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Rapid endocytosis is triggered upon imbibition in Arabidopsis seeds

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Abbreviations: BFA, brefeldin A; DMSO, dimethylsulphoxide; FM4-64, N-(3-triethylammoniumpropyl)-4-(6-(4-diethylaminophenylhexatrienyl)pyridinium dibromide; RG-II, rhamnogalacturonan-II

During seed imbibition and embryo activation, rapid change from a metabolically resting state to the activation of diverse extracellular and/or membrane bound molecules is essential and, hence, endocytosis could be activated too. In fact, we have documented endocytic internalization of the membrane impermeable endocytic tracer FM4–64 already upon 30 min of imbibition of Arabidopsis seeds. This finding suggest that endocytosis is activated early during seed imbibition in Arabidopsis. Immunolocalization of rhamnogalacturonan-II (RG-II) complexed with boron showed that whereas this pectin is localized only in the cell walls of dry seed embryos, it starts to be intracellular once the imbibition started. Brefeldin A (BFA) exposure resulted in recruitment of the intracellular RG-II pectin complexes into the endocytic BFA-induced compartments, confirming the endocytic origin of the RG-II signal detected intracellularly. Finally, germination was significantly delayed when Arabidopsis seeds were germinated in the presence of inhibitors of endocytic pathways, suggesting that trafficking of extracellular molecules might wield an important role in the overcome of germination. This work constitutes the first demonstration of endocytic processes during germination and opens new perspectives about the role of the extracellular matrix and membrane components in seed germination.

Introduction

Endocytosis is a process whereby portions of the plasma membrane invaginate and bud off to form membrane-bounded vesicles containing extracellular materials. This transport event includes both soluble and membrane-bound cargos. The initial internalization process is followed by a series of vesicle mediated transfer steps, which carry the cargo molecules through internal (endosomal) compartments. As previously described in animal systems, more than one type of endocytosis might exist in plant cells, including clathrin-dependent.[1-3] In fact, endocytosis is considered essential for plant development and adaptation to the environment.[4-6] This process is required to accomplish a wide variety of cellular functions, including the uptake of extracellular molecules, intercellular signal transduction, regulation of receptors-ligand biological activity on the plasma membrane and establishment and maintenance of cell polarity.[6-9] Thus, growing attention has been given in the last decade to the endocytosis in developmental and cellular plant biology.

A variety of inhibitors of the endocytic pathways have been used to unravel this process in plant cells. Among them, brefeldin A (BFA) is a fungal toxin widely used to block endocytosis[10,11] since it interferes with vesicle trafficking and fusion events, causing the aggregation of early endosomes and *trans*-Golgi network in characteristic BFA compartments.[12,13] It has been established that GTP-exchange factors that catalyze the activation of a small GTPases are the primary targets of BFA action.[14,15] Besides BFA, other drugs that directly interfere with vesicular trafficking and fusion events, namely wortmannin, LY294002 and tyrphostin A23, turned out to be very useful tools in determining endocytic networks.[6,16] The effects of wortmannin and LY294002 are achieved through inhibition of phosphatidylinositol-3 phosphate (PI-3P) kinases, enzymes responsible for the production of PI-3P, a lipid characteristic of endosomal membranes.[17,18] Besides, wortmannin and LY294002 are often used to perturb receptor mediated endocytosis, a feature well-documented for plant cells.[19-21] On the other hand, receptor-mediated endocytosed proteins have tyrosine-containing motifs which interact with the clathrin assembly polypeptide (AP complex) mediating recruitment into clathrin-coated vesicles. This interaction is inhibited by tyrphostin A23.[22,23]

Concerning seed physiology, despite the pivotal role of endocytosis in embryo development and post-germinative growth,[8,24,25] the participation of this cellular event during germination is still unknown. It is widely assumed that seed germination *sensu stricto*- involving those events occurring between imbibition of a dry seed and radicle emergence- implies a physiological burst that requires the existence of preformed

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materials such as intracellular proteins and mRNA that accumulate in dry seeds to ensure a fast entrance in germination once this process has been triggered.[26-28] On the other hand, the putative role of extracellular materials and plasma membrane internalized into imbibing seeds has not been analyzed yet. In this context, the aim of this work was to investigate whether endocytic events take place during imbibition and their contribution to Arabidopsis seed germination.

Results and Discussion

FM6–64 is rapidly internalized upon imbibition. FM4–64 is an amphiphilic dye unable to cross membranes thus constituting a powerful tool since it can only enter living cells through endocytic events.[29] In order to determine if endocytosis takes place during early germination, intact Arabidopsis dry seeds were submitted to imbibition in the presence of FM4–64. Afterwards, seed coat was removed and epidermal cells were monitored for the internalization of the fluorescent dye under confocal microscope. As a control of cell viability we performed all incubations in the presence of the vital dye Sytox Green, which only stains the nucleus of dead cells.[30] As shown in **Figure 1a**, in dry seeds incubated for 5 min in water the dye only appears associated to the plasma membrane. On the other hand, after 30 min of imbibition apparent endocytic vesicles were stained with the dye (**Fig. 1b**), and upon 1 h of imbibition FM4–64 was clearly detected intracellularly in epidermal cells (**Fig. 1c**). The size of these early endosomes (approximately 1 μm) suggest that they may be clathrin-coated vesicles.[22,31] The absence of Sytox Green fluorescence in the nucleus of these cells confirmed that they were alive, so that the internalization of the FM dye cannot be attributed to cell death. In fact, cells pretreated with DMSO 10% died and serve as a positive control for Sytox Green stain (**Fig. 1d**). Hence, these results suggest that active endocytosis is a primary response to water imbibition in germinating seeds and thus could be important to initiate germination.

The extracellular pectin RG-II is endocytosed when germination starts. Rhamnogalacturonan II (RG-II) is a highly conserved, complex pectic polysaccharide present in the cell walls of all vascular plants examined to date.[32] RG-II exists in primary walls as a dimer that is covalently cross linked by a Ca^{2+} dependent borate diester bond. Dimerization which results in the cross-linking of the two homogalacturonan chains is accomplished only in cell walls and is required for the formation of a three-dimensional pectic network in muro.[33] By the use of a RG-II antibody that only recognizes the dimeric form of RG-II,[34] it has been demonstrated that RG-II is internalized by endocytosis in meristematic root cells and delivered to the cell plate.[34] If endocytosis is effectively blocked by drugs, then no RG-II dimers are present within the cytoplasm.[35] On the other hand, importantly, the endocytic internalization of RG-II cell wall pectins is associated with their depletion in cell walls.[34] Using the same

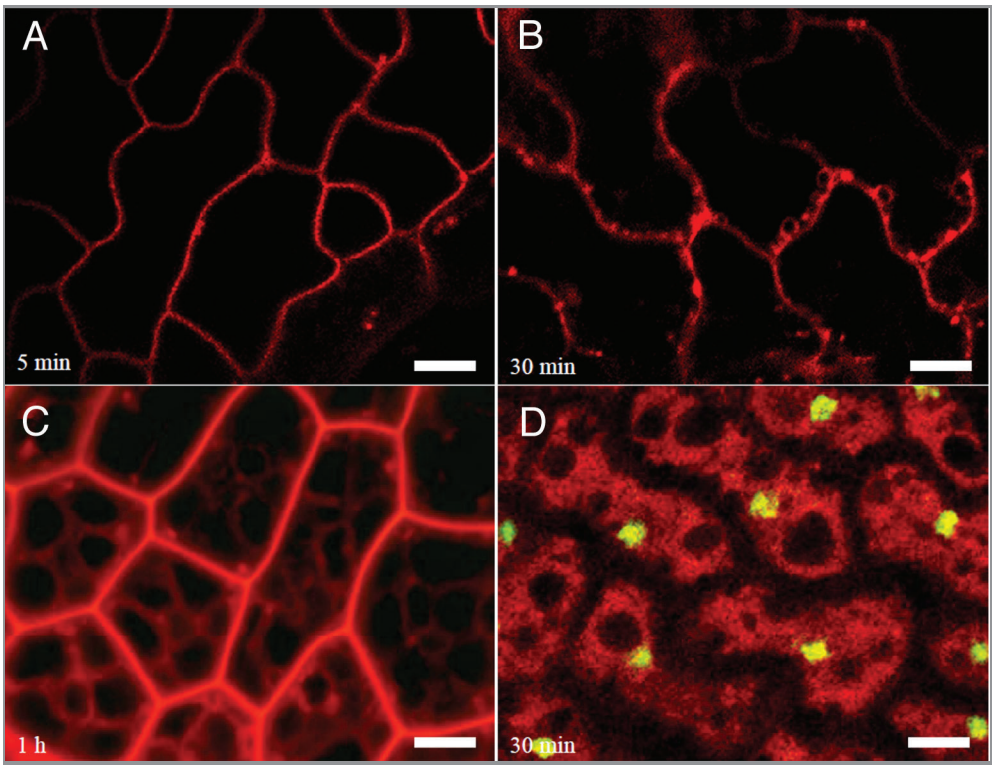

Figure 1. FM4–64 is endocytosed rapidly during early imbibition in Arabidopsis seeds. Dry seeds were imbibed in the presence of 50 μM FM4–64 and 1 μM Sytox Green for different times, washed with distilled water, decoated and observed under confocal microscope. Fluorescent signal from cotyledon cells corresponds to FM4–64 staining after 5 min (a), 30 min (b, d) or 1 h (c) of imbibition. d: Seeds were pre-incubated with 10% DMSO to induce membrane permeability; bright green signal in the nucleus corresponds to Sytox Green staining. Scale bar: 5 μm

antibody, here we have observed that RG-II has only extracellular localization in cells of dry seeds/embryos (**Fig. 2a**) while, already after 1 h of imbibition, the antibody clearly detects the boron-crosslinked RG-II epitope intracellularly (**Fig. 2b**). As the boron-mediated dimerization of RG-II monomers can be accomplished only in the cell wall, this finding clearly demonstrates a rapid endocytic internalization of the RG-II pectin upon the early imbibition.

Further experiments were aimed to confirm if RG-II was endocytosed during imbibition. When Arabidopsis dry seeds were imbibed for 1 h in the presence of 50 μM BFA, intracellular RG-II results in a clearly different distribution (**Fig. 2c**) compared with control seeds imbibed without the inhibitor (**Fig. 2b**). In fact, RG-II accumulates in intracellular aggregates whose size and shape resembles to the typical BFA compartments[10,12] (**Fig. 2c**). These results support the notion that the intracellular RG-II observed upon imbibition was incorporated by endocytosis. The size of the endosomes observed suggest that they may be the result of clathrin-mediated endocytosis.

Embryo axis elongation and radicle emergence are turgor-driven processes that require cell wall loosening in the cells of the embryo. Many cellular and metabolic changes occur in non-dormant seeds before the completion of germination.[35] In fact, completion of germination (radicle emergence) in Arabidopsis has been shown to be the result of cell expansion.[36] In embryo axes of non-dormant *Medicago truncatula* seeds germinating on water, the expression of genes involved in cell wall biosynthesis: xyloglucan endotransglucosylase (XET), cellulose synthase, arabinogalactan protein, two extensions and PPRD (protein with proline-rich domain) increased throughout germination.[37] More importantly, genes known to play a key role in cell-wall loosening and expansion, such as expansins, 1–3 glucanase and XET, were significantly expressed in the embryos as early as 5 or 15 h of imbibition, namely before germination completion and radicle extension.[37] It has been proposed that the endocytic delivery of cell wall pectins represents a ready-to-use reservoir of building blocks for rapid cell wall formation during cytokinesis[38] or cell expansion[39] and emerges to be critical for the polarity of pollen tubes.[40] So, the internalized RG-II observed here could operate as an intracellular pectin reservoir for the rapid cell expansion that proceeds upon imbibition.

Endocytosis inhibitors affect the germination rate. To determine the effect of different vesicular trafficking inhibitors in the germination capabilities of Arabidopsis seeds, germination kinetics was analyzed in the presence of 5 to 50 μM of BFA, or 5 to 30–33 μM wortmannin, LY294002 and tyrphostin A23, according to previous reports.[10,13,21,22] BFA impairs the activation of GNOM-dependent ADP-ribosylation factors (ARFs), affecting GNOM-dependent endocytosis.[41] Since this toxin acts at the late-endosome level, both clathrin dependent and independent endocytosis is affected by BFA. Wortmannin and LY294002 inhibit phospho inositol-3-kinase an enzyme responsable for the production of phosphoinositol-3-phosphate, (PI-3P), a characteristic lipid of endosome membranes.[42] On the other hand, tyrphostin A23 is a tyrosine analog that competes with type YXXΦ protein domains for its interaction with clathrin. In consequence, clathrin coated vesicles in tyrphostin A23 treated cells, are not capable to recruit the proteins to be endosytosed.[43]

Our results revealed that all those inhibitors affect the germination kinetics to different extent (data not shown). **Figure 3** illustrates the results obtained using the concentration of each inhibitor that exerted a maximal effect. It can be seen that the presence of all inhibitors produced a delay in seed germination. In order to compare the treatments, the parameter G_{50} was defined as the time needed for 50% of the seeds to complete germination, which corresponds to 14.3 h for control Arabidopsis seeds. A 2-fold increase in G_{50} value was observed when the seeds were germinated in the presence of tyrphostin A23 (G_{50} = 29.75), BFA (G_{50} = 31.85) and wortmannin (G_{50} = 32.78), while LY294002 exhibit a more pronounced effect on seed

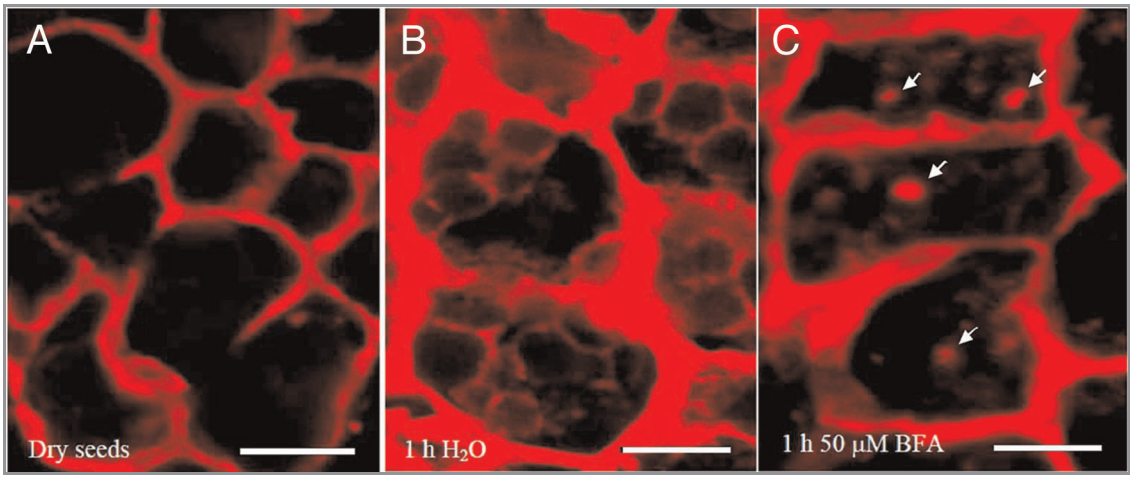

Figure 2. RG-II is internalized by endocytosis upon early imbibition and accumulates in BFA compartments. Arabidopsis dry seeds or seeds incubated for 1 h in water or 50 μM BFA were wax embedded, sectioned and labeled with RG-II antibodies followed by Alexa Fluor 546-conjugated goat anti-rabbit IgG detection. Confocal laser scanning microscopy shows immunolocalization of RG-II in cotyledons from dry seed (a), 1 h water imbibed seeds (b) or seeds imbibed for 1 h in the presence of 50 μM BFA (c). Arrows point BFA compartments. Scale bar: 5 μm

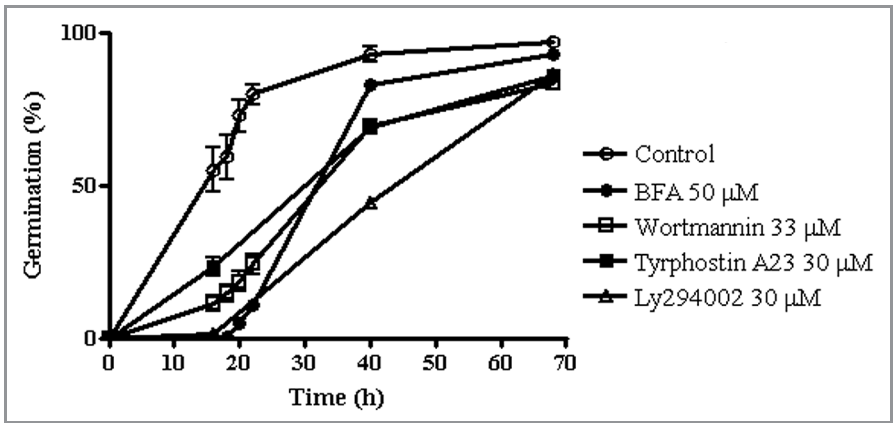

Figure 3. Inhibitors of endocytosis and endocytic versicle recycling reduce the rate of seed germination. Arabidopsis seeds were surface-sterilized and plated in solid medium in the presence of 50 μM BFA, 33 μM wortmannin, 30 μM tyrphostin A23, 30 μM LY94002 or equivalent volumes of DMSO (control). Germination kinetics was determined by measuring the time needed for radicle emergence. Error bars represent SE of means of three independent experiments.

germination showing a $G_{50} = 44.68$. So, the rate of seed germination is clearly affected when endocytosis is restricted.

In conclusion, this report demonstrates a remarkably rapid activation of endocytic processes when seed imbibition starts. Taking into account that the major endo[illegible] mechanism in plants depends on the coat protein clathrin[2] and the fact that inhibitors known to affect clathrin-dependent endocytosis provoked a remarkable delay in the rate of seed germination, the early endocytosis reported here seems to be mediated, at least in part, by chlatrin-dependent endocytosis. However, non-clathrin endocytosis has recently been r[illegible]ed for the internalization of glucose into BY-2 protoplasts[43] and our earlier study reported on myosin VIII-dependent fluid phase endocytosis in root cells.[11] Therefore, further studies are needed on the further analysis of the nature of plant endocytosis supporting the plant embryo germination. The rapid change from a metabolically resting state to the active germinating condition triggered by imbibition may require not only de novo synthesis - a comparative slow process - but also the recruitment of several molecules that have been strategically set aside from its target localization during seed maturation. Our results point out that endocytosis participates in the first stages of seed germination highlighting a still unknown role for endocytic internalization of membrane and/or extracellular components to achieve rapid radicle emergence.

Materials and Methods

Plant material and measurement of germination. *Arabidopsis thaliana* seeds Columbia ecotype (Col-0), were surface-sterilized and transferred to Murashige and Skoog medium with vitamins in the presence of different inhibitors of endocytic pathways, or equivalent volumes of 100% DMSO. Plates were incubated in the dark for 4 d at 4°C to break dormancy and then transferred to light at 24°C. All cultures were grown with a photoperiod of 14 h of light (250 μmoles $m^{-2} s^{-1}$, Sylvania Bio-lux fluorescent tubes) and 10 h of dark. Germination kinetics was determined after 16 h from the transfer to light by measuring the time required for radicle emergence from repeated experiments with triplicate plates of approximately 100 seeds. Statistical analyses were performed by a two way ANOVA test and further Bonfernoni test to determine differences between treatments.

RG-II localization, FM4–64 staining and confocal microscopy. Dry or water imbibed Arabidopsis seeds were fixed in 50 mM phosphate buffer pH 7.4, 10% DMSO, 4% paraformaldehyde, 1% glutaraldehyde at 4°C, and dehydrated by running them through ethanol series as follows: 15 min 30% ethanol, 15 min 50% ethanol, 15 min 70% ethanol, 15 min 96% ethanol. When the samples were thorough infiltrated with alcohol, they were embedded in Steedman wax,[38] placed into plastic molds and allowed to solidify overnight at room temperature. Sections (10 μm) were cut from these blocks using a rotary microtome, collected on albumin-coated slides and dried for 24 h. Seed sections were dewaxed, rehydrated through ethanol series and blocked as follows: 15 min 100% ethanol, 15 min 96% ethanol, 15 min 90% ethanol, 15 min 70% ethanol, 15 min 50% ethanol, 15 min 30% ethanol, 15 min in Phosphate Buffered Saline (PBS) and finally 15 min in PBS with 3% bovine serum albumin. Labeling with RG-II antiserum (1:200) and Alexa 546-coupled goat anti-rabbit IgG as secondary antibody (1:500) was performed according to Gillespie et al. (2005). For FM4–64 staining, dry seeds were imbibed in the presence of 50 μM FM4–64 and 1 μM Sytox Green for different times, washed with distilled water, decoated and observed under confocal microscope. Microscopic analysis of labeled seeds was performed using an Nikon C1 confocal laser scanning microscope. All images were acquired with 60x/1.40/0.13 oil-immersion lens. Alexa 546-coupled antibody was excited at 543 nm and detected at 550–650 nm. FM4–64 was excited at 488 nm and detected at 650–750 nm. The post-processing of images was performed with the aid of EZ-C1 FreeViewer versión 3.2 software.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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