted synergistically with these genes in regulating stomatal patterning.

Fig. 2. GVG-NRPB3RNAi transgenic plants display severe stomatal development defects. (A-C) SEM images of the abaxial epidermis of the sixth immature rosette leaf of vector control (A) and GVG-NRPB3RNAi (B,C) transgenic plants. Arrows indicate caterpillar-like structures in B and clustered stomata in C, bracket, meristemoid-like cell cluster. (D-H) The expression of TMM<sub>pro::</sub>nucGFP in the abaxial epidermis of the sixth immature rosette leaf of vector control (D) and GVG-NRPB3RNAi (E-H) transgenic plants. (F-H) Close-up of clusters of small, highly divided meristemoid-like cells expressing TMM<sub>pro::</sub>nucGFP in GVG-NRPB3RNAi transgenic plants. (I-M) The expression of MUTE<sub>pro::</sub>GFP in the abaxial epidermis of the sixth immature rosette leaf of vector control (I) and GVG-NRPB3RNAi (J-M) transgenic plants. (K-M) Close-up of multiple adjacent cells or caterpillar-like structures expressing MUTE<sub>pro::</sub>GFP in GVG-NRPB3RNAi transgenic plants. Scale bars: 50 µm in A-C; 20 µm in D-M.

Fig. 3. Expression patterns of NRBP3. (A-C) NRBP3<sub>pro::</sub>NRBP3-GUS expression in 1 dag (A), 4 dag (B) and 8 dag (C) seedlings. (D-G) Stronger NRBP3<sub>pro::</sub>NRBP3-GUS expression in the meristematic and elongation zone of root tip (D), stele (E), lateral root primordium (F) and newly formed lateral root (G). (H-K) GUS activity in inflorescences and axillaries. (H) NRBP3<sub>pro::</sub>NRBP3-GUS expression in the whole developing inflorescence. (I) Inflorescence apex that shows strong GUS expression. (J) GUS activity in a single flower. (K) GUS activity in a newly formed siliquae. Scale bars: 1 mm in A-C,J; 0.5 mm in H; 100 µm in D-G,I; 200 µm in K.

Fig. 5. Expression patterns of NRBP3. (A-C) NRBP3<sub>pro::</sub>NRBP3-GUS expression in 1 dag (A), 4 dag (B) and 8 dag (C) seedlings. (D-G) Stronger NRBP3<sub>pro::</sub>NRBP3-GUS expression in the meristematic and elongation zone of root tip (D), stele (E), lateral root primordium (F) and newly formed lateral root (G). (H-K) GUS activity in inflorescences and axillaries. (H) NRBP3<sub>pro::</sub>NRBP3-GUS expression in the whole developing inflorescence. (I) Inflorescence apex that shows strong GUS expression. (J) GUS activity in a single flower. (K) GUS activity in a newly formed siliquae. Scale bars: 1 mm in A-C,J; 0.5 mm in H; 100 µm in D-G,I; 200 µm in K.
NRPB3 genetically interacts with FAMA, FLP, ICE1 and MUTE in restraining stomatal lineage cell divisions

The molecular character of NRPB3 led us to investigate its genetic interactions with transcription factors, including FLP, MYB88, FAMA, ICE1, SCRM2, MUTE and SPCH (Fig. 6 and see Figs. S10,S11). The flp-1 mutants typically had two laterally aligned stomata. Severe phenotypes with a larger size and greater frequency of clusters were observed in nrbp3-1 flp-1 and amiR-NRPB3-1 flp-1 (Fig. 6C,D,M,N and see Fig. S10). In fama, caterpillar-like structures were produced in the normal positions of stomata. Those structures were larger in nrbp3-1 fama, strongly exaggerating the phenotype of fama (Fig. 6E,F,O). In nrbp3-1 ice1-2, larger clusters of meristemoid-like cells were evident, and the number of meristemoid-like cells and paired stomata increased dramatically (Fig. 6G,H,P,Q). Neither myb88 (SALK_068691) nor scrm2-1 exhibited any visible defects in stomatal development. The phenotypes of nrbp3-1 myb88 and nrbp3-1 scrm2-1 were similar to nrbp3-1 (see Fig. S11). In nrbp3-1 mute, a higher density of undifferentiated meristemoid-like cells was observed (Fig. 6I,J,R). In nrbp3-1 spch, the epidermis was only composed of pavement cells and no stomatal lineage was initiated (Fig. 6K,L), suggesting that the involvement of NRPB3 in stomatal development is dependent on SPCH. In summary, spch, mute, fama and ice1 were epistatic to nrbp3-1 with regard to stomatal differentiation. Evidently, NRPB3 genetically interacts with FAMA, FLP, ICE1 and MUTE in restraining stomatal lineage cell divisions.

NRPB3 physically interacts with FAMA and ICE1

The genetic interactions between NRPB3 and the transcription factors involved in stomatal development led us to investigate their interactions at the molecular level. The yeast two-hybrid (Y2H) system was initially used. When NRPB3 was fused with the Gal4 DNA binding domain (BD), transcriptional activation itself was detected. However, it disappeared when the N-terminal 67 amino acids of NRPB3 were deleted (Fig. 7A). The results showed that NRPB3 strongly interacted with FAMA and ICE1, but not interacted with FLP, MYB88, SCRM2, MUTE or SPCH (Fig. 7A). In addition, FAMA was also identified in a Y2H screen, further demonstrating the interactions between NRPB3 and FAMA. In agreement with the Y2H results, functional associations of NRPB3 with FAMA and ICE1 were detected in bimolecular fluorescent complementation (BiFC) assays (Fig. 7C). These results indicated that NRPB3 physically interacts with FAMA and ICE1, both in vitro and in planta.

To investigate whether the mutation in nrbp3-1 influenced the physical interactions of NRPB3 with FAMA and ICE1, another Y2H system in which protein-protein binding capability could be measured with yeast growth was used (Fig. 7B and see Fig. S12A). Remarkably, binding affinities of NRPB3 with both FAMA and ICE1 were decreased for the mutation (Fig. 7B), suggesting that this site (residue 172) is crucial for their physical interactions. Clear interactions of nrbp3 with both FAMA and ICE1 were detected in the BiFC system (Fig. 7C). However, it was difficult to conclude whether the mutation influenced their binding abilities in this system, because multiple factors affected the reconstitution of complementary YFP molecules (Lalonde et al., 2008). Additionally, CYCLIN-DEPENDENT KINASE B1:1 (CDKB1:1), which is directly repressed by FAMA (Ohashi-Ito and Bergmann, 2006; Hachez et al., 2011), was upregulated in nrbp3-1 (see Fig. S13), suggesting that the suppression of FAMA on its target gene CDKB1:1 was impaired by the NRPB3 mutation.

Previous studies revealed that the RETINOBLASTOMA RELATED (RBR) protein represses entry asymmetric cell divisions by binding directly to the SPCH promoter and ensures irreversible stomatal terminal differentiation by interacting with FLP, MYB88 and FAMA (Borghi et al., 2010; Weimer et al., 2012; Lee et al., 2014; Matos et al., 2014). This led us to investigate whether RBR was a potential candidate for connecting stomatal signals to Pol II via NRPB3. However, direct interactions between these proteins were not detected in the Y2H system (see Fig. S12B).

NRPB3 works together with FAMA, ICE1 and FLP/MYB88 to limit GMC division during terminal GC differentiation

To confirm the molecular interactions of NRPB3 with FAMA and ICE1, the stomatal cell-specific markers FAMA, which marks GMCs and GCs (Ohashi-Ito and Bergmann, 2006), and E361, which marks mature GCs (Gardner et al., 2009), were used to determine the cell identity in caterpillar-like structures in nrbp3 mutants. In GVG-NRPB3RNAi amiR-NRPB3-1 plants transformed with FAMApro::nucGFP, strong GFP signals were observed in the parallel-aligned stomata and caterpillar-like structures (Fig. 8A-F and see Fig. S14A-D). In addition, aberrant GMCs or GCs were occasionally observed (Fig. 8G,H and see Fig. S14E). In GVGAmer-NRPB3RNAi plants marked with E361, GFP signals could be observed in clustered stomata and
unpaired guard cells (Fig. 8I-L), but not in caterpillar-like structures (Fig. 8M). These results indicated that the same caterpillar-like structures as those in \textit{fama} or \textit{ice1} were produced in \textit{nrpb3} mutants. The function of \textit{NRPB3} in terminal GC differentiation was further investigated using \textit{amiR-NRPB3-2} driven by the \textit{FAMA} promoter (\textit{FAMA}$_\text{pro}$::\textit{amiR-NRPB3-2}). The \textit{FAMA}$_\text{pro}$::\textit{amiR-NRPB3-2} construct induced clusters of stomata and small, highly divided meristemoid-like cells and dramatically
exaggerated the *flp-1* phenotype (Fig. 9A-C and see Fig. S15). More importantly, caterpillar-like structures expressing *FAMApro::nucGFP* were observed in *FAMApro::amiR-NRPB3-2* plants (Fig. 9D-F). Altogether, these results suggest that *NRPB3* works together with *FAMA, ICE1* and *FLP/MYB88* to limit GMC division during terminal GC differentiation.

Fig. 6. Genetic interaction analysis between *NRPB3* and stomatal differentiation genes. (A-D,G,H) SEM images of abaxial epidermis of the seventh fully expanded rosette leaf of Col (A), *nrpb3-1* (B), *flp-1* (C), *nrpb3-1 flp-1* (D), *ice1-2* (G) and *nrpb3-1 ice1-2* (H). (E,F,I-L) SEM images of the abaxial epidermis of 2-week-old cotyledons of *fama* (E), *nrpb3-1 fama* (F), *mute* (I), *nrpb3-1 mute* (J), *spch* (K) and *nrpb3-1 spch* (L). (M) Frequency of clusters per area. (N) The relative means of cells per cluster and of normal stomata in each genotype. (O) The relative means of cells per cluster in each genotype. (P,Q) Density of meristemoid-like cells (P) and paired stomata (Q) on the abaxial surface of mature cotyledons. Arrows indicate paired stomata (B,C), stomatal clusters (D), caterpillar-like structures (E,F) and meristemoid-like cell (I,J); bracket indicates meristemoid-like cell clusters in G,H. Error bars indicate s.e.m.; **P < 0.01 by Student’s t-test; n = 30 per genotype. Scale bars: 50 µm.
NRPB2, the second largest subunit of Pol II, is also required for stomatal development

The requirement of NRPB3 for stomatal development indicates that functional Pol II might be crucial for this process. All of the null mutants of Pol II genes identified to date are lethal (Onodera et al., 2008; Ream et al., 2009). However, a weak allele of the second largest subunit of Pol II (NRPB2) has been isolated as nrpb2-3 (Zheng et al., 2009). Increased stomatal cell density and paired stomata were observed in nrpb2-3 and nrpb3-1 nrpb2-3 (see Fig. S16A-D,I,J). Additionally, nrpb2-3 dramatically enhanced the phenotypes of both tmm-1 and flp-1 (see Fig. S16E-H,K-N). These results indicate that NRPB2 is involved in stomatal patterning and differentiation. Taken together, we concluded that Pol II plays an essential role in stomatal development.

DISCUSSION

The partial loss-of-function mutants of NRPB3 exhibit pleiotropic phenotypes and its homozygous T-DNA mutants are lethal, indicating that functional NRPB3 is essential for plant viability and development. NRPB3 is strongly expressed in the tissues and cells that show high mitotic activity, suggesting that its function is closely related to cell division. Furthermore, a much higher number of both stomatal and non-stomatal cells were produced upon its mutation, indicating that NRPB3 largely affects cell division and cell cycle regulators may be its targets.

Developmental signals are transmitted to Pol II, regulating the transcription of target genes. Thus, the mutation of NRPB3 could cause widespread effects on the stomatal signaling pathway. Consistent with this view, the expression of several stomatal development genes was indeed changed in nrpb3 mutants and severe stomatal development defects were observed. In this sense, it is not surprising that NRPB3 synergistically interacts with the known stomatal regulators genetically. It has been reported that several factors, such as plasmodesmatal permeability, sterols, auxin transport and the microRNA pathway, regulate stomatal development in parallel to the TMM-MAPK signaling pathway (Kutter et al., 2007; Guseman et al., 2010; Kong et al., 2012; Qian et al., 2013; Le et al., 2014; Yang et al., 2014). Therefore, we cannot exclude the possibility that NRPB3 regulates another independent pathway in stomatal development.

Pol II receives genetic regulatory information from tens of thousands of sequence-specific DNA binding transcription factors (Kadonaga, 2004). Signal transmission from these transcription factors to Pol II is extremely complicated. During this process, the multisubunit Mediator complex, which is broadly required for transcription by Pol II, bridges between gene-specific transcription factors and the general Pol II machinery (Conaway and Conaway, 2011; Larivière et al., 2012). It can directly integrate inputs from multiple signal-regulated transcription factors through its specialized subunits, recruit Pol II to target promoters and regulate the assembly of the Pol II initiation complex (Carrera and Treisman, 2008; Conaway and Conaway, 2011). Previous research has found that the Pol II subunit RPB3 directly interacts with the Mediator subunit Med17 and mutations in RPB3 (C92R, A159G)
transmission from SPCH and MUTE to Pol II might partially depend on their separate interactions with the shared protein ICE1. During terminal GC differentiation, both ICE1 and FAMA could directly transmit their mediated signals to Pol II by associating with NRBP3, whereas signal transmission from FLP/MYB88 to Pol II might rely on unknown proteins (Fig. 10). Therefore, mutation of NRBP3 would disrupt the proper function of these transcription factors, especially that of FAMA and ICE1. Consistent with this view, the nrpb3 mutants produced caterpillar-like structures similar to those of *fama* or *ice1* and caused large genetic exaggerations of the phenotypes of *flp-1*, *fama*, *ice1* and *mute*. Recent studies have shown that SPCH, together with SCRMs (ICE1/SCRM1 and SCRM2), can directly activate the expression of *TMM*, which in turn inhibits SPCH and SCRMs (Lau et al., 2014; Horst et al., 2015). Hence, in the case of *SPCH* upregulation in the *nrpb3* mutants, it is likely that the mutation of NRBP3 disrupts the function of unidentified inhibitors, which could interact with NRBP3 and directly repress *SPCH*. Future work is required to elucidate the underlying mechanisms.

Some parallels between the muscle cell and stomata differentiation are emerging (Pillitteri and Torii, 2007; Serna, 2009; Matos and Bergmann, 2014). Four tissue-specific bHLH regulators (MyoD, myogenin, Myf5 and MRF4), which are sequentially expressed, function as heterodimers with ubiquitously expressed bHLH factors (E-like proteins) and specify successive cell fate transitional steps in myoblast differentiation (Lassar et al., 1991; Weintraub, 1993). Analogously, three consecutive cell fate...
transitional steps in stomatal differentiation are directed by three specifically expressed bHLH transcription factors (SPCH, MUTE and FAMA), which also form heterodimers with broadly expressed bHLH-LZ proteins (ICE1 and SCM2). Similar to RPB3, which is involved in myogenesis by interacting with the bHLH regulator myogenin, NRPB3 participates in stomatal differentiation by associating with the bHLH transcription factors, FAMA and ICE1. This further highlights the surprisingly similar mechanisms for muscle cell and stomata differentiation.

MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis thaliana Col-0 was used as the wild type. The mutants and transgenic lines used in this study were as follows: tmm-1, flp-1, er105, er105 erl1-2 erl2-1, erl1-2 erl2-1, er105 erl2-1, mute, scrm2-1, nrpb3-2, ef2-1, icle1-2, myb88 (SALK_068691), sdal1-1, fama-1, spch-1, nrpb2-3, TMMpro::TMM-GFP, TMMpro::nucGFP, MUTEpro::GFP, FAMApro::nucGFP and E361. Details of sources are provided in supplementary Materials and Methods. All nrpb3-1 genotypes used for genetic analysis were generated by crossing and were confirmed using the primers listed in Table S1. Seedlings were grown initially on 1/2 MS medium and then were generated by crossing and were confirmed using the primers listed in Materials and Methods. All nucGFP and E361. Details of sources are provided in supplementary Materials and Methods.

Map-based cloning of NRPB3

Plants with the nrpb3-1 phenotype were isolated as recombinants from F2 plants of a cross between the nrpb3-1 (Col-0 ecotype) and Landsberg erecta (Ler). Approximately 10,000 F2 plants were used for mapping the NRBP3 locus. DNA markers that were used for detecting polymorphisms between ecotypes (Col-0 and Ler) were obtained from an Arabidopsis mapping platform (AMP) (Hou et al., 2010). The nrpb3-1 mutation was mapped to a 110 kb genomic region on chromosome 2. All candidate genes in this region were sequenced and a G/A mutation in At2g15430 was identified. The authors declare no competing or financial interests.

Fig. 10. A proposed model for the function of NRPB3 during stomatal development. During MMC to meristemoid and meristemoid to GMC transitions, signal transmission from SPCH and MUTE to Pol II might partially depend on their separate interactions with the shared protein ICE1. During terminal GC differentiation, both ICE1 and FAMA could directly transmit their mediated signals to Pol II by associating with NRPB3, whereas signal transmission from FLP/MYB88 to Pol II might rely on unknown proteins.

GUS staining assays

The approach for GUS staining has been described previously (Qian et al., 2013). The T2 transgenic plants of six independent lines carrying the NRPB3pro::NRPB3-GUS construct were used for analysis.

Plasmid construction and generation of transgenic plants

The Gateway cloning system (Invitrogen) was used to construct plasmids as detailed in supplementary Materials and Methods. All the expression constructs were transferred into appropriate Arabidopsis plants by the floral dip method (Clough and Bent, 1998).

Transient expression

Transient expression in Arabidopsis protoplasts was performed as described previously (Yoo et al., 2007).

Real-time PCR analysis

The method used for real-time PCR has been described previously (Qian et al., 2013). For each real-time PCR experiment, at least three biological replicates were conducted. See Table S1 for DNA primer sequences.

Yeast two-hybrid assay and two-hybrid screen with N-terminally deleted NRPB3

Yeast two-hybrid assay was carried out using the MATCHMAKER two-hybrid system 3 (Clontech) as detailed in the supplementary Materials and Methods. For the yeast two-hybrid screen, yeast strain Y190 transformed with bait pGBK-DN-NRPB3 was retransformed with a prey library made from 3-day-old seedlings in pACT (ABRC stock CD4-22) and β-gal activity was assayed according to the manufacturer’s protocol (Clontech) as described in more detail in supplementary Materials and Methods.

BiFC

Leaves of 3-week-old Nicotiana benthamiana were transformed by injection of Agrobacterium GV3101 strains containing BiFC constructs (Lavy, 2002) as described in supplementary Materials and Methods. Leaves were incubated with 0.2 mg/l DAPI to stain nuclei and YFP signal was examined 2 days after injection using an Olympus FV1000MPE2 confocal fluorescence microscope. Each interaction was tested at least three times.

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Competing interests

The authors declare no competing or financial interests.
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