The Transcription Factor NIN-LIKE PROTEIN7 Controls Border-Like Cell Release

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The root cap covers the tip of the root and functions to protect the root from environmental stress. Cells in the last layer of the root cap are known as border cells, or border-like cells (BLCs) in Arabidopsis (Arabidopsis thaliana). These cells separate from the rest of the root cap and are released from its edge as a layer of living cells. BLC release is developmentally regulated, but the mechanism is largely unknown. Here, we show that the transcription factor NIN-LIKE PROTEIN7 (NLP7) is required for the proper release of BLCs in Arabidopsis. Mutations in NLP7 lead to BLCs that are released as single cells instead of an entire layer. NLP7 is highly expressed in BLCs and is activated by exposure to low pH, a condition that causes BLCs to be released as single cells. Mutations in NLP7 lead to decreased levels of cellulose and pectin. Cell wall-loosening enzymes such as CELLULASE5 (CEL5) and a pectin lyase-like gene, as well as the root cap regulators SOMBRERO and BEARSKIN1/2, are activated in nlp7-1 seedlings. Double mutant analysis revealed that the nlp7-1 phenotype depends on the expression level of CEL5. Mutations in NLP7 lead to an increase in susceptibility to a root-infecting fungal pathogen. Together, these data suggest that NLP7 controls the release of BLCs by acting through the cell wall-loosening enzyme CEL5.

The root cap surrounds the root tips of nearly all angiosperms, gymnosperms, and pteridophytes (Barlow, 2003) and is fundamental to root-environment interactions. This tissue is critical to plant health as it protects the delicate root meristem (Barlow, 2003), senses gravity (Blancaflor et al., 1998, 1999) and water (Eapen et al., 2005), and secretes antimicrobial compounds and secondary metabolites that defend the root against soil-borne pathogens and abiotic stress (Hawes et al., 1998, 2000; Vicré et al., 2005; Driouich et al., 2013; Watson et al., 2015). The root cap is composed of two cell types that surround the meristem: the lateral root cap, which flanks the meristem, and the columella root cap located at the root tip (Fig. 1). In Arabidopsis (Arabidopsis thaliana), the columella is composed of five to six layers of rectangular cells (Benfey and Scheres, 2000; Fig. 1). New columella cells are produced from columella stem cells, and as they age they progressively differentiate from amyloplast-filled cells that sense gravity to mucilage-secreting cells that separate from the rest of the root cap and are released from its edges (Vicré et al., 2005; Durand et al., 2009; Bennet et al., 2010, 2014; Driouich et al., 2013). In Arabidopsis, cells of the columella root cap are released as a layer of living cells that are attached to each other as well as to one or two lateral root cap cells (Fig. 1; Vicré et al., 2005; Durand et al., 2009; Kumpf and Nowack, 2015). Together, the columella and lateral root cap cells that are released in this last layer are called border-like cells (BLCs; Vicré et al., 2005; Kumpf and Nowack, 2015). Despite their simultaneous release and physical attachment, the mechanism of release for columella BLCs and lateral root cap BLCs is very different. BLCs of the lateral root cap transition to programmed cell death at the time of their release (Fendrych et al., 2014). In contrast, BLCs of the columella do not undergo programmed cell death (Fendrych et al., 2014; Kumpf and Nowack, 2015) and remain alive for at least 72 h after release (Plancot et al., 2013). How columella BLC release occurs is not well understood.

Border cells and BLCs secrete root exudates including antimicrobial compounds and are an important part of the root’s defense against both biotic and abiotic rhizosphere stresses (Hawes et al., 1998, 2000; Vicré et al., 2005; Driouich et al., 2013; Plancot et al., 2013; Watson et al., 2015). Both border cell and BLC release require cell wall-degrading enzymes (Hawes and Lin, 1990; Wen et al., 1999; Vicré et al., 2005; Durand et al., 2009). In Arabidopsis, BLC release occurs through the action of enzymes that modify components of the cell wall, including cellulases and pectin methyltransferases (Bouton et al., 2002; del Campillo et al., 2004; Durand et al., 2009). Mutants deficient in cell wall homogalacturonan (HG), a major component of pectin (Wolf et al., 2009), release BLCs as single cells instead of an intact layer (Bouton et al., 2002; Durand et al., 2009). BLC release also depends on the proper degradation of...

1This work was funded by Purdue University.
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R.K. performed most of the experiments; F.S.-R. provided technical assistance; R.K. and A.S.I.-P. designed the experiments and analyzed the data; A.S.I.-P. supervised the experiments and wrote the article with input from all authors. Articles can be viewed without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.16.00453
cellulose, as a mutant defective in CELLULASE5 (CEL5) has a sticky root cap, with extra root cap cells stuck to the root tip (del Campillo et al., 2004). Furthermore, a triple mutant defective in the transcription factors SOMBRERO (SMB), BEARSKIN1 (BRN1), and BRN2 has an extremely sticky root cap with masses of cells remaining attached and has reduced CEL5 expression (Bennett et al., 2010). Although cell wall-loosening enzymes are known to be necessary for BLC release, how their expression is controlled to ensure release of an intact layer of BLCs is not clear.

Here, we show that the transcription factor NIN-LIKE PROTEIN7 (NLP7) is required for the release of an intact layer of BLCs in Arabidopsis. Low pH stress causes the release of BLCs as single cells from the root tip of wild-type plants, and a mutation in NLP7 significantly enhances this single cell release in both standard pH (pH 5.7) and low pH (pH 4.0) conditions. NLP7 encodes an Arabidopsis homolog of the nodule inception (NIN) transcription factor from Lotus japonicas and has been previously described in Arabidopsis for its role in nitrate signaling (Castaings et al., 2009; Marchive et al., 2013). NLP7 expression is activated by low pH conditions and is highly expressed in BLCs. The root of the nlp7 mutant shows a decrease in cellulose and pectin content. Gene expression of SMB and BRN1/2 is activated in the mutant, as is the expression of several cell wall-loosening enzymes, such as CEL5, XYLOGLUCAN ENDOTRANSGLUCOSYLASE (XTH5), and a PECTIN LYASE-like gene (PL). Double mutant analysis reveals that the nlp7 phenotype depends on the expression level of CEL5. Consistent with a role for BLCs in biotic stress, mutations in NLP7 lead to increased susceptibility to the soil-borne fungus Fusarium oxysporum f. sp. conglutinans (Foc). Together, our data show that NLP7 expression maintains pectin and cellulose levels in the root and represses the expression of CEL5, XTH5, PL, SMB, and BRN1/2, thereby preventing single cell border-like cell release.

RESULTS

Low pH Stress Promotes Single-Cell BLC Release in Arabidopsis

We previously found that among five different cell types in the Arabidopsis root, low pH conditions most significantly affected gene expression in the columella root cap (Iyer-Pascuzzi et al., 2011). Given that BCs are important for tolerance to Al toxicity (Li et al., 2000), a major problem in acid soils (Kochian et al., 2015), we reasoned that BLCs may be affected by low pH conditions. Under standard conditions (pH 5.7), at 7 d after imbibition (dai) over 85% of wild-type Arabidopsis roots release BLCs as intact layers of physically attached cells, and <15% of wild-type roots release BLCs as single cells (Fig. 2, A and G). In contrast, examination of BLCs in plants grown under low pH stress (pH 4.0) showed that only 45% of wild-type roots release BLCs as intact layers, while approximately 55% of roots release BLCs as single cells (Fig. 2, B and G). Low pH stress therefore promotes the release of BLCs as single cells in Arabidopsis.

NLP7 Is Required for Columella Border-Like Cell Adhesion

To search for genes that may play a role in BLC release, we examined a time course of gene expression in whole roots after exposure to low pH (Iyer-Pascuzzi et al., 2011). We reasoned that genes coexpressed with known regulators of the low pH response may be fundamental to low pH-induced phenotypes. K-means clustering of 1848 genes differentially expressed throughout the time course (Iyer-Pascuzzi et al., 2011) identified one cluster that contained the transcription factor (TF) Sensitive to Proton Rhizotoxicity (STOP1). Genes in this cluster were activated 6 to 12 h after exposure to low pH stress (Supplemental Fig. S1). Mutations in STOP1 lead to hypersensitivity to acidic pH and severe root growth inhibition under low pH (Iuchi et al., 2007). We hypothesized that the 10 other TFs in this cluster may also play a role in the low pH response. Because we were specifically interested in the BLC phenotype, we examined the root cap in mutant lines for each of these 10 TFs.

One of these lines (SALK_026134), with a T-DNA insertion in NLP7, showed defective BLC release (Fig. 2,
Quantitative RT-PCR (qRT-PCR) confirmed that NLP7 was activated by low pH (Supplemental Fig. S2). Examination of the BLCs in SALK_026134 (hereafter nlp7-1) seedlings showed that under standard pH, approximately 44% of roots released BLCs as single cells, compared to just 12% in wild-type plants and 19% in roots of the complemented line pNLP7:NLP7:GFP/nlp7-1 (Fig. 2G). Under low pH conditions, which loosen cells in the root cap and promote single cell BLC release, over 75% of nlp7-1 mutant roots released BLC as single cells, compared to 55% of wild-type plants and 42% of pNLP7:NLP7:GFP/nlp7-1 roots (Fig. 2G). These results show that NLP7 is necessary for BLC adhesion.

In addition to the border-like cell phenotype, the nlp7-1 plant is smaller than the wild type, with a shorter root and fewer elongated lateral roots (Supplemental Fig. S3).

We identified another T-DNA insertion mutant line (SALK_114886) within the last exon of the coding region of NLP7. This allele, nlp7-2, expressed the NLP7 transcript in the root at 85% of wild-type levels (15% reduction; Supplemental Fig. S4). Because nlp7-2 expressed NLP7 at near wild-type levels and did not have a defective BLC phenotype, this line was not further pursued. Thus, the complemented line pNLP7:NLP7:GFP/nlp7-1 was used in addition to nlp7-1 for our experiments.

NLP7 Is Strongly Expressed in BLCs

The above results suggested that NLP7 functions in BLC release and may be important for BLC adhesion.

To further understand the role of NLP7 in BLC release, we examined the expression of NLP7. Plants expressing pNLP7:GUS showed that NLP7 is strongly expressed in the columella root cap and maturation zone of the root (Castaings et al., 2009; Fig. 3A). Examination of the translational fusion of pNLP7:NLP7:GFP demonstrated that NLP7 is expressed in the BLCs under both standard and low pH conditions (Fig. 3, B and C; standard pH shown; low pH in Supplemental Fig. S5). Previous work showed that NLP7 localizes to both the cytoplasm and nucleus (Castaings et al., 2009; Marchive et al., 2013). We observed NLP7 in the cytoplasm and the nucleus of BLCs, and in the nucleus of cells in the root differentiation zone, in both standard (Fig. 3, B–D) and low pH conditions (Supplemental Fig. S5).

Mutations in NLP7 Lead to Reduced Pectin, HG, and Cellulose Content

Because the nlp7-1 mutant had altered BLC release, and BLC release in Arabidopsis depends on cellulose and pectin content (del Campillo et al., 2004; Durand et al., 2009), we analyzed the levels of cell wall polysaccharides in the nlp7-1 mutant. We first stained roots of nlp7-1, the wild type, and the pNLP7:NLP7:GFP/nlp7-1 complemented line with the histochemical stain ruthenium red, which stains unesterified (acidic) pectin (Sterling, 1970; Sabba and Lulai, 2002). Roots of the nlp7-1 mutant showed less pectin compared to the wild type at standard pH (Fig. 4, top panel). At low pH, pectin levels in the wild-type root decrease and are similar to those in the nlp7-1 mutant (Fig. 4, bottom panel).
decreased pectin in wild type and showed no difference in labeling between roots of the CDPK32 (Calcium-Dependent Protein Kinase 32) the two genotypes, we used a positive control. Anti-not due to differences in antibody penetration between and JIM7 labeling between the wild type and primary antibody). To ensure that the differences in JIM5 labeling compared to the wild type at standard pH (Fig. 5; see Supplemental Fig. S6) for negative control with no antibody (Supplemental Fig. S7).

Consistent with our experiments demonstrate that the nlp7-1 mutant has decreased levels of the cell wall polysaccharides pectin and cellulose. This suggests that defective BLC release in nlp7-1 is due to defects in cell adhesion.

Transcriptional Profiling of nlp7-1 Roots Identifies Cell Wall-Loosening Enzymes

Given that BLCs were released as single cells in nlp7-1, we hypothesized that NLP7 may function in cell adhesion in the root cap. We used microarray analysis to investigate whether NLP7 modulated this process. Transcriptional profiling of whole roots of 5 dai nlp7-1 plants identified 194 genes differentially expressed (false discovery rate < 0.0001, fold change > 1.5) compared to the wild type (Supplemental Table S1). A total of 160 genes were up-regulated, and 34 were repressed in the root of nlp7-1. Significant Gene Ontology (GO) categories (Provart et al., 2003) for all differentially expressed genes, as well as the up-regulated and down-regulated genes separately, are listed in Supplemental Table S2. Highly significant GO categories for biological

Figure 3. NLP7 expression in root. A, Transgenic plants expressing pNLP7-GUS. NLP7 is expressed in the columella root cap as well as the elongation and maturation zone of the root. Bar = 100 μm; 20× magnification. B and C, nlp7-1 mutant complemented with pNLP7:NLP7:GFP showing that NLP7 is expressed in cytoplasm (B) and nucleus (C) of the BLCs. D, NLP7 expression in the nucleus of the cells in differentiation zone of root. White arrow indicates the nucleus and the scale bar indicates 50 μm. B to D are 40× magnification.

nlp7-1 had decreased JIM7 labeling compared to the wild type, but there was no significant difference in labeling between the wild type and nlp7-1 with the JIM5 antibody (Supplemental Fig. S7).

Cellulose, formed by β-1,4-linked glucan chains, is another major component of plant cell walls. Cellulose degradation is necessary for proper BLC release (del Campillo et al., 2004). We therefore examined cellulose levels in nlp7-1 seedlings grown at standard pH using an acid hydrolysis assay (Dubois et al., 1956). Compared to the wild type, the nlp7-1 mutant contains significantly less cellulose (Fig. 6). Together, these experiments demonstrate that the nlp7-1 mutant has decreased levels of the cell wall polysaccharides pectin and cellulose. This suggests that defective BLC release in nlp7-1 is due to defects in cell adhesion.

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Figure 4. Mutations in NLP7 lead to a decrease in pectin in roots at both standard and low pH. Ruthenium red staining of the wild type, nlp7-1, and complemented line (pNLP7:NLP7:GFP/nlp7-1). All roots are 7 dai. Bar = 100 μm; magnification is 20×. The experiment was repeated twice with n > 10 for each genotype and treatment in each replicate.
process included transport ($P = 7.68 \times 10^{-6}$), response to abiotic/biotic stimulus ($P = 1.33 \times 10^{-6}$), response to stress ($P = 3.99 \times 10^{-6}$), and cell organization/biogenesis ($P = 1.72 \times 10^{-5}$). For molecular function GO categories, transporter activity ($P = 7.35 \times 10^{-4}$) and DNA/RNA binding ($P = 5.60 \times 10^{-4}$) were most significant. In the cellular component category, the category with the highest significance (other than unknown) was the Golgi apparatus ($P = 5.29 \times 10^{-5}$). This was interesting because cell wall polysaccharides such as hemicelluloses are synthesized in the Golgi and then transported by vesicles to the cell wall. Genes encoding proteins operating in the cell wall were present but not significant ($P = 0.059$).

Previously, Marchive et al. (2013) identified direct transcriptional targets of NLP7 in whole nlp7-1 seedlings that had been resupplied with nitrate for 10 min after a period of nitrate starvation. We examined whether any genes differentially expressed in the nlp7-1 root were also direct targets of NLP7 in their work and identified 14 genes (Supplemental Table S3). This number is not higher likely because of the differences in growth and environmental conditions in the two data sets: 13-d-old whole nlp7-1 seedlings grown in liquid medium under N starvation followed by resupply by Marchive et al. (2013) and 5-d-old nlp7-1 roots grown on agar with full nutrient supply in our study. Several of the genes that overlapped between the two data sets had roles in nutrient transport (Supplemental Table S3).

Because of our interest in the nlp7-1 border-like cell release phenotype, we focused on cell wall-related processes and identified 11 genes related to the cell wall in our data set. These included CEL5, XTH5, two exostosins, and a PL-like gene (Supplemental Table S4). All but one of the 11 genes was up-regulated in nlp7-1.

We next used the Arabidopsis root expression map (Brady et al., 2007) to examine the cell-type-specific expression pattern of these 11 genes. Due to the defective root cap phenotype, we looked for genes highly expressed in the columella and/or lateral root cap. Two genes, CEL5 (At1g22880) and PL (At1g65570), were highly expressed in the columella root cap (Supplemental Figure S8), while another, XTH5 (At5g13870), was highly expressed in the lateral root cap.

We used qRT-PCR to confirm the expression of these three genes in nlp7-1 (Fig. 7). We also examined expression of QUA1 (QUA1) and QUA2. Although these genes were not identified in our microarray analysis, QUA1 and QUA2 encode proteins that control pectin biosynthesis and are important for proper border-like cell release (Bouton et al., 2002; Mouille et al., 2007; Durand et al., 2009). As shown in Figure 7A, consistent with our microarray results, CEL5, PL, and XTH5 expression were up-regulated in the nlp7-1 root, while QUA1 and QUA2 remained unchanged.

We next used mutant analysis to determine whether mutations in CEL5, XTH5, or PL altered border-like cell release. Mutations in each of these genes lead to increased root cap layers (Fig. 7B). The cel5 mutant had

**Figure 5.** Mutations in NLP7 lead to decreased levels of HG in roots. A and C, Wild-type; B and D, nlp7-1. Whole-mount immunolabeling of wild-type and nlp7-1 roots grown at pH 5.7. JIM5 (top panel) and JIM7 (middle panel). E and F, Average corrected total fluorescence (CTF) for labeling intensity of JIM5 (left) and JIM7 (right). nlp7-1 roots show significantly ($P < 0.005$) decreased labeling for each. Bar = 50 μm. Similar numbers of slices per z-stack were used for the wild type and nlp7-1 for each antibody, with each slice 5 μm (see “Materials and Methods” for details). The area extending from the root tip to 200 μm was selected for fluorescence quantification. The experiment was repeated twice, and the average of at least nine seedlings for each genotype and each antibody is shown. Magnification is 40×.

**Figure 6.** Mutations in NLP7 decrease cellulose content in roots. Acid hydrolysis assay showing decreased cellulose content in 5 dai nlp7-1 roots grown at standard pH. **P < 0.001 (two-tailed paired t test). Bars indicate so for two biological replicates.
the “stickiest” root cap, and we focused on this gene for further analysis. Mutations in CEL5 have been previously shown to lead to defective border-like cell release in Arabidopsis under standard growth conditions (del Campillo et al., 2004). CEL5 encodes an endo-1,4-β-D-glucanase that hydrolyzes the β-1,4-linked glucan chains that comprise cellulose, and CEL5 expression is highly enriched in the columella compared to other cell types within the root (Supplemental Fig. S8; del Campillo et al., 2004). As previously observed (del Campillo et al., 2004), analysis of the cel5 mutant (T-DNA insertion line SALK_079921) showed that BLC are not properly released and extra cells stick to the root cap when grown under standard conditions (del Campillo et al., 2004). We searched for additional cel5 alleles, but found no other lines with significantly reduced CEL5 transcript (Supplemental Fig. S9). Since wild-type plants show an increase in release of single BLCs when grown under low pH conditions (Fig. 2), we used confocal microscopy to examine the root cap of the cel5 mutant after exposure to low pH. In contrast to the wild type, when the cel5 mutant was grown at low pH, the root cap retained its stickiness; BLCs adhered to the tip of the cel5 root, and intact root cap layers were clearly observed adjacent to the root tip (Fig. 7, C and D). Only 14% of cel5 roots showed single border-like cell release at low pH compared to 55% of wild-type plants at low pH. Thus, even in a root cap loosening environment, cells of the cel5 root cap do not correctly detach.

**Figure 7.** NLP7 regulates border-like cell release by altering expression of cell wall modifying enzymes. A, Relative expression levels of cell wall-modifying enzymes in nlp7-1 mutant by qRT-PCR. B, Total number of root cap layers in various mutants involved in cell wall modification. C and D, BLCs adhere more tightly to the root cap of the cel5 mutant at pH 4.0. C, The 7 dai wild-type root growing at pH 4.0 showing release of single border-like cells (white arrow). D, The 7 dai cel5 mutant growing at pH 4.0 showing cells adhering to the root cap (white arrow) and several released root cap layers adjacent to the root cap (white arrowheads). Bar = 50 μm. C and D are 40× magnification. *P < 0.05 and **P < 0.001.

**Single Border-Like Cell Release in nlp7-1 Depends on the Expression Level of CEL5**

Because NLP7 encodes a transcription factor, and expression of CEL5 is activated in the nlp7-1 mutant, we hypothesized that CEL5 may be required for the nlp7-1 phenotype. To test this, we generated the nlp7-1 cel5 double mutant. In roots of the double mutant, BLC release occurs in layers, similar to wild-type plants (Fig. 8A), and the average number of root cap layers is not different compared to the wild type (Fig. 8B). Out of 13 double mutant lines examined, none showed single BLC release. Thus, the single BLC release phenotype observed in nlp7-1 depends on the expression level of CEL5.

**Expression of Root Cap Regulators Is Altered in nlp7-1**

The NAC-domain family transcription factors SMB, BRN1, and BRN2 redundantly regulate root cap maturation and cell wall modifications in the root cap (Willemsen et al., 2008; Bennett et al., 2010). The smb-3 brn1-1 brn2-1 triple mutant has a very sticky root cap, similar to that of cel5 (del Campillo et al., 2004; Bennett et al., 2010). We used qRT-PCR to assess expression levels of these genes in the root of the nlp7-1 mutant. We found that expression of SMB, BRN1, and BRN2 is activated (Fig. 9). Expression of CEL5 is reduced in the smb-3 brn1-1 brn2-1 triple mutant (Bennett et al., 2010). Thus, in mutants with a sticky columella root cap, CEL5 expression is reduced, while in the nlp7-1 mutant, which releases BLCs as single cells, CEL5 expression is activated.

**Mutations in NLP7 Lead to Increased Susceptibility to the Soil-Borne Fungus Foc**

We hypothesized that the impaired border-like cell release in the nlp7-1 mutant may lead to increased...
susceptibility to soil-borne pathogens. To test this, we inoculated the soil-borne fungal pathogen Foc directly on to the tip of \textit{nlp7-1} and wild-type roots. Foc first invades root systems and then enters the vasculature and eventually causes wilting of the aboveground portion of the plant (Tjamos and Beckman, 1989). As shown in Figure 10, \textit{nlp7-1} has more Foc growth around the root tips than wild-type or the complementation line (Supplemental Fig. S10) due to increased susceptibility to Foc. Also, fewer \textit{nlp7-1} roots grew through the Foc inoculation site (Fig. 10C). Furthermore, \textit{nlp7-1} showed increased anthocyanin accumulation in the shoots compared to the wild type at 5 d after infection (Fig. 10D). Thus, in keeping with a role for BLCs in plant defense, mutations in \textit{NLP7} lead to increased susceptibility to a root-invading pathogen.

**DISCUSSION**

Collectively, our data support a model in which the transcription factor \textit{NLP7} regulates BLC release by suppressing expression of cell wall-loosening enzymes as well as key transcription factors involved in root cap maintenance and maturation (Fig. 11). This promotes cellulose and pectin maintenance, preventing single-cell BLC release in wild-type plants. Whether NLP7 directly regulates these genes or does so indirectly through the transcription factors \textit{SMB}, \textit{BRN1}, or \textit{BRN2} is not clear.

In wild-type plants, environmental conditions that lead to cell wall loosening, such as those encountered under low pH, result in an increase in \textit{NLP7} expression. Increased \textit{NLP7} expression suppresses cell wall loosening by repressing expression of cell wall modification enzymes, which results in proper cell adhesion under stressful conditions. In the \textit{nlp7-1} mutant, an increase in expression of cell wall modification enzymes in the root cap coupled with a decrease in HG and cellulose leads to release of BLCs as single cells.

Border cells and BLCs are known for their protective roles in plant defense (Hawes et al., 2000; Watson et al., 2015). For example, border cells in pea protect the root tip from infection by the pathogenic fungus \textit{Nectria hematococca} (Gunawardena and Hawes, 2002), while those of maize (\textit{Zea mays}) exude a compound that promotes branching in the arbuscular mycorrhizal fungus \textit{Gigaspora gigantea} (Nagahashi and Douds, 2004). In \textit{Medicago truncatula}, border cells have elevated levels of defense compounds (Watson et al., 2015). In Arabidopsis, BLCs perceive and activate defense signaling in response to microbe-associated molecular patterns, such as flagellin22 and peptidoglycan (Plancot et al., 2013). Consistent with a role for BLCs in plant defense mechanisms, mutations in \textit{NLP7} lead to an increase in susceptibility to the soil-borne, root-infecting fungal pathogen Foc. The increased release of single BLCs from the root cap and the decrease in pectin and cellulose content may facilitate fungal entrance and increase colonization of the mutant root. Given the ubiquity of soil-borne pathogens (Lewis and Papavizas, 1991; Lumsden et al., 1995), this suggests that \textit{NLP7} may act to protect the root from biotic stress.

The low pH-induced release of BLCs as single cells may be a result of changes to the cell wall polysaccharide pectin (Koyama et al., 2001; Fig. 4). Low pH changes the availability of nutrients in growth media and soils, causing a major decrease in the availability of Ca$$^{++}$$ (Truog, 1946). Arabidopsis roots grown in minimal media at low pH had decreased viability after 2 h, but this effect was ameliorated if the minimal...
media was supplemented with Ca\textsuperscript{++} (Koyama et al., 2001). Ca\textsuperscript{++} is a divalent cation with a large ionic radius that can cross-link and stabilize pectic polysaccharides (Carpita and Gibeaut, 1993). Although our plants are grown on full nutrient agar, since low pH decreases the availability of Ca\textsuperscript{++}, the low pH-induced release of single BLCs in wild-type plants may be due to a decrease in available Ca\textsuperscript{++} and, thus, a decrease in pectin stabilization. This phenotype is exacerbated at low pH in the \textit{nlp7-1} mutant, in which the expression of cell wall-loosening enzymes, including PL, is activated.

\textit{NLP7} is part of the RWP-RK transcription factor family, so named for the conserved RWPYRK protein motif present in all members (Schauser et al., 2005; Chardin et al., 2014). Members of this family bind to a nitrogen response cis-element to control nitrate-regulated transcription (Konishi and Yanagisawa, 2013). The \textit{NLP7} protein moves from the cytoplasm to the nucleus after resupply of nitrate to N-starved seedlings (Marchive et al., 2013), and nitrate sensing is defective in the \textit{nlp7} mutant (Castaings et al., 2009). In addition to its role in the N response, \textit{nlp7} mutants have high drought tolerance (Castaings et al., 2009), suggesting that this transcription factor has additional unexplored roles in environmental stress responses.

Here, we show that \textit{NLP7} controls BLC release by regulating cell wall loosening in the root cap. The role of \textit{NLP7} in BLC release appears to be primarily through suppression of the enzymes that control cellulose and pectin degradation. For example, a pectin lyase-like gene, which degrades pectin, is up-regulated in the \textit{nlp7-1} mutant, but there is no change in expression of the pectin biosynthetic enzymes \textit{QUA1} and \textit{QUA2}. Interestingly, cell wall loosening and degradation are important parts of the nodulation process, and recent work has demonstrated that NIN, a \textit{NLP7} homolog, binds to the promoter and activates the expression of a pectate lyase required for nodulation in \textit{L. japonicus} (Xie et al., 2012). Together, these results suggest that the regulation of cell wall modification may be a common feature of NIN and NLPs, yet employed in different developmental contexts. It will be intriguing to determine whether additional members of the NLP family have roles in BLC release or cell wall modification in different tissues.

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**Figure 10.** Mutations in \textit{NLP7} lead to increased susceptibility to Foc infection. \textbf{A}, Foc growth around the root of root-tip inoculated wild-type and \textit{nlp7-1} at 48 hpi. \textbf{B}, Quantification of the area of Foc growth around the root tip at 48 hpi. \textit{nlp7-1} shows significantly more growth of Foc around the root tip. \textit{P} < 0.05. \textbf{C}, Percentage of roots growing through the inoculation site. \textbf{D}, Graph showing total anthocyanin content from the shoots of the wild type and \textit{nlp7-1} at 6 d postinoculation. Letters indicate significant difference using ANOVA and Tukey’s HSD test.

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**Figure 11.** Model for \textit{NLP7} control of border-like cell release. See text for details.
MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of Arabidopsis (Arabidopsis thaliana) wild type (Col-0), nlp7-1 (Salk_026134), nlp7-2 (SALK_148866), cel7s (SALK_079921), xth5 (SALK_057512), and a lycC-lyase-less gene (GK100005R) were obtained from the Arabidopsis Biological Resource Center. Homozygous nlp7-1 plants were backcrossed once to Col-0. The seeds were surface sterilized in 50% bleach and stratified at 4°C for 48 h. The seeds were plated on standard Murashige and Skoog (MS; pH = 5.7) or on low pH MS (pH = 4.0) plates. The seedlings were grown in a growth chamber at 22°C under 50% relative humidity with a 16-h/8-h day/night schedule. Unless otherwise noted, plants in all experiments were examined 7 d after imbibition, and error bars in figures show SD.

To generate complementation lines, 3 kb upstream of the NLP7 start site (NLP7 promoter) was first cloned into vector pDONR-P41 using Gateway technology (Life Technologies) to generate pENTR:pNLP7. The coding region of At4g24020 (NLP7) was amplified from cDNA and cloned into Gateway vector pDONR221 to generate pENTR:NLP7. The vectors pENTR: pNLP7, pENTR:NLP7, and GFP in pDONR2P3 were recombined with pDEST43-43 using Multisite Gateway cloning technology (Life Technologies). The final construct of pNLP7:NLP7:GFP was transformed into Arabidopsis by the floral dip method (Clough and Bent, 1998). Transgenic plants were confirmed by PCR using GFP-specific primers, confocal microscopy for GFP expression, and qRT-PCR for NLP7 expression. The seeds of pNLP7:GUS from Castaings et al. (2009) were obtained as a gift from Dr. Anne Krapp (INRA, France).

RNA Isolation, cDNA Synthesis, and Quantitative Real-Time PCR

For RNA extraction, seedlings were either grown continuously on control MS plates (pH = 5.7) or transferred to low pH 4.0 plates for 24 h after initial growth on control MS as specified for each experiment. Whole seedlings or only roots were frozen in liquid N2 and stored at −80°C until further use at the time specified for each experiment. Total RNA was isolated using the plant RNA purification kit (Norgen Biotek). On-column DNAse treatment was performed with DNase 1 in DNase digestion buffer (Omega Bio-tek). The cDNA was synthesized using 1 μg of total RNA with oligo(dT) primers using AMV first-strand cDNA synthesis kit (New England Biolabs) according to the manufacturer’s protocol. The cDNA was diluted 1:5 (v/v) and 2 μL of diluted cDNA was used for qRT-PCR. The qRT-PCR was performed using Taq Fast Eva Green Supermix (Bio-Rad) according to the manufacturer’s instructions. The melting temperature of each primer pair set was evaluated using a cDNA dilution series, and the relative expression was calculated using the delta-delta Ct method. Gene expression was calculated relative to At1G13320 (Czechowski et al., 2004) as described by Iyer-Pascuzzi et al. (2011). A total of three to five independent biological replicates, each with three technical replicates, were performed to calculate relative fold difference in gene expression. Primers used are listed in Supplemental Table S5. In all qRT-PCR figures, error bars show SD.

Microarray Sample Preparation and Analysis

Sample preparation and microarray analysis was performed as described (Iyer-Pascuzzi et al., 2011). Briefly, seedlings of the wild type (Col-0) and nlp7-1 were grown on MS, pH 5.7, for 5 d and roots cut just below the root-hypocotyl junction. Cut roots were immediately placed into RLT buffer (Qiagen) and RNA extraction using the RNeasy plant mini kit (Qiagen). Two biological replicates were performed. Probes for array analysis were prepared using the one-cycle amplification protocol from Affymetrix. Samples were hybridized to ATH1 microarrays by Expression Analysis. All arrays were normalized and differentially expressed probe sets identified using a mixed-model ANOVA Perl script as described (Levesque et al., 2006). Differentially expressed probe sets were identified using an 1.5 fold change cutoff and a false discovery rate (q-value) of 1 × 10−6 as described (Iyer-Pascuzzi et al., 2011). Heat maps were created using TMV microarray software (www.tm4.org).

GUS and Ruthenium Red Staining

The seedlings expressing pNLP7:GUS were grown on control MS plates (pH = 5.7) for 6 to 8 d. The seedlings were transferred to either low pH plates (pH 4.0) or to control plates (pH 5.7). After 24 h, seedlings were incubated in GUS staining buffer containing 50 mM NaPO4, pH 7.0, 2.5 mM K3CN, 2.5 mM K3CN, 0.1%, Triton X-100, and 2.5 mM X-gluc for 1.5 h at 30°C and washed with the GUS staining buffer without X-gluc. To analyze pectin content in the roots of wild-type and nlp7-1 seedlings, 0.05% (w/v) solution of ruthenium red (Sigma-Aldrich) was prepared and seedlings were stained for 10 min as described by Durand et al. (2009). The pictures were taken with a Nikon Eclipse 800 under 20× magnification.

Confocal Microscopy and Immunofluorescence

Seeds of the wild type (Col-0), nlp7-1, cel7s, nlp7-1 cel7s, xth5, and pl were sterilized as described and seedlings were grown for 7 d. Seedlings were stained with propidium iodide (Sigma-Aldrich) for about 1 min and mounted on a glass slide, and roots caps were visualized under a confocal microscope. For all experiments, images were taken using Nikon A1Rsi confocal microscope, and images were edited using NIS elements software (Nikon). For immunofluorescence experiments, 7 dai wild-type and nlp7-1 seedlings were fixed in 4% N,N-dimethylformamide for 30 min following three washes with 1× PBS. To prevent nonspecific antibody binding, seedlings were incubated in blocking solution (3% nonfat dairy milk in 1× PBS) for 30 min followed by 3 washes with 1× PBS. Seedlings were incubated overnight with a primary antibody JIM5 or JIM7 (Carboxyls) diluted 1:6 in 1× PBS with 3% nonfat dairy milk in 1× PBS, washed with TBSB (0.05% Tween in 1× PBS) for 20 min with 4× 5-min washes, and then incubated with FITC-conjugated secondary antibody diluted 1:50 in TBSB for 1 h at room temperature. Finally, the seedlings were washed four times with TBSB for a total of 20 min followed by a last wash with PBS. Seedlings were left in PBS until confocal microscopy was done. “No primary antibody” control was used in each biological replicate as a negative control to check for specificity of antibodies. As a positive control, both wild-type and nlp7-1 roots were labeled with anti-CDPK23 custom made by Yenzym Antibodies). The images of roots were taken using a Nikon confocal A1Rsi with Z-stack sectioning, and a 3D image was reconstructed using NIS elements. Two independent biological replicates were performed. Z-stack projections for the wild type and nlp7-1 were composed of similar numbers of slices, and each slice was 5 μm. For fluorescence measurements, the maximum intensity projections were generated using NIS elements ND2 viewer, and fluorescence intensities were measured using Imagej software. For measuring fluorescence, the area between the root tip and 200 μm upwards was selected in each root and the outline for the area of the root was drawn in Image J. The corrected total fluorescence (CTF) was calculated as CTF = integrated density − (area of selected root tip × mean fluorescence of background readings). The average of at least nine roots with similar number of Z-stack sections was used for each wild type and nlp7-1 for each antibody. Error bars show SD.

Quantification of Root Cap Layers

Root cap layers were counted from the root cap images taken using a confocal microscope. The number of root cap layers was counted from the columella initials to the last attached layer of BLCs still adhered to the primary root. A minimum of 30 roots was examined per genotype with at least two biological replicates.

Cellulose Assay

Cellulose content in the nlp7-1 mutant and wild type was determined by the acid hydrolysis method modified from Dubos et al. (1956). Briefly, the roots of 7 dai nlp7-1 and the wild type were ground in liquid N, and 3.5 mg each of the freeze dried powder was used for further analysis. The ground tissue was treated with trifluoroacetic acid for 90 min at 120°C. To determine the cellulose content as a Glc derivative, the phenol-sulfuric acid hydrolysis method was performed, and the Glc concentration was determined using a standard curve of 0 to 1,000 nmol of Glc at 500 nm. The entire experiment was repeated twice. Error bars show SD.

Pathogen Assay

nlp7-1 and wild-type plants were grown on standard MS plates for 7 d. The root tips of nlp7-1 and the wild type were inoculated with 2 μL of the 1 × 103 microconidia suspension in sterile water of Foc (NRRL #38297). Foc cultures were grown on potato dextrose agar plates for 3 to 4 d to obtain conidia. The
plates inoculated with Foc were scanned at 24 and 48 h postinoculation (hpi). Mycelial growth was measured at 48 hpi using ImageJ. The area of mycelial growth around each root of the wild type and nlp7-1 was selected in ImageJ and measured. Root growth inhibition was measured by counting the number of roots passing through the infection site. Anthocyanin concentrations were measured by standard anthocyanin spectrophotometric assay as described by Sims and Gamon (2002). Briefly, shoot tissue of the infected plants was harvested in liquid N₂ and ground in acidified methanol (99% methanol and 1% HCl). The extracts were centrifuged at 4,000 rpm for 5 min at 4°C and the supernatant was used for spectrophotometry. Absorbance for anthocyanin was measured at 530 nm. The total anthocyanin content was calculated by accounting for degraded chlorophyll content.

Accession Numbers

Sequence data from this article can be found in the EMBL/GenBank data libraries under accession number GSE63474.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Time course of gene expression of genes in the STOP1 cluster after exposure to pH 4.6.

Supplemental Figure S2. qRT-PCR of NLP7 expression in whole roots 24 h after exposure to pH 4.0 or pH 5.7 shows that NLP7 is activated by low pH.

Supplemental Figure S3. nlp7-1 mutant phenotype in the whole plant.

Supplemental Figure S4. Levels of NLP7 transcript in nlp7-1 (SALK_026134) and nlp7-2 (SALK_114886) mutants and in the complemented line.

Supplemental Figure S5. NLP7 localization at low pH.

Supplemental Figure S6. Negative and positive controls for immunolabeling experiment.

Supplemental Figure S7. Immunolabeling of 5 dai root tips of wild-type and nlp7-1 seedlings grown at pH 4.0.

Supplemental Figure S8. Heat map of root cell-type-specific expression of 11 genes with roles in cell wall-related processes and differentially expressed in nlp7-1 roots compared to the wild type.

Supplemental Figure S9. Semiquantitative RT-PCR for reduction in CEL5 transcript in two SALK T-DNA insertion lines.

Supplemental Figure S10. nlp7-1 and the complementation line (pNLP7: NLP7::GFP) 48 hpi with Foc.

Supplemental Table S1. One hundred and ninety four genes differentially expressed between whole roots of nlp7-1 and wild-type Col-0, ATH1 microarray, pH 5.7.

Supplemental Table S2. GO categories of the genes differentially expressed in the nlp7-1 root.

Supplemental Table S3. Genes differentially expressed in the nlp7-1 root and direct targets of NLP7 after nitrate resupply in Marchev et al. (2013).

Supplemental Table S4. Eleven genes with roles in cell wall-related processes and differentially expressed in the nlp7-1 root.

Supplemental Table S5. Primers used in this study.

Supplemental Methods.

ACKNOWLEDGMENTS

We thank members of the Syed-Pascuzzi lab for critical reading of the manuscript, members of the Carpita lab for technical help with the cellulose acid hydrolysis assay, Anne Krapp for use of the pNLP7::GUS line, and Philip Benfey for supporting early experiments with NLP7.

Received March 20, 2016; accepted May 19, 2016; published May 24, 2016.

LITERATURE CITED


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Plant Physiol. Vol. 171, 2016 2111


Plant Physiol. Vol. 171, 2016 2111


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