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A novel mutation in *KNOPF* uncovers the role of α-glucosidase I during post-embryonic development in *Arabidopsis thaliana*

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Abstract ***N*-glycosylation is a common protein modification. Joining of polypeptide and carbohydrate elements into hybrid molecules provides an opportunity to fine-tune protein properties. However, the role of *N*-glycosylation on the development of multicellular organisms remains elusive. Here we report a hypomorphic allele of *KNOPF/GLUCOSIDASE 1*, which allows us to describe the effects of impaired α-glucosidase I on post-embryonic development of plants for the first time. This *knf-101* mutation alters cell shape but does not affect cell arrangements, except for the patterning of specialized epidermal cells, delineating the significance of *N*-glycan processing during epidermal development in *Arabidopsis*.**

Keywords: Epidermal development; *KNOPF/GLUCOSIDASE 1*; *N*-glycan processing; α-Glucosidase I; *Arabidopsis thaliana*

1. Introduction

N-glycosylation is a common covalent protein modification (for a review, see [1]). It occurs through addition of a core oligosaccharide to the asparagine residue of growing, nascent polypeptides, in which the Asn-X-Ser/Thr motif (X can be any amino acid except proline) is necessary for oligosaccharide transfer. The core oligosaccharide consists of a branched oligosaccharide unit comprised of three glucoses, nine mannoses, and two *N*-acetylglucosamines. After transfer to the protein, the oligosaccharide undergoes several modifications, leading to the diverse array of N-linked carbohydrates on mature glycoproteins.

Despite the extensive knowledge regarding the biosynthesis, transfer, and maturation of N-linked glycans, the biological function of *N*-glycosylation remains obscure. In yeast and mammalian cells, studies indicate that N-linked glycans exert their functions by facilitating molecular and cellular interactions (for a review, see [2]). However, the mechanisms of N-linked glycans in these processes are unclear. In *Arabidopsis thaliana*, mutants defective in *N*-glycosylation have been shown to impact cell wall synthesis, anisotropic cell growth, and seed storage protein synthesis [3–7]. These mutants exhibit pleiotropic phenotypes, and many of them are lethal during early development, making it difficult to discern the roles of N-linked glycans in specific developmental stages. New genetic resources will allow us to further investigate the roles of *N*-glycosylation in the development of multicellular organisms.

In multicellular organisms, the epidermis differentiates during embryogenesis and supports further development. In *Arabidopsis*, cells in the outermost layer are differentiated early in embryogenesis [8]. After germination, the epidermis serves as a communicative boundary, separating the plant from the environment, perceiving stimuli, preventing water loss, and taking up nutrients. Thus, cell types of varying size, shape, and function can form in the epidermis, such as guard cells, root hair cells, and leaf trichome cells. Molecular genetic studies have identified genes that regulate the development of specialized epidermal cells, and a molecular genetics framework for cell fate specification in the *Arabidopsis* epidermis is now available (for reviews, see [9–11]). However, the molecular details of the regulatory mechanisms remain to be addressed.

2. Materials and methods

2.1. Plant materials and growth conditions

Unless otherwise stated, all plants were in the Columbia (Col) background, and Col was used as the wild-type. Plants were grown on rock-wool bricks supplemented with vermiculite under cool white fluorescent light in long day conditions (16 h light/8 h dark) at an average temperature of 22 °C. For observation of root phenotypes, seeds were germinated on nutrient agar plates containing half-strength Murashige and Skoog Plant Salt Mixture (Wako Pure Chemical Industries, Osaka, Japan), 1% sucrose, and 0.3% Gelrite (Wako Pure Chemical Industries). The pH of the medium was adjusted to 5.7 with KOH. The plates were incubated at 22 °C in a near vertical position.

The *epf1-1* was described previously [12]. The *knf-14* mutant was in the Landsberg *erecta* (L*er*) background, and *knf-14* seed stock (CS6119) was obtained from the Arabidopsis Biological Resource Center (ABRC) at Ohio State University (Columbus, OH). The *GL2::GUS* transgenic line was in the Wassilewskija (WS) background [13].

2.2. Map-based cloning and PCR-based genotyping

The *muc* mutant was crossed to L*er*, and F2 seedlings with the *muc* phenotype were examined for recombination between the mutation and PCR-based polymorphic markers. For details, see Supplementary Methods.

dCAPS markers were designed for *muc/knf-101* and *knf-14* alleles based on the mutations in *knf-101* and *knf-14* [6], respectively. The primer sequences and details are available in Supplementary Table S1. PCR amplification was performed under standard conditions.

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Abbreviations: GUS, β-glucuronidase; RT-PCR, reverse transcription-PCR; 35S, cauliflower mosaic virus 35S RNA promoter; UTR, untranslated region

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2.3. Complementation of muc/knf-101 mutant

For complementation of *muc*, *35S::KNF* (pCF602) was generated, and *muc* plants were transformed using *Agrobacterium*-mediated transformation. For details, see Supplementary Methods.

2.4. RNA extraction and RT-PCR

For details on RNA extraction and RT-PCR, see Supplementary Methods.

2.5. Morphological analyses

Detailed protocols, microscopy, and histochmical GUS staining are available in Supplementary Methods.

3. Results

3.1. Isolation of munchkin mutant

To identify new loci involving the epidermal development in *Arabidopsis*, we conducted a visible screen of ethyl methanesulfonate-mutagenized seeds. Previous studies revealed that mutants defective in shoot epidermal development often result in dwarf stature, therefore, we limited our search to mutants smaller than wild type [14,15]. Among candidate mutants, *munchkin* (*muc*) was isolated by its semi-dwarf phenotype (Fig. 1A and B). The Fl offspring from *muc* and wild-type plants produced wild-type plants, and 22% of F2 offspring were mutant plants ($n = 264$), indicating that *muc* is a single nuclear recessive mutation.

3.2. Epidermal cell patterning is disturbed in the muc fruits

In wild-type *Arabidopsis* fruits, the outer epidermis of the valve has two types of cells, typical long-shaped cells and stomatal lineage cells (Fig. 1C). In the outer valve epidermis of *muc* fruits, clusters of small cells surround the stomata, and the stomatal density is increased (Fig. 1D and E). Because these small cells around stomata are not seen in wild-type fruits, the stomatal index does not greatly increase in the *muc* mutant (Fig. 1F). Although the *muc* stomata are indistinguishable from the wild-type stomata in their size and shape, the *muc* long-shaped cells are shorter than wild-type and increase in diameter. These indicate that *MUC* is required for proper cell differentiation in the fruit epidermis.

3.3. muc is a novel allele of knf/gcs1

The *muc* mutation was mapped to a 122-kb region, and sequencing candidate genes revealed a single base mutation in the Atlg67490 locus (Fig. 2A and B). This G-to-A substitution replaces Gly with Asp at amino acid residue 504. The *muc* phenotype was complemented by constitutive expression of the Atlg67490 cDNA (Supplementary Fig. S1). Thus, the identified mutation accounts for the *muc* phenotype.

The Atlg67490 gene has been previously described as *KNOPF* (*KNF*)/*GLUCOSIDASE 1* (*GCS1*), which encodes α-glucosidase I (E.C. 3.2.1.106) that specifically cleaves the outermost glucose residue from the N-linked core oligosaccharide [3,6]. While the *muc* mutant is viable and fertile, all other *knf/gcs1* mutants have been embryonic lethal [3,6,16]. In order to determine whether *muc* is allelic to *knf/gcs1* mutations, we crossed *muc* homozygous plants with *knf-14* heterozygous plants. The resulting Fl progeny produced wild-type and severe-dwarf plants at a 1:1 ratio, indicating that *muc* is allelic to *knf/gcs1* mutations. Therefore, the *muc* allele was renamed *knf-101*. Like *knf-101* homozygous plants, *knf-14/knf-101 trans*-heterozygous plants are viable and fertile. However, *knf-14/knf-101* plants displayed more severe phenotypes than *knf-101* plants (Fig. 2C–F). In contrast to other *knf/gcs1* mutants, embryo development is not severely affected in *knf-101* (Supplementary Fig. S2). RT-PCR analysis revealed that the *knf-101* mutation does not affect accumulation of the *KNF* transcript (Supplementary Fig. S3).

3.4. Genetic interaction between epf1-1 and knf-101 mutations

Recently, *EPIDERMAL PATTERNING FACTOR 1* (*EPF1*) was identified as a negative regulator of stomatal patterning

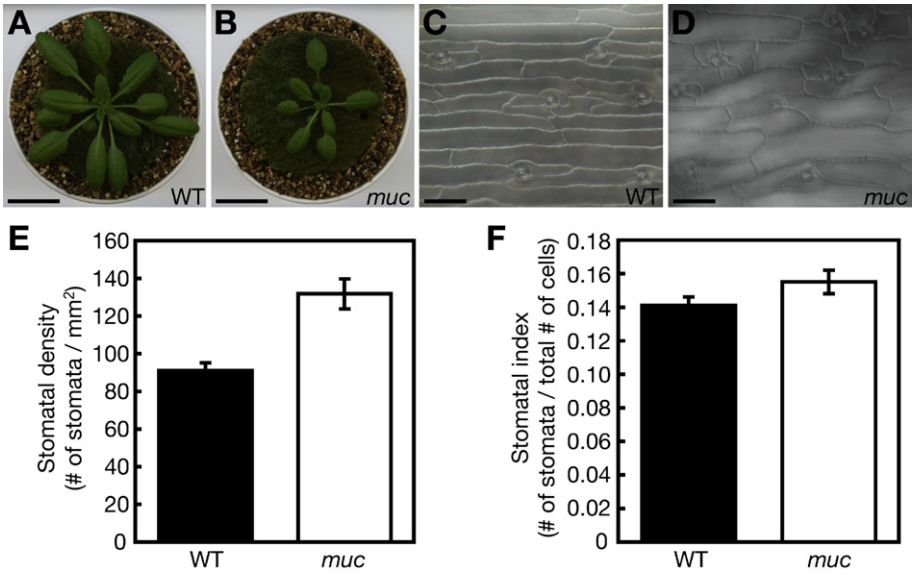

Fig. 1. The shoot phenotype of *muc* mutant. (A, B) Plants grown for 25 days after germination (dag). (C, D) The morphology of the outer epidermal cells in the valve of fully elongated fruits. (A, C) Wild-type (WT). (B, D) *muc* mutant. (E, F) Number of stomata in the abaxial valve epidermis of wild-type and *muc* fruits. (E) Stomatal density ($n = 9$; three measurements per plant). (F) Stomatal index ($n = 9$; three measurements per plant). Each value represents the average, and bars indicate standard errors. Scale bars: 2 cm (A, B) and 50 μm (C, D).