NAI1 Gene Encodes a Basic-Helix-Loop-Helix–Type Putative Transcription Factor That Regulates the Formation of an Endoplasmic Reticulum–Derived Structure, the ER Body

Ryo Matsushima,a Yoichiro Fukao,a,c Mikio Nishimura,b and Ikuko Hara-Nishimura,a,c,1

Department of Botany, Graduate School of Science, Kyoto University, Kyoto 606-8502 Japan
Department of Cell Biology, National Institute for Basic Biology, Okazaki, 444-8585, Japan
Core Research for Evolutionary Science and Technology, Japan Science and Technology Agency, Kawaguchi, 322-0012, Japan

Plant cells develop various types of endoplasmic reticulum (ER)–derived structures with specific functions. ER body, an ER-derived compartment in Arabidopsis thaliana, is a spindle-shaped structure. The NAI1 gene regulates the development of ER bodies because mutation of NAI1 abolishes the formation of ER bodies. To better understand the role of NAI1, we cloned the NAI1 gene using a positional cloning strategy. The nai1-1 mutant had a single nucleotide change at an intron acceptor site of At2g22770 (NAI1 gene). Because of this mutation, aberrant splicing of NAI1 mRNA occurs in the nai1-1 mutant. NAI1 encodes a transcription factor that has a basic-helix-loop-helix (bHLH) domain. Transient expression of NAI1 induced ER bodies in the nai1-1 mutant. Two-dimensional electrophoresis and RT-PCR analyses showed that a putative lectin was depressed at both the mRNA and protein levels in nai1 mutants, as was a β-glucosidase (PYK10). Our results provide direct evidence that a bHLH protein plays a role in the formation of ER bodies.

INTRODUCTION

Endoplasmic reticulum (ER) is an extensive, morphologically continuous network of membrane tubes and flattened cisternae. Classically, the ER is subdivided into three compartments: rough ER, smooth ER, and the nuclear envelope (Baumann and Walz, 2001). In addition to these compartments, many ER-derived structures with specific functions have been identified in plant cells (Okita and Rogers, 1996; Staehelin, 1997; Chrispeels and Herman, 2000). The protein bodies in the endosperm of maize (Zea mays) and rice (Oryza sativa) are responsible for the accumulation of seed storage proteins (Herman and Larkins, 1999). Precursor-accumulating vesicles in maturing cotyledons of pumpkin (Cucurbita maxima) mediate the transport of seed storage protein to protein storage vacuoles directly from ER (Hara-Nishimura et al., 1998). KDEL (Lys-Asp-Glu-Leu)-tailed Cys proteinase-accumulating vesicles and ricinosomes are also ER-derived structures that are found in vegetative organs of black gram (Vigna mungo) and castor bean (Ricinus communis), respectively (Schmid et al., 1998; Toyooka et al., 2000).

In transgenic Arabidopsis thaliana expressing green fluorescent protein (GFP) with an ER-retention signal (GFP-HDEL, His-Asp-Glu-Leu), spindle-shaped GFP-fluorescent structures (∼10 μm long and ∼1 μm wide) have been visualized together with the ER networks (Haseloff et al., 1997; Ridge et al., 1999; Hawes et al., 2001; Hayashi et al., 2001). Electron microscopic studies show that the structures have a fibrous pattern inside, and they are surrounded by ribosomes (Hayashi et al., 2001). The presence of ribosomes on the surface of the structures indicates that they are directly derived from the ER. Therefore, we recently proposed to call them ER bodies (Hayashi et al., 2001). ER bodies develop in nontransgenic Arabidopsis, indicating that they are not artificial structures caused by overexpression of the transgene but, rather, accumulate some endogenous materials inside and have some specific role in plant cells (Matsushima et al., 2003a). Similar structures have been reported in the cells of various organs of Brassicaceae plants (Bonnett and Newcomb, 1965; Iversen, 1970; Behnke and Eschelbeck, 1978; Bones et al., 1989).

Transgenic Arabidopsis expressing GFP-HDEL (GFP-h) enabled us to study the unique distribution of ER bodies in living plants. A large number of ER bodies are observed in cotyledons, hypocotyls, and roots of young seedlings (Matsushima et al., 2002). Cotyledons decrease the number of ER bodies during senescence, whereas hypocotyls and roots maintain the ER bodies (Matsushima et al., 2002). In contrast with seedlings, rosette leaves of mature plants have few ER bodies and have GFP fluorescence only in ER networks (Matsushima et al., 2002). However, many spindle-shaped structures can be induced in rosette leaves by wounding them or treating them with exogenous methyl jasmonate (MeJA), a plant hormone that induces a defense response against wounding and chewing by insects (Matsushima et al., 2002). The induced structures have shapes and sizes similar to those of ER bodies of seedlings. We...
named them induced ER (i-ER) bodies because rosette leaves have no ER bodies under normal conditions (Matsushima et al., 2003b). These observations suggest that i-ER bodies play some roles in plant defense systems when wounding or insect feeding damages plants.

To study the molecular mechanisms responsible for the biogenesis of ER bodies, we previously screened Arabidopsis mutants from chemically mutagenized GFP-h plants and isolated a nai1 mutant in which fluorescent ER bodies were hardly detected (Matsushima et al., 2003a). The nai1 mutant shows no visual defects under normal conditions, other than the absence of ER bodies. However, GFP-h and nai1 seedlings exhibit different protein compositions. ER bodies are concentrated in a 1000g pellet (P1) fraction obtained from GFP-h seedlings (Hayashi et al., 2001), whereas no ER bodies were detected in the P1 fraction from nai1 (Matsushima et al., 2003a). A comparison of proteins in the two P1 fractions showed that a 65-kD protein (p65) is present in GFP-h seedlings but not in nai1 (Matsushima et al., 2003a). p65 is PYK10, a β-glucosidase with an ER-retention signal, KDEL. Immunofluorescent staining and immunoelectron microscopy confirmed that PYK10 is specifically localized in ER bodies (Matsushima et al., 2003a). The accumulation of PYK10 in wild-type seedlings is high; Coomassie blue staining can detect it in crude extracts of cotyledons, hypocotyls, and roots (Matsushima et al., 2003a). Therefore, PYK10 is a major component in ER bodies. The physiological role of PYK10 has not been determined. On the other hand, BGL1, a PYK10 homolog in Arabidopsis (70% identity), has been suggested to play a role in the defense against herbivores because it is induced after feeding by diamondback moth (Plutella xylostella) (Stotz et al., 2000). Therefore, we previously suggested that PYK10 also is involved in defense systems against herbivores and pathogens.

The nai1 mutant is an ideal tool to investigate the molecular basis of ER body biogenesis. In this study, we performed fine mapping of the NAI1 locus. The nai1 mutant had a single base pair change at the intron splicing acceptor site of the At2g22770 gene. A T-DNA insertion line containing an insertion in the second exon of the At2g22770 gene was allelic to the nai1 mutant (Columbia background) with wild-type Arabidopsis, AtMYC2 (445 to 497, Arabidopsis), AN1 (468 to 520, Petunia x hybrida), DEL (436 to 488, Antirrhinum majus), PG1 (455 to 507, Phaseolus vulgaris), c-myc (351 to 406, Homo sapiens), and MAX (20 to 74, H. sapiens). Alignment of amino acid sequences of the bHLH domain (125 to 177) of At2g22770 protein and other bHLH proteins, PIF3 (340 to 392, Arabidopsis), AtMYC2 (445 to 497, Arabidopsis), AN1 (468 to 520, Petunia x hybrida), DEL (436 to 488, Antirrhinum majus), PG1 (455 to 507, Phaseolus vulgaris), c-myc (351 to 406, Homo sapiens), and MAX (20 to 74, H. sapiens). These bHLH proteins are thought to function as transcription factors. The Glu residue indicated by an arrow is critical for the recognition of the E-box sequence. Identical residues are shown in black, and similar residues are shown in gray.

**RESULTS**

**Fine Mapping of the NAI1 Locus**

We previously showed that the nai1 mutation segregated as a single recessive allele (Matsushima et al., 2003a). We crossed a nai1 mutant (Columbia background) with wild-type Arabidopsis (Landsberg erecta background) and identified 174 homozygous F2 plants. These plants were subsequently scored according to their genetic background with a series of molecular markers.
using the cleaved amplified polymorphic sequences mapping procedure (Konieczny and Ausubel, 1993; Bell and Ecker, 1994) (Figure 1A). Our initial mapping of the NAI1 locus located it to the middle of chromosome 2, between PHYB and ERECTA. For high-resolution mapping, we generated six molecular markers, T9I22-5, T9I22-4, T9I22-7, T30L20-3, T30L20-4, and T20K9-1. The number of chromosomes that showed a Landsberg background represents the number of recombinations that occurred between the NAI1 locus and the position of each molecular marker because the genomic DNA of the nai1 mutant has a Columbia background. Fine mapping revealed that the NAI1 locus was located in the 69-kb region between T9I22-4 and T30L20-4 (Figure 1A). Fourteen putative open reading frames are predicted in this region. Sequence comparison of wild-type Columbia and the nai1 mutant revealed a single G-to-A transition in the At2g22770 gene. This G-to-A transition is consistent with the mode of action of EMS, the mutagen that was used to generate the mutagenized population from which the nai1 mutant was identified.

The At2g22770 gene contains three exons and two introns (DNA in Figure 1B). Determination of the 5′ untranslated region revealed that the At2g22770 gene has two distinct transcription start sites (arrows in Figure 1B). The single G-to-A transition in the nai1 mutant is located at the acceptor site of the first intron. The At2g22770 gene encodes a 320–amino acid protein that contains a basic-helix-loop-helix (bHLH) domain (protein in Figure 1B). This sequence similarity is clearly in alignment with sequences from a variety of organisms (Figure 1C). The bHLH proteins are a superfamily of transcription factors that bind as dimers to specific DNA target sites (Ferre-D’Amare and Burley, 1995; Heim et al., 2003; Toledo-Ortiz et al., 2003). The bHLH domain is comprised of ~50 amino acids with two functionally distinct regions. The basic region, located at the N-terminal end of the domain, is involved in DNA binding (yellow box in Figure 1C). The HLH region functions as a dimerization domain and is constituted mainly of hydrophobic residues that form two amphipathic α-helices separated by a loop region of variable sequence and length (blue and gray boxes in Figure 1C). Outside the bHLH domain, the At2g22770 protein has little sequence similarity to other proteins with proven functions. The At2g22770 protein has one acidic region and two Ser-rich regions (protein in Figure 1B). The acidic region has seven acidic residues out of 13 residues. The Ser-rich regions have 14 and 12 Ser residues out of 24 and 22 residues, respectively. At2g22770 is most similar to the

Figure 2. Aberrant Splicing of At2g22770 mRNA in the nai1 Mutant.

(A) Analyses of At2g22770 expression in wild-type Col, GFP-h, and nai1. Actin was used as an internal control. The lane GFP-hRT− is the negative control in which reverse transcriptase was omitted from the reaction mixture.

(B) Electropherogram of the amplified cDNA fragment of At2g22770 RNA resolved by capillary electrophoresis. The 35-cycle products in (A) were analyzed. The amplified cDNA fragment of nai1 had a smaller size than the amplified cDNA fragments of Col (blue) and GFP-h (black). The peak of nai1 (red) had a shoulder. The peak and shoulder positions were 9 or 16 base pairs smaller, respectively, compared with the peak positions of Col and GFP-h.

(C) Effect of the single nucleotide mutation in nai1 on the translation of predicted proteins. Sequencing of the amplified cDNA fragment revealed that the G-to-A transition at the intron acceptor site resulted in aberrant splicing. Asterisks indicate the mutated nucleotide. Two distinct splicing patterns were detected in nai1 (nai1 cDNA1 and cDNA2). Boxed sequences in the genome are introns that are spliced out in the mature mRNA.
Arabidopsis At2g22750 and At2g22760 genes, both of which encode bHLH proteins.

**Aberrant Splicing of At2g22770 mRNA in the nai1 Mutant**

The point mutation (G to A) in the nai1 mutant disrupted the splicing acceptor site of the first intron. Therefore, the correct splicing of At2g22770 mRNA was probably prevented in nai1 plants. To examine this possibility, we performed RT-PCR analyses with gene-specific primers that cover the first intron. Using these primers, the amplified cDNA fragment should be 517 bp if correct splicing occurs. The amplified cDNA fragment was separated in 1.0% agarose gel and stained with ethidium bromide (Figure 2A). The band intensity of the At2g22770 cDNA fragment from nai1 was almost the same as that from wild-type Columbia (Col) and GFP-h plants. The negative control that omitted reverse transcriptase gave no signal (GFP-h RT/C255). Actin as an internal control yielded constant signals. Next, the amplified cDNA fragments were subjected to capillary electrophoresis to analyze them more precisely (Figure 2B). The signals of the Col and GFP-h cDNA fragments were similar in strength and occurred at the same peak position, whereas the signal of nai1 cDNA fragment was weaker and its peak had a shoulder. The peak and shoulder positions of the nai1 cDNA fragment were ~9 and 16 base pairs shorter, respectively, than the peak position of Col and GFP-h cDNA fragments. These results indicated that the amplified cDNA from the nai1 mutant is a mixture made up of more than one fragment.

Sequencing of the nai1 cDNA fragment revealed two distinct sequences (nai1 cDNA1 and nai1 cDNA2 in Figure 2C). Figure 2C compares the genomic DNA and cDNA sequences and the predicted protein sequences. In the case of nai1 cDNA1, an AG sequence that is eight to nine bases after the point mutation site (indicated by an asterisk) was used as an intron splicing site. This led to a deletion of three amino acids after the mutation site, but it did not introduce a frame shift. In the case of nai1 cDNA2, an AG sequence that is 15 to 16 bases after the point mutation site (indicated by an asterisk) was used as an intron splicing site. This led to a frameshift of the coding sequence that, in turn, led to the creation of a premature stop codon. The lengths of nai1 cDNA1 and cDNA2 corresponded to the shoulder and peak positions in Figure 2B, respectively.

**Characterization of a Mutant with a T-DNA Insertion in the At2g22770 Gene**

Next, we characterized a mutant (CVJ9, Wassilewskija [Ws] background) containing a T-DNA insertion in the At2g22770 gene. CVJ9 had a T-DNA insertion in the second exon of the At2g22770 gene (Figure 3A). To determine allelism, GFP-h and nai1 were crossed with CVJ9. The F1 progeny of GFP-h × CVJ9 exhibited fluorescent ER bodies in cotyledons, hypocotyls, and roots, being representative of the wild-type phenotype (Figure 3B), whereas no ER bodies were detected in the F1 seedlings of nai1 × CVJ9. Bars = 10 μm.

We previously showed that PYK10 is a major component in ER bodies and that the accumulation of PYK10 is decreased in nai1-1 (Matsumisha et al., 2003a). We examined whether this phenotype was also observed in nai1-2. Crude extracts of 7-d-old GFP-h, nai1-1, Ws, nai1-2, and F1 progeny of GFP-h × nai1-2 and F1 progeny of nai1-1 × nai1-2 were subjected to immunoblot analyses with anti-PYK10, anti-GFP, and anti-BiP antibodies. Molecular masses are given at the left in kD.

**Figure 3. Allelism Test between nai1 and T-DNA Insertion Line.**

(A) Schematic representation of the T-DNA insertion site in CVJ9 line.

(B) Fluorescent images of F1 progeny of a cross between GFP-h × CVJ9 and nai1 × CVJ9. No ER bodies were detected in the F1 seedlings of nai1 × CVJ9. Bars = 10 μm.

(C) Absence of PYK10 in nai1-1, nai1-2, or F1 progeny of nai1-1 × nai1-2. Extracts prepared from 7-d-old GFP-h, nai1-1, Ws, nai1-2, and F1 progeny of GFP-h × nai1-2 and F1 progeny of nai1-1 × nai1-2 were subjected to immunoblot analyses with anti-PYK10, anti-GFP, and anti-BiP antibodies. Molecular masses are given at the left in kD.
ER, used as a loading control yielded almost the same signals among the analyzed plants. These results indicate that nau1-1 and nau1-2 are also allelic with respect to PYK10 accumulation.

Induction of ER Bodies by Transient Expression of At2g22770 Protein

To examine whether At2g22770 protein is responsible for the formation of ER bodies, we expressed At2g22770 protein transiently in nau1-1 cells by biolistic transformation. We introduced a plasmid (monomeric red fluorescent protein 1 [mRFP1] + At2g22770/pBI221) in 7-d-old nau1-1 seedlings to coexpress At2g22770 protein and mRFP1 (Campbell et al., 2002) under the control of 35S promoter (Figure 4A); mRFP1 was used to identify the bombarded cells. In these cells, many ER bodies were observed 48 to 53 h after bombardment. The percentage of bombarded cells that developed ER bodies was 83.8% \( (n = 74) \). As a negative control, we bombarded a plasmid (mRFP1/pBI221) from which only mRFP1 is expressed (Figure 4B). In this case, the percentage of bombarded cells that developed ER bodies was 1.4% \( (n = 74) \). These results indicate that expression of At2g22770 cDNA complemented the nau1 phenotype. Therefore, we concluded that At2g22770 is the NAI1 gene.

Two-Dimensional Electrophoresis Analysis Identified Proteins Depressed in nau1-1 Mutant

Previously, we compared proteins of the P1 fraction of GFP-h and nau1-1 by one-dimensional SDS-PAGE and identified PYK10 as a component of ER bodies (Matsushima et al., 2003a). To further resolve proteins in P1 fractions, they were subjected to two-dimensional electrophoresis (Figure 5). Protein spots specific to GFP-h (spot numbers 1 to 4) were analyzed by matrix-assisted laser-desorption ionization time of flight mass spectrometry (Table 1), and the results confirmed by the other analyses. The N-terminal sequences of spot numbers 1, 2, and 3 were determined by Edman degradation to be DGPVCPPXNKLXRA, DGPVXPP, and DGPVXPPSNKLSRA, respectively \( (X, \text{not determined}) \). A peptide of spot number 4, having a mass of 1091.63, was identified to have the sequence IGVHVRPLSN by post source decay analysis. Spot numbers 1, 2, and 3 were PYK10. Spot number 4 was putative myrosinase binding protein (At3g16420).

Downregulation of Transcription of PYK10 and At3g16420 Genes in nau1 Mutants

We raised anti-At3g16420 antibodies that are specific to an internal amino acid sequence of At3g16420 protein. Immunoblot analysis of Arabidopsis extracts showed that At3g16420 protein was hardly detected in nau1-1, nau1-2, or the F1 progeny of nau1-1 × nau1-2 (Figure 6A). This result indicated that loss of NAI1 results in the absence of At3g16420 protein. NAI1 is a putative transcription factor because it has a bHLH domain (Figure 1C). Therefore, it is possible that NAI1 regulates the transcription of the PYK10 and At3g16420 genes. The mRNA transcripts of these genes were examined by RT-PCR (Figure 6B). The level of PYK10 and At3g16420 RNAs in nau1-1 was reduced compared to their level in GFP-h, and the level of PYK10 and At3g16420 RNAs in nau1-2 was reduced compared to their level in Ws. The PYK10 and At3g16420 RNA levels in GFP-h and Col were about the same. No PYK10 RNA signal was observed in the negative controls, which did not have reverse transcriptase (GFP-hRT- and WsRT-). The level of Actin RNA, used as an internal control, was the same in all the strains. These results suggest that reduction of PYK10 and At3g16420 proteins in the
**nai1-1** and **nai1-2** mutants is a consequence of lower levels of gene transcription.

**nai1** Mutants Have Lower Activities of \(\beta\)-D-Glucosidase and \(\beta\)-D-Fucosidase than Wild-Type Plants

It was expected that PYK10 has \(\beta\)-D-glucosidase activity because PYK10 has high homology (40 to 70% identity) with experimentally confirmed \(\beta\)-D-glucosidases. We measured \(\beta\)-D-glucosidase activity in crude extracts of GFP-h, **nai1-1**, Ws, and **nai1-2** using a fluorogenic substrate of 4-methylumbelliferyl (4-MU) \(\beta\)-D-glucopyranoside (Figure 7A). The \(\beta\)-D-glucosidase activity of **nai1-1** was 19% of that in GFP-h, and the \(\beta\)-D-glucosidase activity of **nai1-2** was 39% of that in Ws. To confirm the \(\beta\)-D-glucosidase activity of PYK10 more directly, the GFP-h and Ws extracts were incubated with anti-PYK10 antibodies before addition of substrate. Anti-PYK10 antibodies reduced the \(\beta\)-D-glucosidase activities of GFP-h and Ws to 27 and 36%, respectively, of the activities in the presence of preimmune serum (Figure 7B). These results indicated that PYK10 has \(\beta\)-D-glucosidase activity and that the reduced level of PYK10 in **nai1** mutants leads to a sharp reduction of \(\beta\)-D-glucosidase activity in these mutants. Anti-PYK10 antibodies reduced the \(\beta\)-D-glucosidase activity of **nai1-1** by only 2.7 pmol 4-MU/s/mg protein (Figure 7B). This result confirms that the inhibitory effect of the antibodies is mostly because of the specific binding with PYK10, not because of the nonspecific binding.

To identify other glycosidase activities of PYK10, we measured the hydrolytic activities of GFP-h and **nai1-1** extracts against several fluorogenic (4-MU) substrates. A major difference in activities between GFP-h and **nai1-1** was found for 4-MU \(\beta\)-D-fucoside (Figure 7C), but little difference was found for eight other substrates (4-MU \(\alpha\)-D-glucoside, 4-MU \(\alpha\)-L-fucoside, 4-MU \(\beta\)-L-fucoside, 4-MU \(\beta\)-D-lactoside, 4-MU \(\beta\)-D-cellobioside, 4-MU \(\beta\)-D-mannopyranoside, 4-MU 7-\(\beta\)-D-xyloside, and 4-MU \(\beta\)-D-galactopyranoside) (data not shown). The \(\beta\)-D-fucosidase activity in **nai1-1** was 11% of that in GFP-h, and the \(\beta\)-D-fucosidase activity of **nai1-2** was 37% of that in Ws. Anti-PYK10

![Image](https://example.com/image.png)  
**Figure 5.** Two-Dimensional Electrophoresis of Proteins in the P1 Fraction from GFP-h and **nai1-1**.  
Six-day-old seedlings of GFP-h and **nai1-1** were homogenized and centrifuged at 1000g. The P1 fraction was separated by two-dimensional electrophoresis with denaturing isoelectric focusing (IEF) on immobilized pH gradients in the first dimension and SDS-PAGE in the second dimension. Proteins were detected by silver staining. Arrows indicate the protein spots that were specific to GFP-h. The protein spot numbers refer to the spot numbers listed in Table 1. Numbers on the x axis are pl, and numbers on the y axis are molecular mass (kD).

| Spot No. | Identified Protein | Accession Number | Calculated Mass (kD) | Calculated pl | Sequence Coverage (%) |
|----------|-------------------|------------------|----------------------|--------------|----------------------|----------------------|
| 1        | PYK10             | At3g09260        | 60.20                | 6.45         | 12                   |
| 2        | PYK10             | At3g09260        | 60.20                | 6.45         | 36                   |
| 3        | PYK10             | At3g09260        | 60.20                | 6.45         | 23                   |
| 4        | Putative myrosinase binding protein | At3g16420 | 32.14                | 5.46         | 26                   |

Proteins were identified with the MASCOT search engine (http://www.matrixscience.com/). Accession numbers, calculated molecular masses, and calculated pl values are cited from the MASCOT database.
antibodies reduced the β-D-fucosidase activity of GFP-h and Ws to 23 and 33%, respectively, of the activities in the presence of preimmune serum (Figure 7D). Anti-PYK10 antibodies also reduced the β-D-fucosidase activity of nai1-1 by only 6.3 pmol 4-MU/s/mg protein (Figure 7D). These results indicated that PYK10 has β-D-fucosidase activity in addition to β-D-glucosidase activity.

Irregular Shapes of i-ER Bodies in nai1-1 Rosette Leaves and Induction of NAI1 by MeJA Treatment

MeJA induces the formation of ER body-like structures (i-ER bodies) in rosette leaves that have no ER bodies under normal conditions (Matsushima et al., 2002). The shapes and sizes of ER bodies and i-ER bodies are so similar that the molecular mechanism(s) underlying the formation of these structures may be shared. We treated nai1-1 rosette leaves with 50 μM MeJA and studied the formation of i-ER bodies (Figure 8A). nai1-1 rosette leaves, like GFP-h leaves, developed many i-ER bodies. However, the i-ER bodies in nai1-1 had various shapes and sizes and included irregular-shaped and longer structures, whereas most i-ER bodies in GFP-h leaves had uniform shapes and sizes. Such irregular-shaped i-ER bodies (Figure 8A) were also observed in MeJA-treated GFP-h leaves occasionally (data not shown). Water had no effect on the ER network or the development of i-ER bodies.

Figure 8B shows the expression patterns of NAI1, PYK10, and At3g16420 genes in water-treated, MeJA-treated, and MeJA plus ethylene–treated leaves. MeJA and ethylene has an antagonistic effect on the induction of i-ER bodies (Matsushima...
et al., 2002). MeJA treatment induced the expression of NAI1, PYK10, and At3g16420 genes, and this induction was suppressed in the presence of ethylene. The levels of Actin RNA, used as an internal control, were the same in all the treatments. These results showed that expression patterns of NAI1, PYK10, and At3g16420 genes is parallel to the induction pattern of i-ER bodies, suggesting that MeJA-induced NAI1 is needed for the correct formation of i-ER bodies.

**DISCUSSION**

NAI1 Is a bHLH Protein That Regulates the Formation of ER-Derived Structure

In this study, we performed map-based cloning of the NAI1 gene and showed that the nai1-1 mutant has a single nucleotide change in the At2g22770 gene. The hypothesis that At2g22770 is the NAI1 gene is supported by the aberrant splicing of At2g22770 RNA in the nai1-1 mutant (Figure 2), the results of an allelism test between nai1-1 and a T-DNA insertion allele (nai1-2) (Figure 3), and complementation of the nai1 phenotype by transient expression of At2g22770 protein (Figure 4). NAI1 is a bHLH protein that could function as a transcription factor.

bHLH proteins have been well characterized in nonplant eukaryotes, especially in mammalian systems. In these systems, bHLH proteins are important regulatory components in transcriptional networks controlling various processes, from cell proliferation to cell lineage establishments (Grandori et al., 2000; Massari and Murre, 2000). In plants, broad physiological roles of bHLH proteins have been reported, including roles in anthocyanin pigmentation (Goodrich et al., 1992; Spelt et al., 2000), phytochrome signal transduction (Ni et al., 1998; Fairchild et al., 2000), regulation of seed storage protein synthesis (Kawagoe and Murai, 1996), response to stress conditions (Abe et al., 1997; Chinnusamy et al., 2003), and organ development (Heisler et al., 2001). Physiological roles depend on the downstream genes regulated by each bHLH protein.

A transcription factor that is related to the formation of ER-derived structures has been described in maize. Opaque-2 (O2) is a basic Leu zipper-type transcription factor that regulates the expression of α-zein genes, whose product is a major component in protein bodies in maize endosperm (Hartings et al., 1989; Lending and Larkins, 1989; Schmidt et al., 1990, 1992). Therefore, α-zein synthesis is depressed in o2 mutants because of the absence of an upstream transcriptional activator (O2) (Hunter et al., 2002). As a result of the depressed amount of α-zein, protein bodies in o2 remain small (0.1 to 0.3 μm in diameter) compared with the size of wild-type protein bodies (1 to 2 μm in diameter) (Lending and Larkins, 1989; Geetha et al., 1991). We suggest that the absence of ER bodies in nai1 mutants is also attributable to the loss of a transcription factor (NAI1) that functions upstream of a major component (PYK10) (see below).

PYK10 and At3g16420 Genes Are Downstream of NAI1 Regulation

bHLH proteins are known to bind to a consensus hexanucleotide (CANNTG) called an E-box (Ferre-D’Amare and Burley, 1995). The basic region in the bHLH domain determines the DNA binding activity. In mammalian bHLH proteins, the analysis of crystal structures has shown that the Glu residue (arrow in Figure 1C) is critical because it contacts the first CA in the E-box (Ferre-D’Amare et al., 1993; Ma et al., 1994). This Glu residue is also conserved in NAI1 (Glu-137). Therefore, downstream genes of NAI1 may have E-box sequences in their promoter region. Because expression of PYK10 and At3g16420 genes were downregulated in nai1 mutants (Figure 6B), these genes must be
downstream of NAI1. PYK10 has five E-box sequences in its promoter region (CAACTG at 805 to 810 bp, CATTGT at 881 to 886 bp, CATG at 977 to 982 bp, CATG at 986 to 991 bp, and CATATG at 1350 to 1355 bp upstream of the putative first ATG). The PYK10 promoter region has been investigated through a promoter-deletion analysis using a β-glucuronidase reporter gene (Nitz et al., 2001). β-Glucuronidase activity was found to be 8 times higher when the promoter contained the 1457-bp region upstream of the putative start codon (construct C described in Nitz et al., 2001) than when it contained the 797-bp region upstream of the putative start codon (construct B described in Nitz et al., 2001). The former promoter contains the E-box sequences described above, whereas the latter does not. This result suggests that the E-box sequences in the PYK10 promoter have a strong effect on the expression of PYK10. It is possible that some or all E-box sequences of PYK10 are directly recognized by NAI1. The At3g16420 gene has three E-box sequences in the 2000-bp region upstream from the putative translation start site. The effectiveness of these E-box sequences has not been studied. Proteomic analysis of seed germination has shown that the relative abundance of PYK10 and At3g16420 proteins are most increased (>200-fold) just after germination (Gallardo et al., 2001). It is very likely that both genes are regulated by a common factor. MeJA induced expression of NAI1, PYK10, and At3g16420 genes, and ethylene suppressed the induction (Figure 8B). The common expression pattern of these genes is consistent with the idea that PYK10 and At3g16420 genes are regulated by a common factor, NAI1.

Physiological Role of At3g16420 Protein

At3g16420 protein consists of two repeated regions (At3g16420-N and At3g16420-C in Figure 9A). Each of these regions is homologous to the α-chain of jacalin, a carbohydrate binding protein (lectin) isolated from jackfruit (Artocarpus integrifolia) (Yang and Czapla, 1993). The α-chain of jacalin has been shown to bind IgA1 from human serum, specifically through the oligosaccharides of the IgA1 hinge region (Skea et al., 1988; Blewenga et al., 1989; Sankaranarayanan et al., 1996). Myrosinase binding protein 70p (MBP70p, Brassica napus) has three jacalin-homologous regions (repeats 1 to 3 in Figure 9A) (Geshi and Brandt, 1998). At3g16420 protein and MBP70p are homologous sharing the jacalin-homologous regions. These sequences are aligned in Figure 9B.

MBPs are known to form large complexes (250 to 1000 kD) with myrosinase (Lenman et al., 1990; Falk et al., 1995; Eriksson et al., 2002). Myrosinase is a β-glucosidase responsible for the hydrolysis of glucosinolates, which are secondary metabolites found mainly in the Brassicaceae family (Bones and Rossiter, 1996; Rask et al., 2000). MBPs have lectin activity (Taipalensuu et al., 1997; Geshi and Brandt, 1998), so oligosaccharides of glycosylated myrosinase have been suggested to be targets of MBPs. PYK10 is also glycosylated with three high-mannose oligosaccharides (Matsushima et al., 2003a). Therefore, it is possible that At3g16420 protein forms complexes with glycosylated PYK10.
Immuno-gold labeling shows that MBP70p is specifically localized to the luminal side of protein-body-like structures and vacuoles, although a cleavable ER signal peptide was not found at the N terminus of MBP70p (Geshi and Brandt, 1998). Some mammalian proteins are known to be secreted without cleavage of a signal peptide (Palmier et al., 1978; Ye et al., 1988). MBP70p has been suggested to enter the secretary pathway by a mechanism that does not need a cleavable signal peptide (Geshi and Brandt, 1998). Hydropathy analysis shows that At3g16420 protein also has no cleavable signal peptides and no transmembrane domains (data not shown). Further studies are needed to determine the subcellular localization of At3g16420 protein.

Many plant lectins serve as natural plant protectants against herbivores (Chrispeels and Raikhel, 1991; Peumans and Van Damme, 1995a, 1995b). Lectins inhibit growth and increase mortality of insect larvae through binding to gut epithelial cells and/or to the peritrophic membrane (Gatehouse et al., 1984; Eisemann et al., 1994). Jacalin inhibits the growth of larval Southern corn rootworm (Diabrotica undecimpunctata howardi) when applied to artificial diets (Czapla and Lang, 1990). We previously suggested that the biological function of ER bodies and PYK10 is related to plant defense against herbivores and pathogens (Matsushima et al., 2002, 2003a, 2003b). It is possible that At3g16420 protein participates in the ER body-mediated defense systems.

PYK10 Exhibits Both β-D-Glucosidase and β-D-Fucosidase Activities

4-MU β-D-glucopyranoside is a general substrate for a broad range of β-D-glucosidases (Dharmawardhana et al., 1995; Esen and Blanchard, 2000; Kim et al., 2000). Therefore, the results in Figure 7B suggest that PYK10 is a major β-D-glucosidase in Arabidopsis roots. In addition to the β-D-glucosidase activity, we found PYK10 also exhibited β-D-fucosidase activity (Figures 7C and 7D). In the presence of anti-PYK10 antibodies, the β-D-fucosidase activity was reduced by 64 to 69 pmol 4-MU/s/mg protein (Figure 7D), whereas β-D-glucosidase activity was reduced by 24 to 26 pmol 4-MU/s/mg protein (Figure 7B). This indicates that PYK10 hydrolyzes the β-D-fucosidic linkage more effectively than the β-D-glucosidic linkage.

Fucosic residues exist as α-L-fucose in oligosaccharides of cell walls and glycosylated proteins in plant cells (Staudacher et al., 1999; Reiter, 2002). α-L-Fucosidase hydrolyzes α-L-fucosidic linkages (Augur et al., 1993; Torre et al., 2002). However, little is known about the in vivo roles of β-D-fucosidase and β-D-fucosic. Some β-D-glucosidases hydrolyze β-D-fucosidic linkages (Eksittikul and Chulavatnatol, 1988; Babcock and Esen, 1994; Srismosap et al., 1996). The β-D-fucosidase activity of PYK10 may be mainly attributable to the close structural similarity of fucose and glucose.

METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotype Col, Landsberg erecta, and Ws were used as wild-type plants. We also used GFP-h and nai1-1 plants. GFP-h is an Arabidopsis (ecotype Col) transformed with a Pro35S-SP-GFP-HDEL gene encoding SP-GFP-HDEL, which is composed of the signal peptide of pumpkin (Cucurbita maxima) 2S albumin, and GFP followed by an ER-retention signal, HDEL (Mitsuhashi et al., 2000; Hayashi et al., 2001). nai1-1 is an ER body–deficient mutant isolated from mutagenized GFP-h plants (Mitsuhashi et al., 2003a). We also used a T-DNA insertion line (ecotype Ws), named nai1-2 (CV9), in which a T-DNA was inserted into At2g22770 gene. nai1-2 was isolated from the Versailles collection of T-DNA insertion mutants (Samson et al., 2002). Seeds of Arabidopsis were surface sterilized and then sown on soil or onto 0.5% (w/v) Gellan Gum (Wako, Tokyo, Japan) with MS medium (Wako) and 1.5% (w/v) sucrose and were grown at 22°C under continuous light conditions.

Mapping of the NAI1 Locus

To determine to map position of the NAI1 locus, nai1-1 and the wild-type Landsberg erecta plants were crossed. F2 seeds were obtained by self-fertilization of the F1 plants. Six- or seven-day-old seedlings of the F2 progeny were examined with a fluorescence microscope to select nai1-1 mutant plants. The genomic DNA of these nai1-1 mutant plants was individually isolated and analyzed using a combination of cleaved amplified polymorphic sequence markers and simple sequence length polymorphism markers (Konieczny and Ausubel, 1993; Bell and Ecker, 1994) with data obtained from the Arabidopsis Information Resource (http://www.arabidopsis.org). Primers and enzymes used for molecular markers are as follows. T922-2: 5’-ATGCTAGAGCTATTCGGAGT-3’ and 5’-AGATTATCCTCTTCTGGCATCAA-3’; Sfcl; T922-4: 5’-GCTGGAATCTGGAATGTCACT-3’ and 5’-GTACCAACCACAATCTACAGAT-3’. 5’-CTAGAAGGTTCCGAGAATG-3’ and 5’-CCGTGCAAACAAATAAGACAGACA-3’; T30L20-3: 5’-TTCAGCGCCGT-3’ and 5’-TGTGTTACCCGACAGCATAGG-3’. Acc; T30L20-4: 5’-CTTACTCCTGCTCGTCTG-3’ and 5’-CCACAATCTGTTCTTCACTG-3’. 5’-TCAAGATGGAGGCT-3’ and 5’-TCTTACCCGGCAATGCAAA-3’. Ndel.

Determination of the Transcriptional Start Site and 3' Untranslated Region of the At2g22770 Gene

Transcriptional start site analysis based on the capping structure of mRNA was conducted using an Arabidopsis Cap Site cDNA dT kit (Nippon Gene, Toyama, Japan) according to the manufacturer’s instructions. We used 5’-TCCGAAAAGAGAACAGAGAGA-3’ and 5’-CCACCTTCTGTCCATGCTGGTAC-3’ as gene-specific primers for initial PCR and nested PCR, respectively. The first PCR conditions were as follows: 95°C for 2 min; 40 cycles of 95°C for 30 s, 59°C for 30 s, and 72°C for 45 s; and a final elongation step of 2 min at 72°C. The second PCR conditions were as follows: 95°C for 2 min; 30 cycles of 95°C for 30 s, 59°C for 30 s, and 72°C for 30 s; and a final elongation step of 2 min at 72°C. The second PCR gave two products with different sizes. These products were subcloned into pT7Blue T-vector (Novagen, Madison, WI) and subjected to sequencing. The 3’ untranslated region of At2g22770 gene was determined by sequencing of an EST clone (AV527715) obtained from Kazusa DNA Research Institute (Kisarazu, Chiba, Japan).

Laser Scanning Confocal Microscopy

Fluorescent proteins were viewed with a laser scanning confocal microscope (LSM510; Carl Zeiss, Jena, Germany). For imaging GFP (Figure 3B), argon laser excitation lines of 488 nm were used with HFT 488 (Carl Zeiss) as a dichroic beam splitter and a 505/550-nm bandpass filter. For imaging GFP and mRFP1 (Figure 4), argon laser excitation lines of 488 nm for GFP and helium-neon laser lines of 543 nm for mRFP1 were
used with line switching using the multitrack facility of the microscope. Fluorescence was detected using HFT 488/543 (Carl Zeiss) as a main dichroic beam splitter and NFT 545 (Carl Zeiss) as a secondary dichroic beam splitter. A 505/530-nm bandpass filter for GFP and a 560/615-nm bandpass filter for mRFP1 were used. The fluorescent images were analyzed with LSM 5 (Carl Zeiss) and Adobe Photoshop 5.5 (Adobe Systems, Tokyo, Japan).

Specific Antibodies
To prepare anti-At3g16420 antibodies, a peptide derived from At3g16420 protein, CKNGOPEGAPLRGTKG, was chemically synthesized with a peptide synthesizer (model 431A; Applied Biosystems, Tokyo, Japan). Cross-linking of the peptide with BSA and immunization of a rabbit were conducted as described previously (Matsushima et al., 2003a). We also used antibodies against each of GFP (Clontech, Palo Alto, CA), BiP (amino acids 228 to 246), and PYK10 [anti-PYK10(IM) antibody] that we had raised previously (Matsushima et al., 2003a).

Immunoblot Analysis
Extracts were prepared from 7-d-old GFP-h, nai1-1, Ws, nai1-2, F1 progeny of GFP-h × nai1-2, and F1 progeny of nai1-1 × nai1-2. A whole seedling was homogenized in 75 µL of extraction buffer (50 mM Tris-HCl, pH 6.8, 2% [w/v] SDS, 10% [v/v] glycerol, and 5% [v/v] 2-mercaptoethanol). The extracts (8 µL) were subjected to SDS-PAGE. After SDS-PAGE, proteins were transferred electrophoretically to a polyvinylidene difluoride membrane ( Immobilon-P; Millipore, Billerica, MA). The membrane was thoroughly dried for blocking and then incubated in Tris-buffered saline, pH 7.5, plus 0.05% (v/v) Tween 20 and antibodies for 1 h. Dilutions of antibodies were as follows: anti-PYK10(IM) 1:15,000 (v/v), anti-GFP 1:30,000, anti-BIP 1:30,000, and anti-At3g16420 (1:5000). Horse serum peroxidase-conjugated goat antibodies against rabbit IgG (Pierce, Rockford, IL) were diluted (1:5000) to be used as second antibodies. Immunodetection was performed with an ECL kit (Amersham Biosciences, Buckinghamshire, UK).

Plasmid Construction
To construct mRFP1/pBI221 (Figure 4B), a DNA fragment was produced by PCR amplification using mRFP1 in pRETS (Campbell et al., 2002) as template and primers 5'-GCTCGAGATGCGGGTTCTCATCATCATC-3' and 5'-CGAGCTTCTAGGCGCGTGCAGTGCGGC-3'. The fragment produced from this amplification was inserted into Xhol and SacI sites of pBI221 (Clontech).

To construct At2g22770/pBI221, At2g22770 cDNA was produced by PCR using the EST clone as template and primers 5'-GGATGATTCAAGCTTTATG-3' and 5'-TTGGATCCTTATTCAGCTAAC-3'. The amplified fragment was then inserted into the Xhol-BamHI site of SP-GFP-2SC/pBI221 (Tamura et al., 2003).

To create mRFP1-At2g22770/pBI221 (Figure 4A), a HindIII-EcoRI (blunted with Klenow) fragment of mRFP1/pBI221 that contains the 3SS promoter of Cauliflower mosaic virus, the sequence encoding mRFP1, and nopaline synthase terminator were inserted into the SphI site (blunted) of At2g22770/pBI221.

Biologic Transformation
Seven-day-old nai1-1 seedlings were bombarded with 1-µm gold particles coated with mRFP1/pBI221 and mRFP1-At2g22770/pBI221 using a Helios gene gun system (Bio-Rad Laboratories, Hercules, CA) based on the manufacturer’s protocol. The bombarded samples were kept in darkness at 22°C for 48 to 53 h and observed with the laser scanning confocal microscope.

Preparation of P1 Fraction
Six-day-old GFP-h and nai1-1 seedlings (1.3 g) were chopped with a razor blade in a Petri dish on ice in 3.9 mL of chopping buffer that contained 50 mM Hepes-NaOH, pH 7.5, and 400 mM sucrose. The homogenates were filtered through cheese cloth and centrifuged at 1000g at 4°C for 20 min. The pellet was resuspended with 400 µL of 20 mM sodium phosphate, pH 7.0, and sonicated using a sonicator (Sonifier 250D; Branson, Danbury, CT, USA). The supernatant was used for two-dimensional electrophoresis.

Two-Dimensional Electrophoresis
The P1 fractions obtained from GFP-h and nai1-1 seedlings were subjected to two-dimensional electrophoresis. Isoelectric focusing was conducted using dry polyacrylamide gel strips (Immobiline DryStrip, pH 3 to 10, 13 cm; Amersham Biosciences, Uppsala, Sweden) with an immobilized pH gradient from 3 to 10 at 20°C. Strips were rehydrated with 220 µL of reswelling buffer (7 M urea, 2 M thiourea, 2% [v/v] 3-[3-chloro-2-hydroxypropyl]dimethylammonio]-1-propanesulfonic acid, 1.2% [v/v] Destreak reagent (Amersham Biosciences), 0.5% [v/v] immobilized pH gradient buffer, pH 3 to 10 [Amersham Biosciences], and 0.005% [w/v] bromophenol blue) and 30 µL of each P1 fraction for 1 h. Isoelectric focusing was performed at 30 V for 11 h, 500 V for 3 h, 1000 V for 2 h, and 8000 V for 50,000 Vh in an Etan IPGphor isolectric focusing system (Amersham Biosciences). Strips were equilibrated by SDS equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% [v/v] glycerol, and 2% [w/v] SDS) with 1% (w/v) DTT for 15 min and then with 2.5% (w/v) iodoacetamide for 15 min. Equilibrated gel strips were placed on a 12.5% (w/v) polyacrylamide gel, and SDS-PAGE was performed.

Mass Spectrometry
The gel was treated with deamplolyte buffer (50% [v/v] methanol and 7% [v/v] acetic acid) for 12 h. Silver staining was performed by the method of Yan et al. (2000). Spots specific to the GFP-h P1 fraction were excised from the silver-stained gel, and destaining was performed by methods of Gharahdaghi et al. (1999). The sample was digested with 0.1 µg of trypsin (Promega, Tokyo, Japan) in 20 µL of 0.2 M ammonium bicarbonate, pH 8.0, for 12 h at 37°C. Peptides were extracted from gel slices by 20 µL of 5% (v/v) trifluoroacetic acid and 50% (v/v) acetonitrile for 30 min twice, and the solution of extracted peptides was dried using an evaporator. The dried sample was reconstituted by adding 3 µL of 0.1% trifluoroacetic acid and 50% acetonitrile and gently pipetting up and down to dissolve the extracted peptides. The peptides were purified and concentrated with reverse-phase media (C18 ZipTips; Millipore). The peptides and 150 ng of α-cyano-4-hydroxy-cinnamic acid (Bruker Daltonics, Bremen, Germany) were mixed and subjected to peptide mass fingerprinting analysis by matrix-assisted laser-desorption ionization time of flight mass spectrometry (REFLEX III; Bruker Daltonics). Proteins were identified by searching the MASCOT search engine (http://www.matrixscience.com/). Protein identity of spot number 4 was further confirmed from post source decay spectra. The searches with the post source decay data were conducted using the MASCOT search engine. Protein identities of spot numbers 1 to 3 were confirmed by the N-terminal amino acid sequence analysis as described previously (Matsushima et al., 2003a).
RT-PCR Analyses

Total RNA was isolated from 6-d-old Col, GFP-h, nai1-1, Ws, and nai1-2 seedlings using an RNeasy plant mini kit (Qiagen, Valencia, CA). One microgram of total RNA was treated with DNAse (Invitrogen, Carlsbad, CA) and subjected to first-strand cDNA synthesis using SuperScriptII reverse transcriptase (Invitrogen) and oligo(dT)12-18 primer (Invitrogen). Chemical Treatment

The supernatants were filtrated through a 5-μm protein filter and incubated in 100 mM sodium phosphate, pH 7.0, for 10 min at 35°C. The filtrates containing 3-ß-D-glucosidase and preimmune serum using a HiTrap protein A HP column (Amersham Biosciences). The filtrates were incubated in 100 mM sodium phosphate, pH 7.0, together with 25-μg protein were incubated in 100 mM sodium phosphate, pH 7.0, for 10 min at 35°C (total 198 μL). Two microfilters of 100 mM substrate was added to the solution and incubated at 35°C. The fluorescence intensity was measured using GENios (TECAN, Mannedorf, Switzerland) for kinetic analysis. The PCR products were separated by 1.0% agarose gel electrophoresis or analyzed by a capillary electrophoresis (Agilent 2100 Bioanalyzer; Agilent Technologies, Waldbronn, Germany).

Assay of ß-D-Glucosidase and ß-D-Fucosidase Activities

For MeJA treatment, rosette leaves of 16- to 17-d-old plants were floated on 50 μM MeJA solution and incubated at 22°C under continuous light conditions. As a negative control, water was used instead of MeJA. The rosette leaves were inspected with a laser scanning confocal microscope at 100 μM solution of the substrates and monitored as described above.

Chemical Treatment

For MeJA treatment, rosette leaves of 16- to 17-d-old plants were floated on 50 μM MeJA solution and incubated at 22°C under continuous light conditions. As a negative control, water was used instead of MeJA. The rosette leaves were inspected with a laser scanning confocal microscope at 34 to 36 h after the treatments.

To study gene expressions, RT-PCR analyses were performed with chemically treated rosette leaves of 16-d-old plants. For treatment of MeJA and ethylene simultaneously, the floating leaves were transferred to an airtight box containing 20 μL/L of ethylene gas. The procedures of chemical treatments were described above except that the treatment time was 12 h. The procedures of RT-PCR were the same as described above.

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Sequence data from this article have been deposited with the EMBL/GenBank data libraries under the following accession numbers: AC006340 (BAC clone T9122), AC005617 (BAC clone T30L20), AC004786 (BAC clone T20K9), O80536 (Pf3, At1g09530), Q39204 (AtMYC2, At2g32640), AA25928 (AN1), AA32663 (DEL), T10861 (P51), P01106 (c-myc), P25912 (MAX), CABS50792 (PYK10, At3g09260), Q96292 (Actin, At3g18780), 1UGX (α-chain of jacalin), T08080 (MBP 70p), AV527715 (an EST clone of At2g22770 gene), and NP_850594 (Atg16420).

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NAII Gene Encodes a Basic-Helix-Loop-Helix–Type Putative Transcription Factor That Regulates the Formation of an Endoplasmic Reticulum-Derived Structure, the ER Body
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