Actin Monoubiquitylation Is Induced in Plants in Response to Pathogens and Symbionts

Edgar Dantán-González,¹ Yvonne Rosenstein,² Carmen Quinto,¹ and Federico Sánchez¹

¹Departamento de Biología Molecular de Plantas, Instituto de Biotecnología, UNAM, Apartado Postal 510-3, Cuernavaca Morelos 62271, México; ²Departamento de Genética y Fisiología Molecular, Instituto de Biotecnología, UNAM, Apartado Postal 510-3, Cuernavaca Morelos 62271, México

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Most dramatic examples of actin reorganization have been described during host–microbe interactions. Plasticity of actin is, in part, due to posttranslational modifications such as phosphorylation or ubiquitylation. Here, we show for the first time that actins found in root nodules of *Phaseolus vulgaris* are modified transiently during nodule development by monoubiquitylation. This finding was extended to root nodules of other legumes and to other plants infected with mycorrhiza or plant pathogens such as members of the genera *Pseudomonas* and *Phytophthora*. However, neither viral infections nor diverse stressful conditions (heat shock, wounding, or osmotic stress) induced this response. Additionally, this phenomenon was mimicked by the addition of a yeast elicitor or H₂O₂ to *Phaseolus vulgaris* suspension culture cells. This modification seems to provide increased stability of the microfilaments to proteolytic degradation and seems to be found in fractions in which the actin cytoskeleton is associated with membranes. All together, these data suggest that actin monoubiquitylation may be considered an effector mechanism of a general plant response against microbes.

Additional keywords: cortical cytoskeleton, microfilaments stabilization, oxidative burst.

Plants initiate various defense responses when attacked by microbes such as fungi and bacteria. Whereas some of these defense mechanisms are induced only after pathogen attack, others are preformed and constitute generalized physical and physiological barriers that hinder pathogen infection. Similar to animal immune responses following pathogen infection, plant responses to microbes involve a network of signal transduction pathways leading to regulation of gene expression (Yang et al. 1997). Pathogen recognition at the site of infection initiates rapid signaling processes that activate multicomponent defense responses at both local and systemic levels (Cohn et al. 2001; Grant and Mansfield 1999; McDowell and Dangel 2000; Scheel 1998). Similar responses can be triggered by elicitor molecules derived from various fungi or bacteria or from proteins secreted by various microorganisms. The generation of a response to a given stimulus is thought to require

three main events: stimulus perception, generation and transmission of a signal, and subsequent changes in downstream biochemical processes (Yamaguchi et al. 2000). The directed movement of vesicles and the nucleus toward the site of infection, the formation of wall appositions, the elevation of cytosolic calcium levels (Cárdenas et al. 1999; van der Luit et al. 2000), the induction of phytohormone synthesis (Møller and Chua 1999), cell division, the formation of new organs, oxidative burst response (Sandermann 2000), and the reorganization of the actin cytoskeleton (Staiger 2000) are only a few examples of the response a plant can generate after pathogen recognition.

The actin cytoskeleton forms a dynamic network that reorganizes rapidly in response to a variety of stimuli (Staiger 2000). In eukaryotic cells, actin exists in both polymeric, filamentous (F) and monomeric, globular (G) forms. Alterations in the balance of monomeric and polymeric actin results in changes in cell shape, movement, and polarity. In plants, F-actin is considered to provide the driving force for cytoplasmic streaming, as well as to constitute the molecular railroad tracks upon which vesicle transport occurs (Schmidt and Hall 1998). Treatment of barley coleoptile cells with microfilament inhibitors has been shown to suppress the overall polarized defense-related reactions under *Erysiphe pisi* developing appressoria, suggesting that the cytoskeleton might play an important role in directing certain defense-related compounds or organelles or both (Kobayashi et al. 1997). On the other hand, Cárdenas and colleagues 1998, showed that Nod factors appear to induce a rapid and reversible fragmentation in the actin cytoskeleton in *Phaseolus vulgaris* root hair cells, and these events may be a necessary prelude for the formation of infection threads by rhizobia.

Rearrangements of actin microfilaments are coordinated and regulated by a plethora of actin regulatory binding proteins (Puius et al. 1998), the functions of which may be controlled by numerous signals including Ca²⁺, phosphoinositides, pH, or reversible phosphorylation (Kameyama et al. 2000; Schmidt and Hall 1998). In animal systems, rearrangements of the actin cytoskeleton may also be the result of posttranslational modifications such as methylation, phosphorylation, or ubiquitin conjugate formation (Ball et al. 1987; Gauthier et al. 1997; Kalhor et al. 1999).

Recently, polypeptide tags have emerged as important post-translational regulators of protein function in plants (Vierstra and Callis 1999). Ubiquitin (Ub) is one the most conserved proteins identified to date. Amino acid sequences are identical among all higher-plant species examined and differ only by one, two, or three amino acid substitutions from *Chlamydomonas*, yeast, and mammalian ubiquitin sequences, respectively. Degra-

Corresponding author: Federico Sánchez;
E-mail: federico@ibt.unam.mx

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dation of proteins by the ubiquitin system starts by tagging polypeptides through isopeptide bonds linking the carboxy-terminal glycine 76 of ubiquitin to the ϵ -amino group of select lysine residues of the protein substrate (Jentsch and Pyrowolakis 2000). In plants, proteolysis by the ubiquitin-proteasome pathway has also been proposed to play an important role in various fundamental biological processes such as hormone responses, light perception, environmental adaptation, and floral development (Callis and Vierstra 2000; Gray and Estelle 2000). Contrary to polyubiquitylation that tags proteins for degradation, stable monoubiquitin or ubiquitin-like adducts such as those found in nuclear histones, receptors, and arthrin (a stable actin form from *Drosophila* flight muscle) confer stability and differential subcellular localization to proteins; albeit the physiological significance of this is not yet fully understood (Ball et al. 1987; Hicke 2001; Robzyk et al. 2000; Shih et al. 2000; Trausch et al. 1993).

Here, we show for the first time that, in plants, actin filaments are stabilized by monoubiquitylation tagging. In addition to its induction in root nodules, this modification of actin molecules was also triggered in all other plant tissues tested when plants were infected with various pathogens and symbionts. Furthermore, neither viral infections nor diverse stressful conditions triggered this response, suggesting that actin monoubiquitylation may be part of a plant innate immune response specifically directed against bacteria and fungi or related signaling metabolites. This was further supported by the fact that adding elicitors or H_2O_2 to suspension culture cells also induced this actin modification.

Fig. 1. Two-dimensional gel electrophoretic analysis of actin expression during *Phaseolus vulgaris* root nodule development. **A**, Immunodetection of actin isoforms with anti-actin monoclonal antibody N-350 in 10-day-old root (row 1) or root nodule total protein extracts isolated from plants 10, 12, 16, 18, 20, and 27 days postinoculation (rows 2 to 7, respectively). **B**, Actin isoforms are schematized, the pI values of corresponding isoforms are indicated.

RESULTS

Differential expression of actin isoforms during root nodule development.

In plants, actin isoforms are temporally or spatially regulated, or both, during diverse organogenic processes (Barlow and Baluška 2000; McLean et al. 1990). The symbiotic interaction between members of the genus *Rhizobium* and legume roots leads to the formation of a new organ: the root nodule (Spaink 2000). In order to identify root- and root nodule-specific developmental markers, actin isoforms were analyzed by two-dimensional immunoblotting in *P. vulgaris* protein extracts. Interestingly, the pattern of actin isoforms, as detected by the anti-actin monoclonal antibody (MAb) N-350, was different in root and root nodules. Three actin isoforms were identified in extracts from 10-day-old roots, while up to six isoforms were detected in root nodules obtained 10 to 25 days postinoculation (dpi) (Fig. 1). Three of the isoforms were common to both organs (5.5, 5.6, and 5.7 pI), while the other three (5.3, 5.4, and 5.8 pI) were specific to root nodules and were transiently expressed. In senescent root nodules (27 dpi), where nitrogen fixation activity ceases, the actin isoform pattern was identical to that of roots. These data suggested that the novel actin isoforms that we identified participate in root nodule development, nitrogen fixation, or both.

Root nodule actin is modified by covalent-bound ubiquitin.

Surprisingly, the anti-actin MAb N-350 recognized several proteins of a relative molecular mass of approximately 63,000 ($M_r = \sim 63$ kDa). The isoelectric points of these were comprised within the same range as the 42-kDa actin isoforms (Fig. 1). To eliminate possible artifacts, immunopurified MAb N-350 and three additional antibodies reported to cross-react with plant actin (Villanueva et al. 1999) were tested. Consistently, all anti-actin antibodies recognized the same actin and the same 63-kDa protein isoforms (data not shown).

The fact that the 63-kDa proteins recognized by the anti-actin MAbs had a similar isoelectric pattern to 42-kDa actin and that these proteins had a mass difference of 20 K with 42-kDa actin isoforms, even under reducing conditions, suggested to us that they could be a covalent modification of actin. Covalent tagging with monoubiquitin has been demonstrated for arthrin, a form of actin specific to *Drosophila* flight muscle (Ball et al. 1987). We investigated this possibility. All 63-kDa proteins were indeed recognized by both anti-actin and anti-ubiquitin antibodies (Fig. 2A and B). Since ubiquitin monomers have a molecular mass of 8.5 kDa, the 20-kDa difference between the 42-kDa actin and the 63-kDa isoforms could be the result of the binding of two ubiquitin molecules to each actin isoform, a modification known as monoubiquitylation (Hicke 2001). Interestingly, this modification was a transient event during nodule organogenesis, correlating with the time of maximum metabolic activity (Fig. 1).

Actin ubiquitylation is not associated with developmental or nitrogen-fixation processes.

Root nodules are organs in which robust cell division and expansion take place (Spaink 2000). In order to determine if actin-ubiquitylated isoforms were characteristic of growth and developmental processes, different *P. vulgaris* organs were

analyzed. Ubiquitylation of actin was absent in leaves, roots, shoots, and floral and root meristematic zones but present in root nodules (Fig. 2C). These data suggested that, in *P. vulgaris*, actin ubiquitylation is a root nodule-specific phenomenon, apparently unrelated to other plant vegetative growth and developmental processes. Even more, actin modification was exclusively found in root nodules of *Glycine max*, *Lotus japonicus*, and *Medicago sativa* and not in other organs (Fig. 2D). All together, these data suggested that actin ubiquitylation might be important for the development of the root nodule or for the nitrogen-fixation process.

In order to discriminate between these two possibilities, *P. vulgaris* root nodules were induced with wild-type *Rhizobium etli* (CFN3) and two different isogenic ineffective strains (CFN4202 and IFC01) (Tabche et al. 1998). As previously reported (Soberón et al. 1993), in CFN4202 mutant-induced nodules, the infection seemed to proceed up to a certain point, but the nodule lacked bacteroids, while in IFC01-induced nodules, bacteria readily differentiated into the intracellular form. In root nodules lacking bacteroids, the ubiquitylated form of actin was absent (Fig. 2D, lane 11), as opposed to root nodules induced with the wild-type and nonfixing IFC01 strain (Fig. 2D, lanes 10 and 12, respectively), suggesting that this phenomenon is probably more related to the presence of the bacteria in the nodule than to the nitrogen-fixation process.

Actin ubiquitylation is specific to plant bacterial and fungal interactions and not a response to stressful abiotic conditions.

The establishment of symbiosis of members of the genus *Rhizobium* with *P. vulgaris* can be considered a stressful situation for the plant. To test the possibility that actin ubiquitylation might reflect a response to stress, bean plants were exposed to various stressful conditions and were analyzed for the presence of the actin-ubiquitin conjugate. As shown in Figure 3A, none of the conditions tested, osmotic shock, wound, or darkness, resulted in ubiquitylation of actin isoforms.

The possibility that actin ubiquitylation could be related to the infective process was further evaluated. Total protein extracts from several tissues were obtained from different species of plants infected with fungi, bacteria, or a virus and screened for ubiquitin-modified actin by immunoblotting. Interestingly, actin ubiquitylation was not exclusive of *Rhizobium* infection. The 63-kDa actin isoform was consistently detected in plants infected with fungi (*Phytophthora capsici* or *Glomus interadians*) or pathogenic bacteria (*Pseudomonas syringae* pv. *tabaci*) but in none of the plants infected with *Tobacco mosaic virus* (TMV) (Fig. 3B). Additionally, *P. vulgaris* suspension cells were infected with *Pseudomonas syringae* pv. *tabaci*, treated with yeast elicitor, and given a heat shock or an oxidative burst (H_2O_2). Ubiquitin-conjugated actin was detected only when cells were exposed to *Pseudomonas syringae* pv. *tabaci*, yeast elicitor, or H_2O_2 but not in response to heat shock (Fig. 3C).

Bacteria and fungi, but not viruses, produce signaling molecules (Nod factors and elicitors) that have been shown to induce host plant responses such as rearrangements and polarization of the actin cytoskeleton (Cárdenas et al. 1998; Kobayashi 1997; Staiger 2000). Ubiquitylation of actin was only induced when *P. vulgaris* roots were treated with a yeast elicitor but not with nodulation factors (Nod factors) from *R. etli*, even at

10^{-6} M, a much higher concentration than that used to trigger the formation of nodulelike structures in bean roots (10^{-8} M) (Cárdenas et al. 1995) (Fig. 3D). This suggests that specific bacterial molecules (elicitors) but not Nod factors are required to trigger the actin-ubiquitylation response in root nodules.

Monoubiquitylation is not a tag for actin degradation.

Ubiquitin has been associated with the proteasome-protein degradation pathway (Jentsch and Pyrowolakis 2000; Weissman 2001). We explored the possibility that the actin-Ub conjugate we detected could be part of a degradation event. In root nodule protein extracts prepared under conditions in which G-actin was stabilized with actin stabilization buffer (ASB; 0.2 mM $CaCl_2$, 2 mM Tris, pH 7, 0.75 mM mercaptoethanol, and 0.2 mM ATP) (Ren et al. 1997), actin was degraded at 4°C, even in the presence of protease inhibitors. This process was accelerated by 2 M urea. Actin degradation was evaluated in *P. vulgaris* root nodule extracts obtained 16 dpi with *Rhizobium etli*. As shown in Figure 4A, the 42-kDa actin form was rapidly degraded within the first 30 min. However, the ubiquitylated form of actin resisted proteolytic degradation and remained stable for several hours, even in the presence of 2 M urea.

Actin ubiquitylation is preferentially associated with filaments, membranes, or both.

A rapid response to a stimulus is often associated with a massive reorganization of actin cytoskeleton. Cellular mecha-

Fig. 2. The 63-kDa protein corresponds to a monoubiquitylated actin form specific to root nodules. **A**, Two-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis Western blotting of total protein extracts of *Phaseolus vulgaris* 16-days-postinoculation nodules with anti-actin monoclonal antibody N-350 and **B**, anti-ubiquitin polyclonal antibody. **C**, Western blot of total protein extracts from *P. vulgaris*: hypocotyl dividing zones (lane 1), elongation zones (lane 2), and not-growing zones (lane 3); flowers (lane 4); leaves (lane 5); nodules (lane 6); and roots (lane 7). **D**, Different organs from various leguminous plants were tested for the presence of ubiquitylated actin: *Glycine max* leaves, root nodules, and roots (lanes 1 to 3, respectively); *Lotus japonicus* leaves, root nodules, and roots (lanes 4 to 6, respectively); *Medicago sativa* root nodules (lane 7); and *P. vulgaris* root nodules (lane 8) and roots (lane 9). Nodules from *P. vulgaris* inoculated with CFN2402 and IFC01 strains (lanes 10 and 11, respectively). Data shown are representative of three independent experiments. Single arrowheads: 42-kDa actin; double arrowheads: 63-kDa ubiquitylated actin.

nisms that control the F-actin/G-actin ratio are essential to orchestrate this response. Recent reports point out that monoubiquitylated proteins tend to be more stable rather than being rapidly degraded (Hicke 2001). Even more, it has been proposed that monoubiquitylation and ubiquitin-related modifica-

tions of proteins change the function of the targeted protein or promote its relocation to a different subcellular compartment (Hicke 2001; Shih et al. 2000). We investigated the cellular distribution of the ubiquitylated form of actin. In root nodules, bacteroids are enclosed in a peribacteroidal mem-

Fig. 3. Environmental stress does not trigger actin ubiquitylation, and actin ubiquitylation is triggered in response to microbial infections or related signaling molecules. **A**, *Phaseolus vulgaris* plants were subjected to different environmental stressful conditions: leaves from salt-stressed plants (lane 1), total plantlet extract after 72 h of darkness (lane 2), roots from water-deficit-stressed plants (lane 3), wounded leaves (lane 4), and *P. vulgaris* root nodules (16 days postinoculation) (lane 5). **B**, Ubiquitylated actin was detected by immunoblotting total protein extracts from various plant organs locally infected with different microorganisms: *P. vulgaris* root nodules inoculated with *Rhizobium etli* (lane 1); *Nicotiana tabacum* leaves after 72 h of infiltration with *Pseudomonas syringae* pv. *tabaci* (lane 2) or H₂O (lane 3); *Capsicum annuum* leaves and roots infected with *Phytophthora capsici* P1319 (lanes 4 and 5, respectively) or uninfected leaves from control plants (lane 6); *Tobacco mosaic virus* particle-bombarded *N. tabacum* leaves after 72 h of infection (lane 7); and *P. vulgaris* roots inoculated with *Glomus interadians* (lane 8). **C**, *P. vulgaris* suspension culture cells. Control (lane 1) or treated for 2 h at 42°C (heat shock, lane 2), with 4 mM H₂O₂ (lane 3), with yeast elicitor (25 mg/ml) (lane 4), or with 1×10^9 CFU of *Pseudomonas syringae* pv. *tabaci* per ml (lane 5). **D**, Total protein extracts from *P. vulgaris* roots incubated for the indicated times with *R. etli* Nod factors (lanes 1 to 3) or with yeast elicitor (lanes 4 to 6). Equivalent amounts of protein were loaded in each lane. Data shown are representative of three independent experiments.

Fig. 4. Ubiquitylated actin is stable and preferentially associated with membranes or filamentous actin. **A**, *Phaseolus vulgaris* root nodules (16 days postinoculation) frozen in liquid nitrogen, ground, and resuspended in actin stabilization buffer (ASB) with or without 2 M urea and shaken at 30°C for the indicated times. **B**, *P. vulgaris* root nodule endomembranes (lane 1), bacteroids (lane 2), and peribacteroidal membranes (lane 3). **C**, ASB-resuspended root nodule tissue was incubated without (lanes 1 and 2), with 0.7% *N*-octyl- β -D-glucoside (O β G) (lanes 3 and 4), or sonicated for 4 min at 4°C (lanes 5 and 6). Samples were centrifuged at $4,000 \times g$ for 10 min and the soluble (lanes 1, 3, and 5) and insoluble (lanes 2, 4, and 6) fractions were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The soluble fraction from the sonicated extract was further partitioned by centrifugation at $100,000 \times g$ for 45 min, and the supernatant (lane 7) and the pellet (lane 8) were both analyzed. A 16-days-postinoculation root nodule sample was included as control (lane 9).

brane resulting from an invagination process of plant plasma membrane (Verma et al. 1978). When membranes from *P. vulgaris* root nodules were fractionated, we observed that ubiquitin-conjugated actin was detected in the endomembrane (Golgi) as well as in the peribacteroid membrane-enriched fractions, but that it was absent from the bacteroid fraction (Fig. 4B). Additionally, the ubiquitylated form of actin was only present in the insoluble fraction of root nodule extracts obtained under G-actin stabilizing conditions, whereas the 42-kDa actin was only present in the soluble fraction (Fig. 4C, lanes 1 and 2). Treatment of root nodule tissues with *N*-octyl- β -D-glucoside (O β G) extracted about 20% of the actin-Ub conjugate into the soluble fraction (lanes 3 and 4), while sonication extracted up to 80% (lanes 5 and 6). However, after centrifugation at $100,000 \times g$ for 45 min of the soluble fraction of the sonicated extract, the ubiquitin-modified actin was again found in the pellet fraction (lanes 7 and 8). These data suggested that actin-Ub is tightly associated with cell membranes, is in its polymeric form, possibly as subcortical filamentous actin, or both.

DISCUSSION

Actin isoforms have been found to be differentially expressed during various developmental processes in plants (McLean et al. 1990). Here, we show by two-dimensional immunoblotting that three and up to six actin isoforms were clearly detected in *P. vulgaris* root and root nodule extracts, respectively. Three out of the six actin isoforms were transiently and specifically expressed during most of the root nodule organogenesis process, while the other three isoforms were common to root and root nodule extracts. Furthermore, late in root nodule development, when nitrogen fixation ceases (27 dpi), the nodule-specific actin isoforms were no longer detected and the actin isoform pattern of nodules was again identical to that of roots (Fig. 1). The fact that the most acidic actin isoforms (pI = 5.3 and 5.4) and the most basic one (pI = 5.8) were only specifically detected during nodule development suggests that they could have an important role in root nodule organogenesis or nitrogen fixation.

Interestingly, in root nodule extracts obtained between 10 to 18 dpi, the anti-actin MAb N-350 recognized several proteins of 63 kDa that had an isoelectric pattern similar to the 42-kDa actin isoforms (Fig. 1). The fact that these proteins had a mass difference of about 20 K with the 42-kDa actin and that they were readily detected by an anti-ubiquitin polyclonal antibody suggests that they are most probably the result of the binding of two ubiquitin molecules (8.5 kDa each) to each root nodule 42-kDa actin isoform (Fig. 2). Multiubiquitin chains at least four subunits long are required for efficient recognition and degradation of ubiquitylated proteins by the proteasome (Weissman 2001). Covalent protein tagging of one to two ubiquitin molecules to a particular protein is a process known as monoubiquitylation (Hicke 2001). Instead of routing proteins for destruction through the proteasome, monoubiquitylation is thought to regulate the location and activity of diverse proteins. Monoubiquitylation has been shown to be involved in at least three distinct cellular functions: histone regulation, endocytosis, and the budding of retroviruses from the plasma membrane (Hicke 2001). Arthrin, a monoubiquitylated form of actin, specific to *Drosophila* flight muscle, has been shown to be more stable while retaining its capacity to form fila-

ments and to interact with ligands such as DNase I and profilin (Just et al. 1993). The monoubiquitylation of nodule actin isoforms we describe here was a transient event correlating with the period of maximal metabolic activity of the root nodules. Data provided here support the fact that actin monoubiquitylation was not a systemic-induced response since it was absent in leaves, roots, shoots, and floral and root meristematic zones of *P. vulgaris* nodulated plants. Furthermore, actin monoubiquitylation could not be detected in nodules approaching senescence (after 20 dpi) (Figs. 1 and 2C), suggesting that this posttranslational modification is not linked to general plant developmental or proteolytic/degradative processes.

Since we detected monoubiquitylated actin exclusively in symbiotic root nodules of diverse legumes (Fig. 2D), we initially thought that this biochemical modification of actin was necessary for an efficient nitrogen-fixation process. This possibility was discarded since no monoubiquitylated actin was detected in nonfixing root nodules induced by the ineffective strain of *Rhizobium* CFN4202, suggesting that this phenomenon is probably more related to the presence of the bacteria in the nodule than to the nitrogen-fixation process (Fig. 2D). This result was further supported by the fact that actin ubiquitylation was detected in nodules infected with IFC01, a strain of *Rhizobium* defective in nitrogen-fixation activity that contains bacteroids (Tabche et al. 1998) (Fig. 2D). All together, these data suggested that actin monoubiquitylation is part of a mechanism by which plants recognize microbes. Whether live bacteria, associated signaling molecules, or both are important for inducing this response remains to be determined.

Nod factors are pivotal for the capacity of rhizobia to induce root nodules (Spaink 2000). We could detect ubiquitylated actin only when *P. vulgaris* roots were treated with a yeast elicitor but not with *R. etli* Nod factors (Fig. 4D). This result is probably consistent with the fact that Nod factors have been found to be responsible for the fragmentation of actin filaments in the root hair (Cárdenas et al. 1999). Other bacterial signaling molecules, but not Nod factors, might be required for triggering host defense responses in inoculated roots legumes (Spaink 2000). Further investigation will be required to determine which of these molecules are responsible for the monoubiquitylation of actin.

The actin cytoskeleton is a highly dynamic network and is regulated by various stimuli and associated signaling pathways. Modification of actin levels in response to diverse stresses is well documented (Olinevich et al. 2000). The fact that we could not find ubiquitylated actin in plants subjected to heat shock, osmotic, saline, or darkness stress (Fig. 3B) suggests that in response to plant bacterial and fungal infections, which often involve additional osmotic and wounding stresses, monoubiquitylation of actin molecules is not directly related to such stressful conditions.

Pathogenic bacteria and fungi have been found to harbor the ability to control plant actin dynamics and rearrangements (Staiger 2000). In barley coleoptile and flax cells, actin rearrangements occur 3 to 4 h prior to penetration by *Erysiphe* or *Melampsora* spp. (Staiger 2000). Additionally, a *Magnaporthe mps1* strain unable to form a penetration peg on plant epidermal cells still elicits rearrangements of the actin cytoskeleton, thus suggesting that a signal must originate from the microbe (Xu et al. 1998). Since wounding does not induce the

monoubiquitylation of actin (Fig. 3A), our data would support the idea that wounding is not involved in such actin responses.

The fact that monoubiquitylated actin seems to be tightly bound to cell membranes, preferentially organized in microfilaments (Fig. 4B and C), or both rather than tagged to G-actin (soluble form) strongly suggests that the actin cortical cytoskeleton is a preferential target for monoubiquitylation, most probably providing enhanced filament stabilization, directing it to a different subcellular localization, or both. Whether the ubiquitylation of actin could be related to a possible accumulation of cytoplasm and actin filaments at the site of microbe contact, where plant cells also generate elaborate wall appositions or papillae remains to be determined.

Data presented in Figure 3B indicate that actin ubiquitylation is a local, generalized plant response to different bacterial and fungal but not to viral infections. Data obtained when a yeast elicitor was added to *P. vulgaris* roots or suspension culture cells suggested that actin monoubiquitylation is part of a plant primary recognition mechanism. Since H_2O_2 was sufficient to induce actin monoubiquitylation, it is probable that the rapid production and accumulation of reactive oxygen species (ROS), such as O_2^- and H_2O_2 , known as the oxidative burst (Yang et al. 1997), participates in the signaling pathway leading to it (Fig. 3C). All together, data presented here suggested that monoubiquitylation of actin represents a general plant response to bacterial or fungal infections. Further investigation is currently underway to characterize the perceptive and signaling pathways leading to it as well as the biological significance of this phenomenon in the context of a plant innate immune response to bacterial or fungal infections.

MATERIALS AND METHODS

Antibodies and chemicals.

Anti-actin MAb N-350 was purchased from Amersham (Arlington Heights, IL, U.S.A.); anti-actin MAb clone 3AD2-EA hybridoma supernatant and anti-actin polyclonal antibody were purchased from Sigma (St. Louis, MO, U.S.A.). All have been shown to cross-react with plant actin (Villanueva et al. 1999). The rabbit anti-bovine red blood cell ubiquitin was from Sigma and anti-ubiquitin MAb from Zymed Labs (San Francisco). Alkaline-phosphatase-conjugated anti-rabbit and anti-mouse antibodies and substrates were from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Ampholytes were purchased from Sigma. All other chemicals were reagent grade.

Germination of common bean (*P. vulgaris* L. cv. Negro jamapa) seeds and harvesting of root nodules.

Seeds were surface-sterilized in 10% (vol/vol) commercial bleach, rinsed with running tap water, sown on water-saturated towels, and germinated in the dark at $27 \pm 1^\circ\text{C}$ and 100% relative humidity. After 5 to 7 days, seedlings were transferred to vermiculite and grown for 20 days in a greenhouse. Ten-day-old roots were collected in liquid nitrogen and stored at -70°C . Alternatively, germinated seeds were planted in pots containing vermiculite, and half of them were inoculated with *R. etli* CFN3, CFN4202, and IFC01. Root nodules were harvested after 10, 12, 16, 18, 20, and 27 days.

Water-deficit treatment.

After 4 days of germination, seedlings of *P. vulgaris* were selected based on uniform size and transplanted to vermiculite containing different amounts of water. Control plants were irrigated with 5 ml of water per gram of vermiculite ($\Psi_w = -0.074$ MPa), while the water-deficit conditions corresponded to $\Psi_w = -0.35$ MPa, $1/12$ the amount of water of the control. Vermiculite was maintained at constant water potential throughout the experiment.

Plant salt treatment.

At the three-leaf stage, bean seedlings were grown for 3 days in a greenhouse in hydroponics using a standard irrigating solution containing 250 mM NaCl.

Suspension culture cells treatments.

P. vulgaris leaf-cultured cells were maintained as described (García-Gómez et al. 2000). Treatments consisted of 1.5 g of cells being transferred at 50 ml of Linsmaire and Skoog (Fisher and Tsai 1978) medium in 100-ml Erlenmeyer flasks. The yeast elicitor, *Pseudomonas syringae* pv. *tabaci*, or H_2O_2 was applied after 3 days of growth at the concentrations indicated in the corresponding figure. Heat shock treatment was also applied after 3 days of growth.

Isolation of membranes from root nodules.

Membranes were fractionated as described by Verma and colleagues (1978).

Tobacco plant infection with TMV.

Tobacco leaves were inoculated by microprojectile bombardment with TMV or pUC18 (negative control) as described previously (Schaffer et al. 1995).

Protein extraction, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), two-dimensional SDS-PAGE (2-D PAGE), and immunoblotting.

The frozen tissue samples were first ground in liquid nitrogen, resuspended in Laemmli extraction buffer, and boiled immediately for 10 min with shaking. The supernatants were recovered by centrifugation at $14,000 \times g$, and equal amounts of total proteins were analyzed by SDS-PAGE or 2-D PAGE and immunoblotting. For 2-D PAGE, protein extracts in Laemmli sample buffer were diluted 1:1 in O'Farrell's buffer and analyzed by isoelectric focusing for the first dimension and by SDS-PAGE for the second dimension according to O'Farrell (1975). For immunoblotting, following transfer of the proteins to nitrocellulose, the membranes were blocked with 5% milk in phosphate-buffered saline (PBS) (PBST: 10 mM Na-phosphate; 150 mM NaCl, pH 7.4; and 0.05% Triton X-100) for 1 h at 42°C . The membranes were then incubated overnight at 4°C with either a 1/4,000 dilution of anti-actin MAbs or 1/5,000 dilution of anti-ubiquitin antibodies in 0.05% Triton X-100 in PBS (PBST). After three washes in PBST, membranes were incubated with a 1/5,000 dilution of alkaline-phosphatase-conjugated secondary antibodies for 1 h at 25°C . The membranes were then washed three times with PBST and rinsed with PBS, and the proteins were visualized by nitroblue tetrazolium and bromo-chloro indolyl phosphate according to the manufacturer (Boehringer Mannheim). For densitometric scans, the

images of the western blots or photographic films were captured by an Apple Color One Scanner using Adobe Photoshop software, and the bands were quantified according to their pixel value using NIH-Image software.

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