Endoplasmic Reticulum Body–Related Gene Expression in Different Root Zones of Arabidopsis Isolated by Laser-Assisted Microdissection

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Abstract
Endoplasmic reticulum (ER) bodies are important organelles for root defense. However, little is known regarding the genetic control of their formation in root tissues. In the present study, Arabidopsis thaliana (L.) Heynh. roots were dissected using laser-assisted microdissection (LAM) with minimal sample preparation (no fixation or embedding steps) and the expression of genes associated with ER body formation and function was assessed by real-time quantitative reverse-transcription polymerase chain reaction (RT-qRT-PCR) in the presence and absence of the defense phytohormone methyl jasmonate (MeJA). Zones of interest were identified in plants overexpressing a SP-GFP-HDEL fluorescent construct; these being the root cap zone, meristematic zone, elongation zone, and differentiation zone. Given their role in ER body formation, the expression of the genes NAI1, NAI2, BGLU21, BGLU22, and BGLU23 was evaluated in the whole root and in the four dissected root zones using RT-qRT-PCR. Our data show that the expression level of all five genes differs in a root-zone-specific manner in untreated roots. They also reveal that all of them are overexpressed in response to MeJA with the two NAI genes being the most highly overexpressed in the EZ. Finally, the NAI1 gene, encoding for a transcription factor that regulates the expression of the four other genes, is the first to respond to MeJA, supporting its central role in ER body formation and function in root defense.

Core Ideas
• ER body–related genes are specifically regulated in different root zones
• Methyl jasmonate induces ER body formation in Arabidopsis root
• Methyl jasmonate induces the overexpression of ER body–related genes in the root
• Primary root zones can be easily microdissected without any chemical treatment

PLANTS are constantly confronted with a range of biotic and abiotic stresses, and they respond using a variety of strategies to ensure maintenance of homeostasis and survival. Such responses begin by the perception of the stress source (Jones and Dangl, 2006; Boller and Felix, 2009), this is followed by an appropriate hormone-related induced response (Verhage et al., 2010), which, in turn, controls the expression of the appropriate...
modulating genes (van Loon and van Strien, 1999). In the case of biotic stresses, this involves upregulating and downregulating of defense-related genes.

The primary root is a complex organ that is composed of different tissue regions with each of these zones contributing a crucial role in root growth and survival. At the distal tip of the root are the border-like cells (BLCs), which are layers of cells detaching from the root cap that protect the root meristem (Vicré et al., 2005; Driouich et al., 2007, 2010, 2013; Plancot et al., 2013). Above these BLCs is the root cap, which is composed of the lateral root cap and the columella cells (Bennett and Scheres, 2010). Above it, there is the quiescent center surrounded by the stem cells (Scheres et al., 2002), which are the primary site for root growth. The root cap plus BLCs, named as the root cap zone (CZ) in this study, facilitates first contact at the plant–soil interface by secreting exudates to modify the rhizosphere (Jones et al., 2009). Growth is also directed from the root tip into the proximal meristematic zone, where significant cell division occurs (Scheres et al., 2002; Benfey et al., 2010), then cells begin to elongate with lower rate of cell division in the distal meristematic zone, also known as the transition zone (Verbel et al., 2006; Benfey et al., 2010). In this work, both proximal and distal meristematic zones were collected together as the whole meristematic zone (MZ). After several divisions, meristematic cells undergo fast elongation forming the elongation zone (EZ). Finally, the EZ gives rise to the differentiation zone (DZ) accompanied by additional elongation and the development of root hairs to form mature root cells (Verbel et al., 2006).

Brassicaceae species, such as Arabidopsis, have evolved a unique organelle that forms on the ER and called the ER body (Hayashi et al., 2001). The ER bodies are spindle-shaped structures of 5 to 10 μm in size that accumulate β-glucosidases (BGLU) BGLU21, BGLU22, and BGLU23/PYK10 (Nakano et al., 2014). These BGLU proteins are able to form a multiprotein complex with high enzymatic activity named the PYK10 complex. The formation of the PYK10 complex occurs when cells and endomembranes are collapsed or are disrupted (Nagano et al., 2005, 2008). This complex hydrolyzes scopolin into scopoletin, a phytoalexin biologically active against pathogens (Peterson et al., 2003; Ahn et al., 2010; Nakano et al., 2014). The ER bodies can be easily observed in an Arabidopsis transgenic line expressing ER-localized green fluorescent protein (SP-GFP-HDEL; Köhler, 1998; Hayashi et al., 2001). They are distributed in the epidermal cells of cotyledons, hypocotyls, and roots but absent in rosette leaves in Arabidopsis (Matsushima et al., 2002). Furthermore, they are present in large quantities in epidermal and cortical cells of radish (Raphanus sativus L.) roots (Bonnet and Newcomb, 1965; Gottle et al., 2015) and garden cress (Lepidium sativum L.) (Iversen and Flood, 1969).

ER body formation events are under the control of the transcription factor NAI1 that is responsible for BGLU21, BGLU22, and BGLU23/PYK10 and NAI2 expression. NAI2 is a protein believed to physically interact with BGLU23 thus playing a major role in producing the ER bodies on the ER network (Matsushima et al., 2004; Yamada et al., 2008, 2011).

ER bodies are currently believed to be involved in signaling events associated with response to external cues such as wounding and infection (Matsushima et al., 2002; Nagano et al., 2005, 2008; Nakano et al., 2014). Indeed, it is well known that the colonization of Arabidopsis roots by the endophytic fungus Piriformospora indica has beneficial effects on nutrient uptake, plant development, seed production, and provides better resistance against toxins and plant pathogens (Schäfer et al., 2007). It is, however, less well known that Arabidopsis mutants deprived of ER bodies, such as nail and pyk10 mutants, are overcolonized, lose those benefits, and are disrupted by this seemingly beneficial fungus now turned semipathogenic (Sherameti et al., 2008). This leads to the conclusion that ER bodies are required to regulate and control the root colonization by microbes and have a clear role in defense and plant immunity. Furthermore, several studies have shown that ER bodies are extremely sensitive to wounding as well as MeJA treatment (Matsushima et al., 2002). Methyl jasmonate is the volatile form of jasmonic acid, a well-known phytohormone involved in the activation of immune responses to most necrotrophic pathogens, insects, and herbivores (McConn et al., 1997; Ballaré, 2011). As an example, it was recently shown that MeJA treatment on maize (Zea mays L.) leaves induced immune responses and overexpression of defense genes such as a BGLU precursor and the pathogenesis-related protein 1 and increased the production of toxic proteins that impact the larva and pupa of the Asian corn borer, Ostrinia furnacalis (Zhang et al., 2015). Indeed, wounding and MeJA treatment were able to induce the formation of new ER bodies in rosette leaves while unwounded and untreated control leaves did not form ER bodies (Matsushima et al., 2002; Ogasawara et al., 2009). It was also observed that MeJA treatment induced fusion between several ER bodies in radish roots resulting on the formation of very long ER bodies (Gottle et al., 2015). However, little is known regarding ER body-related gene expression in roots and certainly almost nothing at the level of different root tissues.

Nonetheless, several studies have shown that different zones of the root show variability in their susceptibility to attack by pathogens. For instance, when infected by the oomycete Aphanomyces euteiches, the pea root (Pisum sativum L.) produces high concentration of the phytoalexin pisatin in the EZ and the root cap, whereas pisatin production was low in the EZ. Consequently, the oomycete preferentially infects the EZ, whereas the root cap and border cells remain free of zoospores in the early stages of infection (Cannesan et al., 2011). It is, therefore, predicted that the four major root zones (CZ, MZ, EZ, and DZ) show differences in gene expression during development and when challenged with pathogens or elicitors. Furthermore, we have previously shown that ER bodies are not found uniformly throughout the root.
and are enriched in certain tissues and root zones (Gotté et al., 2015). Hence, it is of great interest to study the ER body–related gene expression in the different root zones in response to treatment with compounds able to induce defense mechanisms.

LAM has emerged as a novel, powerful tool that uses a laser beam to dissect and isolate specific tissues from different organs (e.g., floral meristems) (Mantegazza et al., 2014) and has even been shown to be able to excise as many as 40 different cell types dissected from rice (Oryza sativa L.) roots and shoots (Jiao et al., 2009). Improvement in the technology and modulating the power and precision of the laser beam has allowed for the effective cutting through hard and thick tissues such as seeds (Thiel et al., 2011). These improvements have recently facilitated the ability to isolate RNA from different tissues for targeted gene expression studies or transcriptomic analyses (Gautam and Sarkar, 2014). Proteomic and metabolomic approaches have also been demonstrated on the dissected tissue samples (Gautam and Sarkar, 2014). A major limitation of the LAM approaches thus far has been the dependence of chemical fixation and embedding procedures (Gautam and Sarkar, 2014). The approach followed involves time-consuming dehydration, embedding, and microtome sectioning steps before the samples are finally microdissected (Gautam and Sarkar, 2014).

The aim of the present study is to further understand how the formation and functioning of ER bodies is regulated under basal and defense-induced conditions. Here, we report on the differential expression of five genes (NAI1, NAI2, BGLU21, BGLU22, and BGLU23) involved in ER body formation and function in different zones of the root in response to the defense phytohormone MeJA. This was achieved by using an easy and reproducible in vivo method to dissect the four major distinct root zones—CZ, MZ, EZ, and DZ—from living Arabidopsis seedlings without any significant sample preparation. This methodology was optimized and enabled us to show that there is a root-zone-dependent control of the five ER body–related genes under basal and MeJA induced conditions using a targeted RT-qRT-PCR approach. The results also provide evidence supporting that NAI1 is a key gene for ER body formation in root under stressed conditions.

Materials and Methods

Plant Material and Growth Conditions

Wild-type A. thaliana (ecotype Columbia [Col-0]) were used for all the RT-qRT-PCR experiments. Transgenic Arabidopsis SP-GFP-HDEL seeds were used for confocal microscopy (Yamada et al., 2008). Seeds were surface sterilized with 70% (v/v) ethanol for 1 min followed by immersion in 0.87% (v/v) bleach for 1 min. After several washes in sterile distilled water, the seeds were sown onto agar plates (1% [w/v] Bacto Agar) supplemented with Arabidopsis medium (Duchefa Biochemie) (11.83 g L⁻¹) and Ca(NO₃)₂ (2 mM). Plates with seeds were placed vertically in 16-h-day/8-h-night cycle for 11 d at 23°C.

Roots were sprayed with a MeJA solution at a concentration of 50 μM (Matsushima et al., 2002; Gotté et al., 2015) in phosphate buffer (KH₂PO₄ 18 mM and K₂HPO₄ 2 mM, pH 6). The phosphate-buffer-treated roots were used as control when analyzing the MeJA effect. The untreated roots were used as the reference sample condition. The excess solution was allowed to drain off and the plates were incubated in the photoperiodic chamber as above for an appropriate duration.

Observation of Endoplasmic Reticulum Bodies by Confocal Microscopy

Roots expressing the SP-GFP-HDEL were take out gently and mounted on a glass microscope slides in a drop of water and directly examined with a confocal laser scanning microscope (Leica TCS SP2 AOBs; objective, 40× Oil; excitation laser, 488 nm; barrier filter, 495–560 nm; Leica Microsystems, Inc.). Images were processed with ImageJ (National Institutes of Health).

RNA Extraction and Reverse Transcription from Whole Roots over a 24-Hour Time Course

The seedlings were grown under the above-described conditions and treated 1, 3, 6, 12, and 24 h before harvesting the roots. This experiment was conducted on the whole root part. After cutting the aerial parts, ~80 roots (~90 mg) per biological replicate were collected with a piler, put into Lysing matrix D tubes (MP Biomedicals, LLC) and immediately frozen in liquid nitrogen. Each sample is then lysed with FastPrep-24 (MP Biomedicals) in presence of lysis buffer from RNasy Plant Mini Kit (Qiagen) and RNA was subsequently extracted using this kit following the manufacturer’s instructions. Three biological sample replicates were used per time point. The RNA quantities were evaluated with the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc.; http://www.nanodrop.com). Complementary DNAs (cDNAs) were synthesized from 1.7 μg of total RNA using the high-capacity cDNA reverse-transcription kits (Applied Biosystems, Life Technologies, Thermo Fisher Scientific Inc.).

Microdissection of Roots Followed by RNA Extraction and Reverse Transcription

Plants were grown and treated, as described above, 3 to 6 h before excision. Phosphate-buffer-treated roots were used as control and untreated roots were used as reference sample when analyzing the MeJA effect. Each plant was individually carefully removed with pliers from its culture plate and transferred to Leica RNase-free PEN foil slides (Leica Microsystems). A few drops of phosphate buffer were deposited on the aerial part, which was not in contact with the PEN film, to maintain the relative moisture content of the plant during the period of microdissection. A Leica LMD7000 system was used to dissect using the following settings for the DZ and EZ: 5× objective at power 55, aperture 17, speed 10, specimen
balance 27, pulse frequency 420 Hz. Since MZ and CZ get higher number of cells per volume unit, the tissues were denser and microdissection got harder; thus, it required stronger settings: 20× objective at power 55, aperture 12, speed 15, specimen balance 45, and pulse frequency 964 Hz. Thus, two zones from the same root were sequentially microdissected in <2 min and collected in separates tubes as follows: EZ then DZ or CZ then MZ. The distinction between each zone was determined by observation based on Benfey et al. (2010). The microdissected zones were collected into a 0.5-mL RNasefree PCR tube containing RLT lysis buffer (Qiagen) complemented with 1% (v/v) β-mercaptoethanol and with carrier RNA (Qiagen) following the manufacturer’s instructions. Twenty dissected zones represented one biological sample and six biological sample replicates were analyzed. Each biological replicate of 20 dissected zones was collected in one session of microdissection that did not exceed 45 min. The samples were subsequently vortexed for 10 s, spun down, immediately put on ice till the end of the microdissection session, then stored at −20°C within 3 d before RNA extraction. The RNA extraction was performed using the RNeasy Micro Kit (Qiagen) following the manufacturer’s instruction manual. RNA quality estimated by the RNA integrity number (RIN) and quantity were then analyzed with the 2100 Bioanalyzer (Agilent Technologies) tool. Complementary DNAs were synthesized from 3.5 ng of total RNA using the Quantitect Reverse Transcription kit (Qiagen).

Real-Time Quantitative Reverse-Transcription Polymerase Chain Reaction

RT-qRT-PCR was performed using a RT-qRT-PCR ABI PRISM 7500 machine and fast SYBR Green Master Mix (Applied Biosystems). The program used for RT-qRT-PCR was as follows: 20 s at 95°C, 40 cycles of 3 s at 95°C, and 15 s at 95°C, followed by a melt curve of 1 min at 60°C, 15 s at 95°C, and 15 s at 60°C. The gene-specific primers, details in Supplemental Table S1, were synthesized (Eurogentec France SASU).

Quantitative gene expressions were calculated by the efficiency correction method (Pfaffl, 2001; Schmittgen and Livak, 2008), which has been improved using the efficiency correction method (Pfaffl, 2001; Schmitts). The gene-specific primers, details in Supplemental Table S1, were synthesized (Eurogentec France SASU).

The expression ratio (R) of a target gene is calculated based on efficiency of the primers (E), the crossing point (CP) deviation of a sample against a reference sample, and expressed in comparison to several housekeeping genes (hse):

\[ R = \frac{E_{\text{target}}^{\Delta CP_{\text{norm}}(\text{reference sample} - \text{sample})} \times E_{\text{hse}}^{\Delta CP_{\text{norm}}(\text{reference sample} - \text{sample})}}{E_{\text{hse}}^{\Delta CP_{\text{norm}}(\text{reference sample} - \text{sample})}} \]

Expression values obtained from whole roots samples over a 24 h time course were normalized to three housekeeping genes (ACTIN2, GAPDH, and EFI-a). Statistical analyses were performed using the two-way ANOVA with the Sidak’s multiple comparison test (Abdi, 2007).

Expression values obtained from dissected root zones were normalized to two housekeeping genes (ACTIN2 and GAPDH). Statistical significances were calculated with the one-way ANOVA with Bonferroni’s multiple comparison test (Abdi, 2007).

Immunocytochemistry: Immunofluorescent Staining of Endoplasmic Reticulum Bodies

*Arabidopsis thaliana* Col-0 whole plants were fixed for 1 h in 50 mM piperazinediethane sulfonic acid (pH 7), 1 mM CaCl2, paraformaldehyde 4% (v/v), and glutaraldehyde 0.5% (v/v). Plants were washed four times for 10 min each with 50 mM piperazinediethane sulfonic acid (pH 7), 5 mM EGTA, and 2 mM MgSO4 and incubated 20 min at 25°C with enzymes in phosphate buffered saline (PBS; pectolyase Y-23 0.01% (w/v),pectinase 0.6% (v/v), cellulase 1% (v/v). Then plants were rinsed three times for 5 min each with the first rinsing solution plus 10% (v/v) glycerol and 0.5% (v/v) triton X-100. Plants were incubated in frozen methanol (−20°C) for 15 min at −20°C and rehydrated with three baths of 5 min of PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, and 1.5 mM KH2PO4). Aldehydes were saturated with 0.1 M glycine in PBS for 15 min and plants were subsequently washed three times with PBS. Plants were saturated with PBS–Bovine serum albumin (BSA) 1% (w/v). Roots were cut with a razor blade on the distal DZ, allowing for observation of all four root zones. Sectioned roots were disposed on 10-well slides and incubated one night at 4°C with the anti-PYK10 (1M type; Matsushima et al., 2003) antibodies (diluted 1:1000 in PBS-BSA) or with only PBS-BSA (control). After washing with PBS-BSA five times (5 min each), the slides were incubated 2 h and 30 min at 37°C with the secondary antibodies (SIGMA Anti-Rabbit IgG [whole molecule]–TRITC antibody produced in goat IgG fraction of antiserum, buffered aqueous solution [diluted 1:50 in PBS-BSA]). After washing five times for 5 min with PBS-BSA and four times for 5 min with double-distilled water, roots were mounted in antifade solution (Citifluor AF2; Agar Scientific) and examined using an inverted confocal laser scanning microscope (Leica TCS SP2 AOBS; objective, 40× Oil; excitation laser, 561 nm; barrier filter, 570–664 nm). Control experiments were performed by omission of the primary antibody. For each root, Z-stack images were acquired through from the root cap to the DZ. Three-dimensional reconstructions were made and used to count the number of ER bodies in the different zones.

Treatment of Images Prior to Analysis

Autoquant software (MediaCybernetics) using the theoretical pointspread-function (10 iterations) was used to deconvolute the Z-stack images. Three-dimensional volumes were then further analyzed in Imaris (Bitplane).
Counting of Endoplasmic Reticulum Bodies

Counting of ER bodies was performed using Bitplane Imaris software by adding spots automatically on ER bodies using the quality filter. Missing ER bodies and errors related to attribution of points were corrected manually to acquire the most accurate number of ER bodies. The volume of the whole root tissue from each section was measured using the surface tool (surface area detail–grain size level, 1.0 μm; threshold 3). Three plants were used for each condition. The ratio number of ER bodies/volume of root tissue was calculated for each root zone and averaged for the three plants. Statistical analysis was performed using two-way ANOVA analysis with Bonferroni’s multiple comparison test.

Results

Distribution of Fluorescently Tagged (SP-GFP-HDEL) Endoplasmic Reticulum Bodies in the Four Defined Root Zones of Arabidopsis thaliana

The distribution of ER bodies in different root cell types of R. sativus were described previously by Gotté et al. (2015). In this study, we have evaluated the distribution of ER bodies in the different tissue zones of Arabidopsis roots expressing the SP-GFP-HDEL fluorescent protein tags (Fig. 1). The fluorescent ER bodies were observed in the epidermal and cortical cells of the DZ and EZ and in the epidermal cells of the MZ. Furthermore, they were found in the CZ’s columella, lateral root cap, and first layer of BLC. Interestingly, fluorescently tagged ER
Methyl Jasmonate Application at Selected Intervals over 24 Hours following NAI1 body genes, namely, might also change expression patterns of the key ER 2015). Hence, we hypothesized that MeJA treatment of ER bodies (Matsushima et al., 2002; Gotté et al., 2015; Sherameti et al., 2008, Nakano et al., 2008, 2009; Nakano et al., 2014) changes in the number, distribution, and morphol-Previous reports have shown that MeJA induces 6 changes in the ER body pro-duction at a tissue level in the four main root zones: CZ, MZ, EZ, and the DZ within 3 mm after the EZ. We chose to perform a targeted analysis of five important genes (NAI1, NAI2, BGLU21, BGLU22, and BGLU23) that play key roles in the development and function of ER bodies as detailed in Table 1. In Arabidopsis cotyledons, these genes are expressed at basal levels during growth conditions and are not induced neither by local nor systemic wounding (Ogasawara et al., 2009), which may involve the jasmonic acid defense pathway. Therefore, it is interesting to assess their expression in root, underbasal, and MeJA-treated conditions.

Monitoring the Whole Root Expression of NAI1, NAI2, BGLU21, BGLU22, and BGLU23 at Selected Intervals over 24 Hours following Methyl Jasmonate Application

Previous reports have shown that MeJA induces changes in the number, distribution, and morphology of ER bodies (Matsushima et al., 2002; Gotté et al., 2015). Hence, we hypothesized that MeJA treatment might also change expression patterns of the key ER body genes, namely, NAI1, NAI2, BGLU21, BGLU22, and BGLU23, in whole root tissues.

The RT-qRT-PCR expression datasets for each of the five genes at six time intervals (t₀ and 1, 3, 6, 12, and 24 h) following the application of MeJA is shown in Fig. 2. Unsurprisingly, the control conditions (only phosphate buffer added) did not induce significant changes in the gene expression levels when compared with t₀. In contrast, all five ER body–related genes showed strong induction with MeJA treatment, although this was time dependent, with most genes being induced after 3 h, while NAI1 was induced after 1 h of MeJA application (Fig. 2). The maximum levels of overexpression were generally found between 3 and 6 h posttreatment and then slowly decreased, except for BGLU21, where the highest overexpression level was found at 24 h posttreatment (Fig. 2). The early expression of NAI1 at 1 h posttreatment, before BGLU21, BGLU22, BGLU23, and NAI2 at 3 h, is not surprising, as NAI1 is a transcription factor that regulates the expression of the four other genes (Matsushima et al., 2004; Nakano et al., 2014). We also observed that BGLU21 had a markedly different expression profile being overexpressed from 3 h posttreatment to get its maximum at 24 h posttreatment (Fig. 2). These data confirm that MeJA induction of ER body–related genes is relatively short (1–3 h posttreatment) (Fig. 2). Furthermore, to perform microdissection with a view to understand tissue level expression differences in response to a plant defense regulator, such as MeJA, the microdissection should be conducted between 3 and 6 h posttreatment.

Laser-Assisted Microdissection Preparation

LAM has been previously used to dissect tissues or specific cells after chemical fixation followed by tissue embedding. In our conditions, the seedling tissues were exposed to direct LAM procedure (see Materials and Methods section for details) with practically no sample preparation. We optimized the cutting settings of the laser and designed the best dissection area. This procedure obviates the risk of RNA damage by substantially reducing sectioning time. Thus, we have been able to dissect two root zones sequentially in <2 min. Dissected tissues immediately fell into lysis buffer. These conditions allowed for a good preservation of RNA integrity especially in the DZ and in the EZ with RINs of 8.37 ± 0.12 and 8.15 ± 0.13, respectively (Fig. 3A, B, C). However, the RNA quality was less well preserved in the CZ with RINs of 5.29 ± 0.41 and 1.81 ± 0.12, respectively (Fig. 3A, D, E), even with much lower power settings. This could probably be due to the higher ratio of damaged to undamaged cells in these two zones compared with DZ and EZ. Thus, RNA in MZ and CZ suffers more degradation.

Real-Time qRT-PCR Measurements of NAI1, NAI2, BGLU21, BGLU22, and BGLU23 Genes on Laser-Microdissected Tissue from Four Root Zones

After microdissection, samples were processed for RNA extraction followed by reverse transcription to cDNA for RT-qRT-PCR. First, we analyzed the difference of basal expression levels of each of the five genes between

| Table 1 Summary of endoplasmic reticulum (ER) body–related proteins functions. |
|---------------------------|---------------------------|---------------------------|
| Genes        | Nature               | Function in ER bodies |
| BGLU21, AT1G66270 | 3-glucosidase       | Member of PYK10 complex; involved in ER body formation (Nagano et al., 2008, 2009; Nakano et al., 2014) |
| BGLU22, AT1G66280 | 3-glucosidase       | Member of PYK10 complex; involved in ER body formation (Nagano et al., 2008, 2009; Nakano et al., 2014) |
| BGLU23/PYK10, AT3G09260 | 3-glucosidase       | Necessary to ER body formation, member of PYK10 complex, necessary for beneficial interaction with the endophytic fungus Piriformospora indica (Matsushima et al., 2003; Sherameti et al., 2008, Nagano et al., 2008, 2009; Nakano et al., 2014) |
| NAI1, AT2G22770 | Transcription factor | Regulates and leads the ER body formation by controlling the ER body–related genes expression. It also regulates the expression of the other PYK10 complex members. (Matsushima et al., 2004; Nagano et al., 2005, 2008; Nakano et al., 2014) |
| NAI2, AT3G15950 | TSK-associating protein 1 (TSA1)-like | Necessary to ER body formation (Yamada et al., 2008; Nakano et al., 2014) |

bodies appeared to be absent from the quiescent center, the endodermis, and the stele of the root tissue.

Given these differences, it is of interest to understand some of the genetic factors that regulate ER body pro-duction at a tissue level in the four main root zones: CZ, MZ, EZ, and the DZ within 3 mm after the EZ. We chose to perform a targeted analysis of five important genes (NAI1, NAI2, BGLU21, BGLU22, and BGLU23) that play key roles in the development and function of ER bodies as detailed in Table 1. In Arabidopsis cotyledons, these genes are expressed at basal levels during growth conditions and are not induced neither by local nor systemic wounding (Ogasawara et al., 2009), which may involve the jasmonic acid defense pathway. Therefore, it is interesting to assess their expression in root, underbasal, and MeJA-treated conditions.
Since the DZ is the major zone of the whole root, each relative gene expression was calculated using the DZ crossing point’s mean as reference sample (Fig. 4). We found that \textit{NAI1}, \textit{NAI2}, \textit{BGLU22}, and \textit{BGLU23} basal expression patterns all tended to show an increase from the CZ to the DZ. In contrast, \textit{BGLU21} showed highest expression levels in the MZ and EZ and then decreased in the DZ (Fig. 4).

Second, we analyzed the effect of MeJA treatment on the expression levels of all five genes in comparison with the phosphate buffer condition (control) in each of the four root zones. We noticed that all the genes tended to be overexpressed in the four root zones in the presence of MeJA (Fig. 5A). However, the overexpression levels were much lower than levels found in the whole root dataset (Fig. 2, 5A). By subtracting the mean relative gene expression data obtained from each root zone from DZ expression data we can show that \textit{NAI1}, \textit{NAI2}, and \textit{BGLU23} were the most overexpressed in the EZ, lower in the CZ, lower still in the DZ, and finally lowest in the MZ. \textit{BGLU22} overexpression is higher in the DZ than in the EZ and CZ and lowest in the MZ (Fig. 5B). As previously noted, \textit{BGLU21} had a different profile with an overexpression quite linear in the CZ, MZ, and EZ and higher in the DZ (Fig. 5B).

Together, these results suggest that there is a subtle modulation of ER body gene expression in the different root zones under basal growth conditions. This slight differential gene expression control is probably linked to the adjustment of the production of ER body in the appropriate cell types. Furthermore, MeJA treatment induces local overexpression of these genes, albeit at lower levels than expected, which in turn, controls the production of ER bodies in the different tissues. These differences may reflect the capacity of different cell types to cope with external stimuli related to biotic stress.

**Methyl Jasmonate Treatment Impacts the Density of Endoplasmic Reticulum Bodies**

\textit{Arabidopsis thaliana} Col-0 roots treated with phosphate buffer (control) and MeJA were immunolabeled with anti-PYK10 antibodies 72 h after treatment to allow for the formation of ER bodies. Labeled ER bodies were counted in the four root zones (Fig. 6). We show that the density of ER bodies (number per volume) was quite low...
in the CZ, whereas it is the highest in the MZ, as it was observed in our previous work on radish (Gotté et al., 2015), then it was observed that the density decreased in the EZ and again in the DZ (Fig. 6). After MeJA treatment, the density of ER bodies significantly increased in the MZ, whereas it had not significantly changed in the other zones, although it tended to be slightly higher in the EZ (Fig. 6). Statistical analyses using two-way ANOVA indicated that there is a little interaction between the two factors: treatment and root zones \((P = 0.120)\). This suggests that MeJA induces localized and root-zone-specific production of ER bodies. We had not noticed any change in the ER body shape (i.e., morphology) or size in our treated samples vs. the controls.

**DISCUSSION**

To date, most of the studies on ER bodies have been limited to cotyledons and leaves, while almost nothing is known about their regulation and likely diverse functioning in roots.

The present study demonstrates that the expression of several genes involved in ER body formation and functioning is differentially regulated in different root zones in response to the defense phytohormone MeJA. The \(NAI1\) gene encoding for a transcription factor that regulates the expression of the four other genes (i.e., \(NAI2\), \(BGLU21\), \(BGLU22\), and \(BGLU23\)) was the most highly expressed in early times after MeJA treatment, supporting its important role in ER body formation and ER body-mediated defense in root cells. The study also demonstrates an easy and reproducible method to dissect various parts of the root (four zones in this study) using LAM without the need for any significant pretreatment steps. This method permits the isolation of tissue samples for the measurement of gene expression in localized zones of the root.

Roots are crucial organs for the survival of plants with a very different development system and an immune response distinct from that observed in leaves (Balmer et al., 2013). Here, we show that roots expressing the SP-GFP-HDEL fluorescent construct demonstrated differential ER body distribution in the four root zones.
delineated using confocal microscopy imaging (Fig. 1). However, the regulation and the expression of associated genes for ER bodies in roots are largely unknown. In fact, previous studies, mainly conducted on shoots, have provided important information regarding their composition (Matsushima et al., 2003, 2004; Nagano et al., 2008; Yamada et al., 2008; Nakano et al., 2014). It is known that one of the major regulatory and functional molecular components include BGLU23/PYK10 along with NAI2 protein (Matsushima et al., 2003; Yamada et al., 2008). In addition, ER bodies also contain at least two other BGLU, BGLU21 and BGLU22, involved in their formation (Nakano et al., 2014). These four compounds are under the control of the transcription factor NAI1.

Fig. 4. Relative expression of five endoplasmic reticulum (ER) body–related genes in the four root zones in untreated plants. Real-time quantitative reverse-transcription polymerase chain reaction (RT-qRT-PCR) diagram showing the relative expression of ER body–related genes (NAI1, NAI2, BGLU21, BGLU22, and BGLU23) in untreated condition. Each gene expression was calculated for border-like cells plus root cap (CZ), whole meristematic zone (MZ), elongation zone (EZ), and differentiation zone (DZ). The expression values were normalized to the GAPDH and ACTIN2 endogenous housekeeping genes. The relative quantity of each mRNA was calibrated against the DZ crossing point’s mean, which was used as reference sample. Error bars indicate the standard errors of the means. Six biological replicates were used.

Fig. 5. Overexpression of endoplasmic reticulum (ER) body–related genes in the four root zones after methyl jasmonate (MeJA) treatment. (A) Real-time quantitative reverse-transcription polymerase chain reaction (RT-qRT-PCR) diagram showing the relative expression of ER body–related genes (NAI1, NAI2, BGLU21, BGLU22, and BGLU23) in control condition with phosphate buffer (black bars) and after MeJA treatment (gray bars) in the microdissected root zones: border-like cells plus root cap (CZ), whole meristematic zone (MZ), elongation zone (EZ), and differentiation zone (DZ). The expression values were normalized to the GAPDH and ACTIN2 endogenous housekeeping genes. The relative quantity of each mRNA was calibrated against the amount in untreated roots used as reference sample. Asterisks (*) indicate statistical significance: *P < 0.05; **P < 0.01; ***P < 0.001 vs. phosphate buffer attested by the Bonferroni’s multiple comparison test. Error bars indicate the standard errors of the means. Six biological replicates were used. (B) Changes in the overexpression of the ER body–related genes along the four root zones. Values were obtained by subtracting the genes mean relative expression in MeJA condition of each zone with the DZ. Each zone is represented on a drawing (adapted from Benfey et al., 2010) showing the microdissection sites (red bars).
whereas the other genes did not exceed an overexpression (9× than control) from ~1 h posttreatment, MeJA initially resulted in the NAI1 discussed by Balmer et al. (2013). Three-dimensional reconstructions were used to determine the number of ER bodies. The results are given as a number of ER bodies per cubic millimeter of root tissue in the four root zones. Asterisk (*) indicates statistical significance: * P < 0.05 vs. phosphate buffer control attested by the Bonferroni’s multiple comparison test.

(Nakano et al., 2014). A study conducted on Arabidopsis cotyledons has shown that BGLU21, BGLU22, and BGLU23 expression were not affected by local or systemic wounding events (Ogasawara et al., 2009). It is well known that jasmonic acid is mainly involved in wound responses and that the phytohormone MeJA induces the production of new ER bodies (McConn et al., 1997; Matsushima et al., 2002; Gotté et al., 2015). Thus, these three genes were not affected by wounds (Ogasawara et al., 2009) and so probably neither by MeJA treatment in cotyledons. Interestingly, in roots, we showed that the genes encoding the NAI1, NAI2, BGLU21, BGLU22, and BGLU23 proteins were strongly overexpressed after a 50 μM MeJA treatment (Fig. 2), which clearly demonstrates that hormonal regulation related to ER body formation is different between roots and shoots (leaves, cotyledons) as discussed by Balmer et al. (2013).

From the whole-root perspective, the application of MeJA initially resulted in the NAI1 gene being highly overexpressed (9× than control) from ~1 h posttreatment, whereas the other genes did not exceed an overexpression of four times at this same stage. All five genes then increased to their maximum overexpression levels seemingly between 3 and 6 h posttreatment. This makes sense, since the BGLU21, BGLU22, BGLU23, and NAI2 genes are known to be expressed in a NAI1-dependent manner (Nakano et al., 2014). Thus, NAI1 expression is probably slightly repressed in basal growth conditions, but this constraint is released when MeJA is perceived (Ballaré, 2011). NAI1 is then able to induce a strong and proportional overexpression of its target genes as suggested by Ballaré (2011).

In comparison, very little is known about the local control of these genes at the root tissue level under basal growth conditions and after induction with MeJA. Hence, it is of considerable interest to understand their regulation in the four different root zones defined (e.g., DZ, EZ, MZ, and CZ). To achieve this, the application of LAM has proven to be an effective tool to excise tissues very rapidly from the four root zones. Indeed, we have developed a procedure with minimal sample preparation to overcome molecular alteration resulting from chemical preparation of tissue (e.g., RNA degradation by cross-linking with formalin) (Farragher et al., 2008). In this study, we demonstrate that our simpler technique can be used to isolate different root tissue zones without the need for harsh chemical treatments and long tissue preparation times. Therefore, we were able to dissect two zones sequentially in a very limited amount of time (i.e., <2 min). Our method combines rapid excision and isolation without any significant tissue preparation, allowing sampling of living plants for subsequent analysis for gene expression. Although this method gave excellent RNA quality in the DZ and EZ with RIN over 8 (Fig. 3A, B, C), the quality was lower in the MZ and CZ with RIN values of 5.29 ± 0.41 (Fig. 3D) and 1.81 ± 0.12 (Fig. 3E), respectively. As MZ and CZ are significantly smaller zones than their EZ and DZ counterparts, they would have smaller but more numerous cells for the same volume of tissue. Consequently, the ratio of wounded and damaged cells (RNA degraded) to undamaged cells may be higher in MZ and CZ, thus the RNA could be in a more damaged state in these two zones. However, as the amplicon sizes are small (<150 bp), it is still possible to perform reliable RT-qRT-PCR analysis (Kashafer et al., 2013). The RT-qRT-PCR results indicate that the relative expression of NAI1, NAI2, BGLU22, and BGLU23 in untreated conditions all tended to increase from the CZ to the DZ (Fig. 4), whereas BGLU21 showed higher expression levels in the EZ and MZ. This suggests that the ER bodies produced in the four different zones have a distinct composition, since the ratio of BGLU21/(BGLU22 + BGLU23 + NAI12) proteins contained in ER bodies may differ from one zone to another. Furthermore, the NAI1, NAI2 and BGLU23 profiles suggest that BGLU23 and NAI2 expression is probably under the strong influence of NAI1 control, while the BGLU21 and BGLU22 genes are probably influenced by other factors in addition to NAI1 levels. Apart from showing that BGLU23 and NAI2 proteins as being critical factors in ER body formation, their gene expression profile also suggests that ER body production increases from the CZ to the DZ. In a previous paper, we demonstrated that the number of ER bodies per cubic millimeter decreased from the MZ to the DZ in radish roots (Gotté et al., 2015), since the MZ cells are smaller than DZ and EZ cells, it suggests that the number of ER bodies per cell could be higher in the EZ or DZ than in the MZ. In the present study, we demonstrate that Arabidopsis roots have the same ER body density profile as that found in radish roots (Fig. 6). This observation would suggest that the production of ER bodies is controlled by the NAI1, NAI2, and BGLU23 genes. Under such conditions, ER bodies are regulated at a spatial level, as the influence of these genes...
is limited in the small root epidermal cells but increases along the length of root in the direction of larger elongated cells found in the EZ and DZ. It may be that a root gradient repression feedback control is present in the MZ and CZ cells, where the genes for ER body formation are suppressed as sufficient ER bodies are formed during growth and as smaller volumetric space in these cells limit the production of new ER bodies.

Finally, it was demonstrated that MeJA treatment caused the five key genes involved in ER body formation to be overexpressed in all four root zones except for NAII in the DZ where levels remained low (Fig. 5). Surprisingly, the density of ER bodies significantly increased only in the MZ and tended to increase in the EZ, whereas it remains almost unchanged in the CZ and DZ (Fig. 6). The difference between the gene expression profile and the number of ER bodies could be due to posttranscriptional regulation mechanisms (Li and Zhang, 2016; Filichkin et al., 2015). Further imaging analyses of ER body morphology, number, and content using antibodies such as anti-BGLU21, BGLU22 or BGLU18 would help to clarify this regulation more accurately.

Furthermore, the overexpression of BGLU23, NAII, and NAII2 tended to be high in the EZ as compared with the other zones (Fig. 5B), leading to slightly more ER bodies per cell in this zone, which is particularly susceptible to infection (Fig. 6). As observed for basal conditions, BGLU21 and BGLU22 present particular profiles after MeJA application. BGLU21 seems to be overexpressed equally in the CZ, MZ, and EZ and highly expressed in the DZ. While BGLU22 seems to follow the same profile as BGLU21 except that the level of overexpression is lower in MZ. All these data seem to indicate that the ER body–related genes were consistently overexpressed at lower levels in the MZ rather than other zones. This could be due to the high number of ER bodies per unit of volume constitutively present in the MZ epidermal cells (Fig. 6). Thus, this zone requires less ER bodies and hence much lower levels of expression. However, our data indicate that the MZ presents much higher levels of ER body production after MeJA treatment. In fact, it is known that the density of cells in this particular zone is extremely higher than the EZ or DZ, which have more bulky cells. Thus, the number of cells for the same volume of root tissue is much higher in the MZ than in the EZ and DZ. Therefore, if each cell induces the same production of ER bodies with an equivalent transcripts level, the subtle differences in this transcript levels between the MZ, EZ, and DZ could lead to strong differences in the zonal density of the ER bodies. It would thus be interesting, although difficult, to determine the exact number of ER bodies per cell in the different zones to support (or not) this assumption.

It is of crucial importance to study and elucidate the defense mechanisms that have evolved in plants to develop natural solutions to agricultural crop losses caused by diseases. To achieve these objectives, the involvement of the root component of the plant preformed and innate immune system is becoming more appreciated and studied. However, much regarding the root immune response remains still to be elucidated. Plants have evolved common and organ-specific responses for protecting roots, shoots, and leaves from damage (Balmer et al., 2013). Furthermore, the immune response is not uniform throughout the different root tissues (Millet et al., 2010). This is probably in response to the variety of root pathogens that exist in nature and their various entry strategies for penetration and colonization. The EZ seems to be the main point of entry for many pathogens such as the oomycetes Phytophthora parasitica and Aphanomyces euteiches (Attard et al., 2010; Cannesan et al., 2011). Thus, there is a real need for more detailed study of the localized and compartmentalized immune response pathways and processes. Our protocol is ideally suited to study the response of defense genes using external elicitor treatments as demonstrated by MeJA application in this study. The absence of sample preparation, along with rapid excision and isolation of tissues, helps to avoid most of the previous methodological issues that would likely induce an artifactual overexpression response of the defense genes. Furthermore, the method could also be used for targeted protein or untargeted proteomic analysis from the various root zones. Proteomic analysis of microdissected samples is clearly more complicated, since protein samples cannot be amplified like DNA and RNA, thus requiring a much larger amount of material to be collected (Gautam and Sarkar, 2014). Our technique avoids the long steps involved in regular sample preparation and consequently reduces the total length of time required to collect all the samples for a single study.

Conclusion
In summary, we demonstrated that the expression of the five genes associated with the production and functioning of ER bodies is root-tissue-zone specific and is dependent on the basal growth stage and external factors such as phytohormone application (e.g., MeJA). Furthermore, we showed that BGLU23 and NAII2 seem to be strongly regulated by transcription factor (NAII1). Whereas BGLU21 and BGLU22 seem to not be solely controlled by NAII1 but additional factors currently unknown and not evaluated in this targeted method study. Finally, we have developed an easy and rapid method to study the level of gene expression in different root zones with LAM, and we have shown that this method can be employed to analyze defense genes and probably also protein expression levels before and after elicitor treatment in plant immune system studies.

Supplemental Information Available
Supplemental Table S1: Specifications of housekeeping and ER body–related genes primers.

Conflicts of Interest
The authors declare no conflicts of interest.
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