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Actin marker lines in grapevine reveal a gatekeeper function of guard cells

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ABSTRACT

Resistance to abiotic and biotic stress is a central topic for sustainable agriculture, especially in grapevine, one of the field crops with the highest economic output per acreage. As early cellular factors for plant defense, actin microfilaments (AF) are of high relevance. We therefore generated a transgenic actin marker line for grapevine by expressing a fusion protein between green fluorescent protein and the second actin-binding domain of *Arabidopsis* (*Arabidopsis thaliana*) fimbrin, AtFIM1. Based on this first cytoskeletal-marker line in grapevine, the response of AFs to phytopathogenic microorganisms could be followed *in vivo*. Upon inoculation with fluorescently labeled strains of phytopathogenic bacteria, actin responses were confined to the guard cells. In contrast, upon contact with zoospores of *Plasmopara viticola*, not only the guard cells, but also epidermal pavement cells, where no zoospores had attached responded with the formation of a perinuclear actin basket. Our data support the hypothesis that guard cells act as pacemakers of defense, dominating the responses of the remaining epidermal cells.

Introduction

Linked with their ability for rapid remodeling, AFs play important roles in plant defense (for review, see Day et al., 2011). Plant immunity comprises two layers: the pathogen-associated molecular patterns (PAMPs) triggered immunity (PTI) is evolutionarily ancient and can be triggered by conserved pathogen structures binding to receptors at the plasma membrane. The second layer is termed effector-triggered immunity (ETI), and originated from coevolution of specific pathogen strains with their hosts. These pathogens produce effectors that can quell the basal PTI, and the host has developed additional receptors that recognize these effectors in the cytoplasm and restore defense (Jones and Dangl, 2006).

AFs appear to be involved in both levels of immunity, PTI and ETI (for review see, Day et al., 2011). AFs participate in callose deposition and organelle clustering around fungal penetration sites, as an important element of basal defense (Bestwick et al., 1995; Opalski et al., 2005; for review see, Schmelzer, 2002). Recently, an actin response to micro-wounding leading to a recruitment of vesicle flow toward the penetration site has been identified as an important element of penetration resistance (Kobayashi and Kobayashi, 2013). In addition, the endocytotic recycling of plant PAMP receptors depends on AFs, as first discovered for *Arabidopsis* FLS2, the receptor for the bacterial PAMP flagellin. This actin-mediated endocytosis of receptors is often necessary for signaling from intracellular compartments (Robatzek et al., 2006). Thus, AFs are implicated in vesicle trafficking, organelle movements, cell wall deposition, and receptor recycling in the context of PTI. Evidence for a role of actin in ETI is emerging as well. Certain R-proteins (acting as receptors for bacterial effectors that subsequently restore defense signaling culminating in hypersensitive cell death) traffic along actin to the infection site (Wang et al., 2009), and the actin-depolymerizing factor ADF4 is necessary to initiate ETI in response to *Pseudomonas syringae* (Tian et al., 2009).

Actin is also involved in host–pathogen interaction in animal cells, where pathogens use actin-based motility to usurp the motility of the host to invade their victim and to propagate within the host tissue (for review, see Day et al., 2011). Since actin-based motility does not play a role in the walled plant cells, the function of actin must be fundamentally different. In plants, it is mainly the effect of actin on signaling that is significant. In defense signaling, AFs are modified through the activation of a coordinated network involving Rho-GTPase family members and their respective target proteins (Yang and Fu, 2007). As shown for the R-protein

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RPS5 (RESISTANCE TO *PSEUDOMONAS SYRINGAE*-5) and its cognate pathogen effector molecule AvrPphB (a cysteine protease delivered *via* the Type III secretion system (T3SS) of *P. syringae*), AFs participate in the perception of the effector and are thus essential for ETI.

The role of plant AFs as sites of action for bacterial effectors represents important targets. For instance, the pathogen-derived toxin coronatine stimulates the rapid opening of stomata by chemical mimicry of the general plant stress signal jasmonate, facilitating pathogen entry (Melotto et al., 2006). Since the stomatal aperture is linked with reorganization of AFs (Kim et al., 1995), this example indicates a link between general defense and AFs. Whether pathogens directly manipulate AFs *via* the action of secreted effector molecules, or whether the activity of these molecules disrupts the regulatory (*i.e.*, GTPase) or structural (*i.e.*, actin-binding protein, ABP) processes required for actin organization are questions that remain to be elucidated.

Grapevine has emerged as model for applied plant biology because it is well studied at the level of functional genomics, with several completed genome projects available (Jaillon et al., 2007; Moroldo et al., 2008). Moreover, grapevine is the crop with the highest cash yield per area. At present, approximately 7.4 million hectares of vineyards are planted worldwide, which means that 0.5% of the total world arable land is dedicated mainly to wine production (Canada's Michael Smith Genome Sciences Center). Currently, viticulture can only be conducted with the aid of intense protection by fungicides. For instance, in the European Union, fungicides are applied at an average rate of 19.5 kg per hectare with 12–15 applications in each season (Gianessi and Williams, 2011). However, the use of grapevine as a model for plant defense is not only linked to agronomy. The existence of wild species of grapevine that is disease resistant, in contrast to the disease-susceptible cultivated grapes, provides experimental systems to link genetics with resistance. The reason for these differences is connected with the evolutionary history of the genus. Prior to the glacial period, the genus *Vitis* was widely distributed over the entire Northern hemisphere with numerous species in Europe (Kirchheimer, 1938). By the end of the Pleistocene, the genus had declined in Europe with only one fossil record for *Vitis vinifera* ssp. *sylvestris* reported in Southern France (De Lumley, 1988). The cultivated grapevine *V. vinifera* ssp. *vinifera* was thus derived from isolated founder populations that had been freed from their cognate pathogens. In contrast, North America and East Asia have preserved numerous species of the genus *Vitis*. Among the numerous diseases of grapevine, Downy Mildew of Grapevine, caused by the oomycete *Plasmopara viticola*, poses the most serious problems for viticulture in Central Europe. *P. viticola* was introduced from North America to Europe around 1878 with infected wild grape plants to be used as rootstocks for their resistance to the insect pest *Phylloxera* (Gessler et al., 2011). Since then, Downy Mildew has caused substantial losses in viticulture. Since the economic value of grapevine is very high (about 40–50 T€ per ha), the losses amount to some 10 T€ per ha and year, and in some cases even total losses of harvest are reported.

Consumers and society are progressively asking for sustainable forms of agriculture. In viticulture, there is strong demand for so called "ecological wine," produced without the massive use of fungicides. Thus, the key topic for sustainable viticulture is grapevine defense. The cytoskeleton with its relevance for stress adaptation in general, and its role in defense in particular, would provide interesting targets to achieve this goal. What roles are played by the cytoskeleton in the defense of grapevine?

The use of green fluorescent protein (GFP)-tagged actin marker lines (Kost et al., 1998) enabling observation of a given cell over time *in vivo* was a major breakthrough in the field, since the traditional methodology for actin visualization, by fluorescent phalloidin, required fixation of the cells, such that only the bulk changes of the cytoskeleton occurring at the late stages response became detectable. We have used a GFP-tagged actin marker line in tobacco BY-2 to probe for a response of actin to elicitors (Guan et al., 2013). A synthetic 22-amino-acid peptide (flg22) from a conserved flagellin domain was used to induce PTI (Felix et al., 1999); whereas HrpZ originating from the bean halo-blight pathogen *P. syringae* pv. *phaseolicola* was used to induce a response that in several aspects resembled ETI (Lee et al., 2001). The results of this study indicate that actin remodeling represents an early event that might partition early signaling between HrpZ-triggered ETI-like defense and flg22-triggered PTI (Guan et al., 2013).

To date, GFP-tagged marker lines for the cytoskeleton have not been available for grapevine. We therefore generated a fluorescently tagged actin marker line in grapevine using the non-invasive tag fimbrin actin-binding domain 2 (FABD2) in fusion with GFP. Using this novel tool in combination with state-of-the-art spinning disk confocal microscopy, we were able to observe actin remodeling in a defense context *in planta*. To trigger defense, we first used phytopathogenic bacteria that can produce elicitors. With this approach, we corroborated previous findings on elicitor-triggered actin responses in the tobacco cell system (Guan et al., 2013) and verified that the transgenic grapevine not only truly reports the tissue-dependent organization of actin, but also truly shows the defense response of actin. In the next step, we turned to the cognate pathogen of grapevine, *i.e.* Downy Mildew of Grapevine. Our observations point to a scenario in which guard cells act as gatekeepers and, upon attachment of the pathogen, release signals targeted on the actin of the neighboring pavement cells. These findings integrate well into a growing body of evidence that, during plant evolution, the structural function of actin that dominates in animal cells has been complemented by a sensory function of actin.

Materials and methods

Agrobacterium-mediated transformation of grapevine and molecular detection

The period of time from transformation to transfer of the transgenic plants to the greenhouse after *ex vitro* acclimation took more than one year. A screen of different independent lines for a physiological actin organization yielded two lines (5a and 10a), showing discernable fluorescent structures (Method S1; Fig. S1). PCR detection and Southern blot of genomic DNA were conducted (Method S2).

Quantitative phenotyping of transformed *Vitis* leaves (see Method S3; Fig. S2B).

Inoculation with dTomato tagged phytopathogenic Gram-negative bacteria

Marker lines of the phytopathogenic bacteria *Erwinia amylovora*, *Agrobacterium vitis* S4, and *A. tumefaciens* strain EHA105 expressing the red fluorescent protein (RFP) dTomato (in case of *A. vitis* S4 and *A. tumefaciens* strain EHA105, fusions with GFP were tested in addition) were used to inoculate the transgenic grapevine plants expressing the *GFP-AtFABD2* marker. For bacterial inoculation, the entire plant (line 10a, raised in the greenhouse) was placed under a mild vacuum immersing the target leaves into a suspension of the respective bacteria for infiltration. As negative control, a parallel sample was infiltrated with buffer [0.02 M MOPS, 2 mM sodium acetate trihydrate, 1 mM disodium EDTA, to final pH of 7.0] 3 days after inoculation, and the samples were examined by spinning disk microscopy. For this purpose, the fourth and fifth expanded leaves counted from the apex of the shoot were excised and rinsed under deionized water. Discs of 5 mm diameter were excised from the leaves with a cork borer and placed on wet filter paper in Petri dishes with the abaxial side up for microscopic examination.

Inoculation and staining of Plasmopara viticola

Fully expanded leaves of line 10a were used for this experiment, using leaves 4 and 5 counted from the apex of plants raised in the greenhouse. Leaves were excised and rinsed under deionized water. Mature sporangia of *Plasmopara viticola* (using an isolate collected in 2012 Bühl/Baden and maintained at AlPlanta) frozen at −20 °C were suspended and shaken in sterile distilled water. Motile zoospores released 1.5–2.5 h after addition of distilled water were separated from sporangia by a custom-made column stowed with 300 mg polyester wool in a 5-ml pipette tip. The filtrate was adjusted to a final concentration of ~50,000 cells per ml as determined by a hematocytometer (Fuchs-Rosenthal, Germany), and used for inoculation. To propagate the *P. viticola* isolate in the greenhouse, zoospore suspensions at a concentration of 5000–10,000 cells per ml were used for inoculation of potted vines.

3 days post-inoculation, leaf discs of 5 mm diameter were excised from the inoculation sites using a cork borer and placed on wet filter paper in Petri dishes with the abaxial side up, before infiltration in Perfluorodecalin (PFD, Sigma–Aldrich, Germany) for 3–5 min to increase transparency of the specimen. Subsequently, samples were stained for 5 min with 0.1% Calcofluor (Fluka), supplemented with 0.01% BREAK-THRU S240 (Evonik Industries, Germany), and subsequently washed three times with 0.067% M KH_2PO_4, according to Díez-Navajas et al. (2007). Some samples were stained for 5 min with 0.1% (w/v) Diphenyl Brilliant Flavine dissolved in water (Sigma–Aldrich, Germany) and were subsequently washed three times with distilled water.

Microscopy (see Method S4).

Quantitative image analysis of actin filaments (see Method S5; Fig. S3).

Results

Phenotypic analysis of transformed plants

To the best of our knowledge, in higher plants, stable fluorescent marker lines for AFs have been established only for *Arabidopsis thaliana* (Kost et al., 1998; Voigt et al., 2005), and rice (Nick et al., 2009), as well as for the tobacco cell line BY-2 (Sano et al., 2005). Grapevine genetic transformation is considered to be a risky and time-consuming procedure. However, establishing a grapevine AF fluorescent protein marker line was necessary for a better understanding of the role of the cytoskeleton in plant–pathogen interaction. Thus far, only few transgenic grapevines have been obtained by *Agrobacterium*-mediated genetic transformation originating from different organs *via* organogenesis or *via* somatic embryogenesis (Mullins et al., 1990; Colby and Meredith, 1990; Martinelli et al., 1993; Gambino et al., 2007; Fan et al., 2008). We therefore ventured on to generate a fluorescent AF marker line in the cultivar 'Chardonnay' using the non-invasive marker fimbrin actin-binding domain 2 (FABD2) representing the state-of-the art marker for plant actin (Voigt et al., 2005).

Lines 5a and 10a were selected from a total of 38 independent transgenic lines expressing the fluorescence of *GFP-AtFABD2* because they showed the normal structures of the GFP-labeled AFs. Unfortunately, line 5a grew very slowly and could not be rescued, and later died off. Details of the selection and regeneration process are provided in Supplementary Results 1.

One month after line 10a had been cultivated in the green house, GFP-expression was visually detected by irradiation with a long-wave UV lamp (UVP Blak Ray B-100 AP, Upland, CA, USA) as a greenish hue on the red background of chlorophyll fluorescence (Fig. 1A). Already at this stage, specific patterns of fluorescence were detectable in leaf and shoot tips by stereomicroscopic

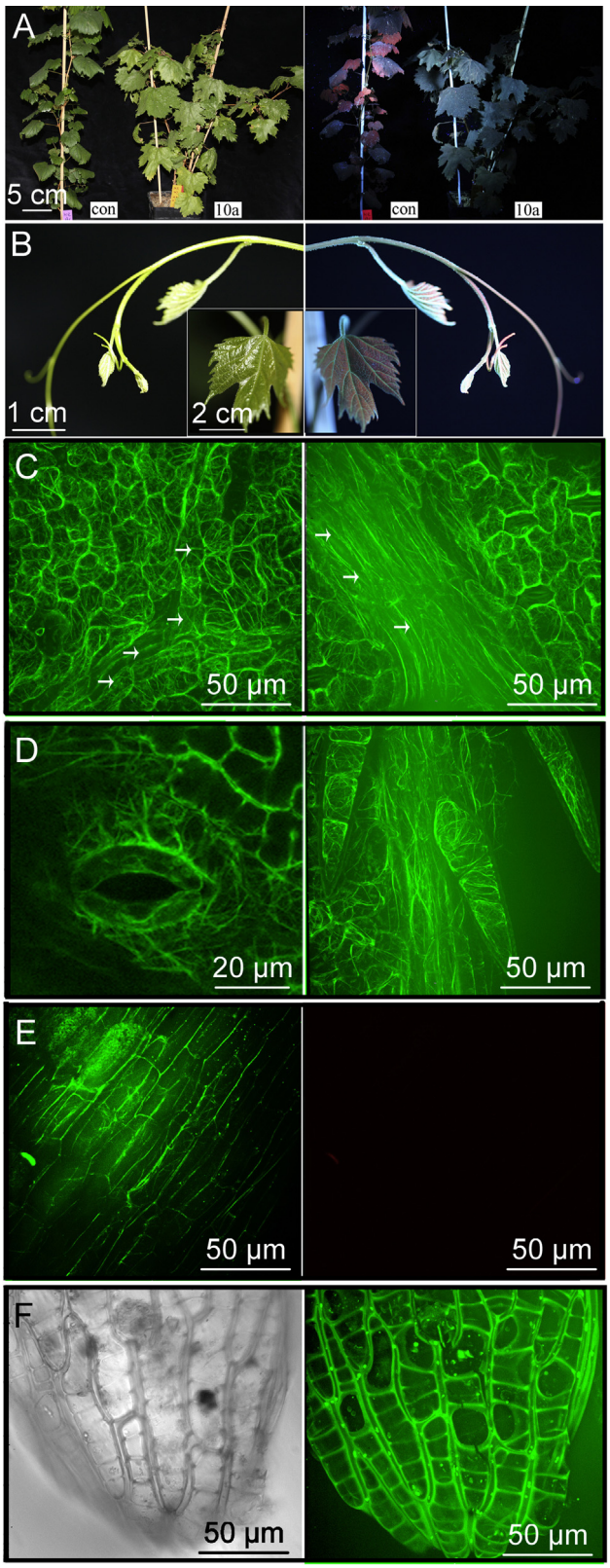

Fig. 1. Overview of transgenic *in vitro*- plants of *V. vinifera* 'Chardonnay' expressing *GFP-AtFABD2*. (A) Control (con) and *V. vinifera* 'Chardonnay' line 10a in greenhouse (white light, left; a long-wave UV lamp, right). (B) Shoot tip and leaves (inset images) (white light, left; a long-wave UV lamp, right). (C) AFs in leaf tissue (adaxial, GFP-filterset left; abaxial, GFP-filterset right). Arrows indicate the leaf vein. (D) Leaf stomata (GFP-filterset, left) and epidermal hairs (GFP-filterset, right). (E) Lateral root cap (GFP-filterset, left; CY3-filterset as negative control to probe for potential autofluorescence, right). (F) Root tip *in vivo* in dic (differential interference contrast) and GFP-filterset.

inspection under UV-A (Fig. 1B). A closer examination by spinning disk confocal microscopy revealed fluorescent filaments that in all aspects of organization, tissue-dependent changes, and morphological details resembled actin (Fig. 1C, D, E and F). For instance, actin in pavement cells (Fig. 1C) was arranged as the typical mesh-work characteristic for these cells, whereas the elongate epidermal cells lining leaf veins showed the actin cables that are aligned with the long cell axis. In developing trichomes (Fig. 1D, right-hand image), the orientation of filaments was oblique in the basal cell of young trichomes, but longitudinal in the apical cell that had completed elongation. This reorientation is a characteristic feature of actin filaments in elongating plant cells and has also been found for actin marker lines in *Arabidopsis* (Kost et al., 1998; Voigt et al., 2005) and rice (Nick et al., 2009). In the cells of the lateral root cap, transvacuolar cables of actin spanned the cell center, whereas the actin in peripheral cells was scarce, which is a characteristic actin configuration for cells that are committed to programmed cell death (important to sustain the root cap protecting the sensitive meristem from mechanical damage). In summary, the fluorescent structures visualized by the GFP-marker in this marker line truly show all of the tissue-specific features and cellular details characteristic for plant actin. To probe for potential autofluorescence that might mimick filamentous structures, the cells were routinely inspected through a CY3-filter set. However, this control was always negative (as representatively shown for the lateral root cap in Fig. 1E, right-hand image) confirming that the filamentous structures observed in the GFP-filter set represent true actin filaments.

The impression of a normal configuration of actin was supported by the normal morphology of the transgenic plants. Only a very close inspection revealed minor phenotypic differences between line 10a and non-transformed controls that had been regenerated in parallel. Leaves were thinner, and their surface appeared rough because the intercostal fields protruded somewhat. Most importantly, leaf venation differed with the main vein converging distally of the petiole joint, indicating a slight fasciation of leaf veins. This leaf phenotype also persisted after line 10a had undergone one period of overwintering and also remained persistent in the newly formed leaves. A quantitative analysis of morphology, using our custom-made program Leafkit (Fig. S2, Table S1), revealed that the angle between the central vein and the outer main veins was significantly steeper in line 10a. The value m0 was significantly larger in 10a. m4-m6 tested by multivariate analysis using one-way ANOSIM (analysis of similarity), based on a non-parametrical Duncan ranking algorithm (Hammer et al., 2001) was significantly smaller, which means that the leaves were significantly shorter in 10a and the proximal lobes more pronounced.

It appears that to obtain stable expression of cytoskeletal markers in grapevine requires a subtle balance between transgene expression, preserved cytoskeletal functionality and viability, since the T-DNA is inserted randomly into the plant genome, and the only strategy is to create a sufficient number of independent lines and to screen them for functionality and viability. To confirm our speculation, several lines exhibited GFP-fluorescence, but aberrant patterns were examined by genomic PCR with primers probing for eGFP, the AtFABD2 insert, the kanamycin resistance gene (nptII), and the left-border region of the vector. Only for the lines 10a and 26a could all four diagnostic bands be detected. A Southern analysis probing for the insert yielded a single band upon digest with *Eco*RI indicating a single insertion of the transgene (see Result S1; Figs. S4 and S5).

Response of actin filaments to different Gram-negative bacteria

Since line 10a exhibited a normal organization of actin, a correct insertion of the transgene, and only minimal morphological changes (details of venation), it qualified as the actin marker line. We therefore asked whether it is possible to observe defense-related actin responses in this line. Such actin responses can be triggered in plant cells by the elicitors HrpN (Qiao et al., 2010) and HrpZ (Guan et al., 2013). Since chemical elicitation with those proteinaceous elicitors is difficult in leaves due to the cuticle impeding the access of proteins, we tried to use the real pathogen, *Erwinia amylovora*, instead. In addition, *Agrobacterium tumefaciens* (as non-host pathogen), *A. vitis* (as host pathogen, but normally not infecting through the leaves) were used for comparison, because here flagellin-triggered immunity is suppressed by bacterial effectors. Leaf discs of *V. vinifera* 'Chardonnay' line 10a were infiltrated with dTomato marker lines of the phytopathogenic bacteria *E. amylovora*, *A. vitis* S4, and *A. tumefaciens* strain EHA105, respectively, and co-cultivated for 3 days. As negative control, leaf discs were infiltrated with buffer. Confocal z-stacks were recorded in focal planes at the level of guard cells. To ensure a comparable status of AFs that reorganize during stomatal closure (Eun and Lee, 1997), only open stomata were considered. In addition, actin was imaged in the pavement cells of the upper and lower epidermis. Concentration series of the inoculum (with OD_{600} ranging from 0.1 to 1.0) were tested in preparatory experiments to adjust optimal conditions. To visualize bacteria and host actin simultaneously, the z-stacks were recorded at dual wavelengths (using both, the RFP- and GFP-filter sets).

As compared to the negative control, inoculation of the bacteria caused significant changes of AFs at 3 dpi at those sites, where the bacteria had attached (Fig. 2A). Since some of these responses involved changes of AF bundling, the image series were analyzed by quantitative image analysis (Fig. 3) based on a method in which the skewness of the fluorescence intensity distribution is used as reporter for the degree of AF bundling (Higaki et al., 2010). As actin in the lip-shaped guard cells, consistent with the published literature on actin, was organized distinctly from the actin mesh-work in the epidermal pavement cells, we asked whether the response of the guard-cell actin differs from that in the pavement cells. Whereas actin filaments in the control guard cells were contiguous, for inoculation with *E. amylovora*, actin was present in short rods or fragments (Fig. 2Ad), but had not altered their bundling state as evident from a constant skewness of 96% in the guard cells (Fig. 3A) compared to the non-infiltrated control (Fig. 2Aa). For inoculation with *A. vitis*, AFs appeared finer (Fig. 2Ai), and were excluded from the nuclear region of guard cells (Fig. 2Al inset image presenting the certain Z stack layers of nuclear region). This impression was confirmed by the quantitative analysis. Here, guard cells showed a reduced skewness of 40% relative to the controls, indicating that the thinning of AFs was significant (Fig. 3A). The same result was obtained upon inoculation with *A. tumefaciens* strain EHA105 (Fig. 2An-p). Here, skewness was reduced to 46% of the control value (Fig. 3A). These changes of actin organization were confined to the guard cells. In the other epidermal cells, neither inoculation with *E. amylovora*, nor with *A. tumefaciens* strain EHA105 and *A. vitis* produced any significant difference (compare Fig. 2Ag, l and q with Fig. 2Ab), which was also confirmed by quantifying skewness (Fig. 3B). Also, the AFs in the pavement cells of the upper epidermis did not display any significant response, although dTomato tagged bacteria also had attached to those cells (compare Fig. 2Ah, m and r with Fig. 2Ac). It should be noted that no indication of necrotic phenomena was observed when the leaves were monitored by stereo microscopy after infiltration. These observations show that (i) AFs are altered in response to contact with phytopathogenic bacteria, (ii) the quality of the AF response depends on the bacterial species used for inoculation (*E. amylovora*: disruption of AFs; *A. vitis* and *A. tumefaciens*: reduced bundling of AFs), and, (iii) the response is confined to the guard cells and not observed in the pavement cells of the lower or the upper epidermis, although the bacteria can attach to those cells as

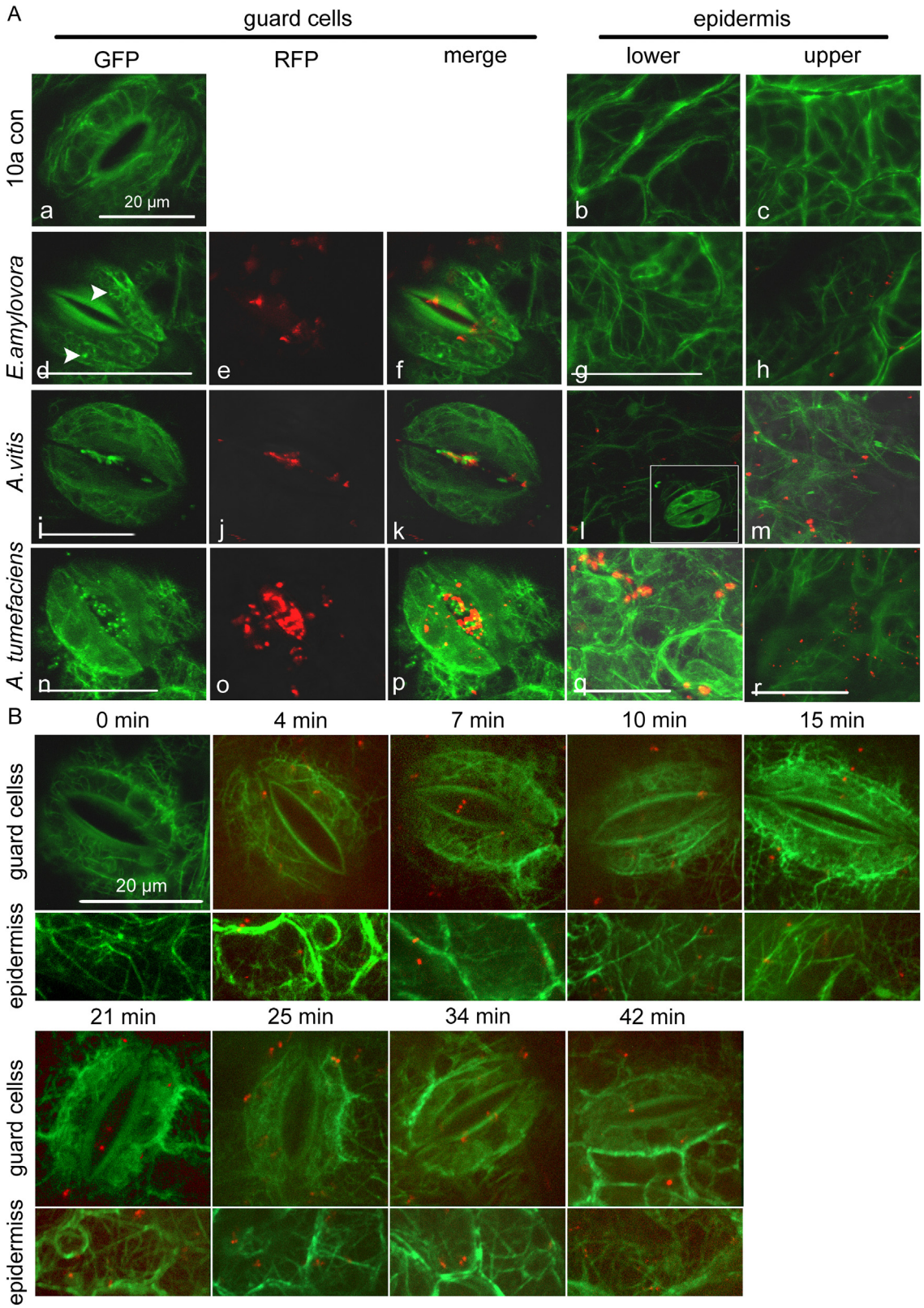

Fig. 2. Response of actin filaments to different Gram-negative bacteria visualized by spinning-disk confocal microscopy. (A) Inoculated after 3 days, AF structures in leaves of *V. vinifera* 'Chardonnay' line 10a expressing *GFP-AtFABD2* shown in green, the bacteria labeled red by the dTomato marker. *Erwinia amylovora* at guard cells (d, GFP, e, RFP, f, merge) and epidermal cells (g, lower; h, upper epidermal cells), *Agrobacterium vitis* S4 at guard cells (i, GFP, j, RFP, k, merge), exclusion of the signal from the nuclear region of guard cells (inset image in l) and epidermal cells (l, lower, m, upper epidermal cells) after inoculation with *A. vitis* S4, *Agrobacterium tumefaciens* strain EHA105 at guard cells (n, GFP, o, RFP, p, merge) and epidermal cells (q, lower; r, upper epidermal cells). Unless otherwise indicated, images were recorded 3d post-inoculation with bacteria grown to OD_{600} ~0.4, Non-inoculated *V. vinifera* 'Chardonnay' GFP-*At*FABD2 line 10a (a, guard cell, b, lower, c, upper epidermal cells) was used as control. White arrow heads indicate the disrupted AFs. (B) Time series after inoculation with *E. amylovora*. The merged images show the AFs responses in guard cells and epidermal cells. Bar = 20 μm.

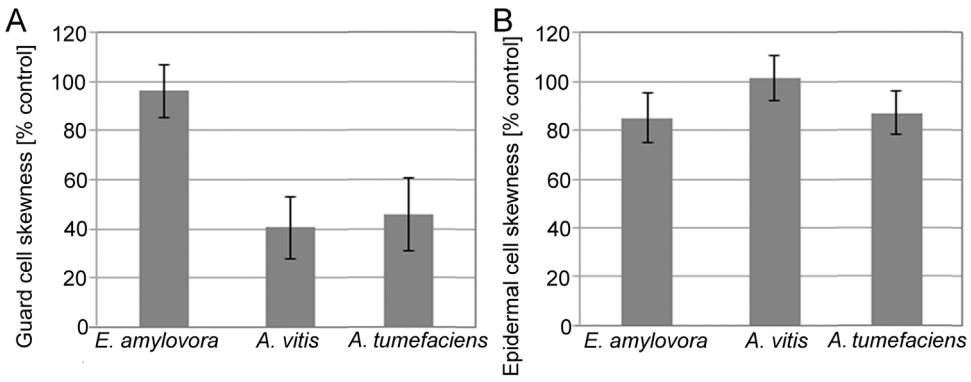

Fig. 3. Actin filaments bundling quantified by quantitative image analysis based on skewness values: in guard cells (A) and epidermal pavement cells (B). Data represent mean values and standard errors from a population of 30–50 individual cells.

well. To understand whether the fragmentation in response to *E. amylovora* was an early response, early time points were followed during the first hour after inoculation (Fig. 2B). Since we could not control to which cell the bacteria would attach, individual frames from different cells had to be recorded over the entire time interval. Fragmentation and elimination of AFs in guard cells could be detected as early as 15 min after inoculation, whereas there was no change in pavement cells over the entire observation period.

Response of actin filaments to Plasmopara viticola

The bacterial inoculation experiment demonstrated an actin response that was specific in quality and specific for a cell type (guard cells). However, this experiment does not report on a realistic scenario, because these bacteria do not infect their hosts through the guard cells. To exploit the full potential of the actin-marker lines for our understanding of plant defense, a pathogen had to be chosen that actually uses guard cells as entrance gate for infection: the oomycete *P. viticola*. A precondition to conduct this experiment was the ability to visualize encysted zoospores, sporangiophores, and mycelium structures within the living leaf tissue. This could be achieved by staining with the fluorescent dyes Calcofluor and Diphenyl Brilliant Flavine in combination with Perfluorodecalin, a compound that renders leaf tissue more translucent. Three days after inoculation with zoospores of *P. viticola*, AFs in guard cells had disassembled (Fig. 4). In contrast to the situation after inoculation with bacteria (Fig. 2), the response was not confined to the guard cells, but had extended to the neighboring epidermal pavement cells, although these were not in contact with the zoospores with respect to their encysted states, (which could be visualized by Calcofluor staining) nor with the early mycelia that could be seen at the substomatal cavity upon staining with Diphenyl Brilliant Flavine. The normal actin network seen in these pavement cells was replaced by a fluorescent cage surrounding the nucleus. These fluorescent structures were not observed upon infection of the non-transformed *V. vinifera* 'Chardonnay' wild type, excluding that they were caused by auto fluorescence of nucleus or plastids (Fig. S6), supporting that actin in these cells is rearranged into a perinuclear structure.

Discussion

A grapevine actin marker line

Methods shape concepts – the availability of fluorescent proteins allowing for imaging of specific organelles in living cells has revealed the seemingly static plant cells as highly dynamic systems. The term "cytoskeleton" was coined at a time when actin filaments and microtubules were accessible only through electron microscopy in ultrathin sections of fixed material. The concept of a static "cellular skeleton" has been replaced by the model of a dynamic equilibrium as evident, when fluorescent markers are followed after local bleaching (so-called fluorescence recovery after photo bleaching, FRAP), or when local changes of fluorescent color are followed, a novel approach made possible by the use of photoconvertible fluorescent proteins (Mathur et al., 2010). Fluorescently tagged transgenic actin-marker lines have been generated for *A. thaliana* (Kost et al., 1998; Voigt et al., 2005), tobacco BY-2 cells (Sano et al., 2005), and rice (Nick et al., 2009), and were useful to analyze actin responses and functions in growing pollen tubes, in root hairs, in stomatal movement, motility of plant organelles, and auxin transport (Ren et al., 1997; Kost et al., 1998; Cárdenas et al., 1998; Higaki et al., 2010; Holweg, 2007; Nick et al., 2009). For grapevine, an important model for perennial woody plants, and at the same time a central fruit crop of high economic impact, these tools have not been available. Such tools would be highly desirable, however, since AFs play a major role in defense against pathogens, a central topic for sustainable viticulture (for review see, Day et al., 2011). We therefore established a fluorescent protein-tagged actin marker line in grapevine. We used the cultivar 'Chardonnay' originating from the Burgundy region, one of the most widespread grape varieties, with over 160,000 hectares worldwide.

This approach was successful for the AF marker line in using embryogenic suspension cells of *V. vinifera* 'Chardonnay'. However, the success rate was low: from 38 plantlets expressing *GFP-AtFABD2* (Fig. S4) that were successfully regenerated, only one line could be obtained that reliably reports AFs and preserved a normal physiology. All successful regenerations were from the embryogenic suspension line of *V. vinifera* 'Chardonnay' (regenerates were neither obtained from parallel attempts with *V. vinifera* 'Müller-Thurgau,' nor from the root stock variety SO4). The regeneration progress of the recovered lines lasted at least 12 months, which is much longer than previous work using other constructs that have no relation with the cytoskeleton. This indicates impaired functionality, likely due to the overexpression of the marker that interferes with cytoskeletal function, giving rise to vital affects in cell development and plant morphogenesis (Holweg, 2007; Voigt et al., 2005). Other factors, such as somatoclonal variation, can modulate somatic embryogenesis as well (Schellenbaum et al., 2008; for review see, Miguel and Marum, 2011), but this would also occur in non-cytoskeletal transgenes, suggesting that marker visibility and the avoidance of cytoskeletal aberrations due to elevated expression of the marker have to be reconciled in a subtle balance, which was achieved only in few of the transformed lines.

Although the FABD2-marker is generally discussed as non-invasive, it has a subtle effect on actin dynamics and cellular processes that depend on actin. Similar to the mouse-talin marker (Ketelaar et al., 2004), it binds to the surface of actin at a site, where

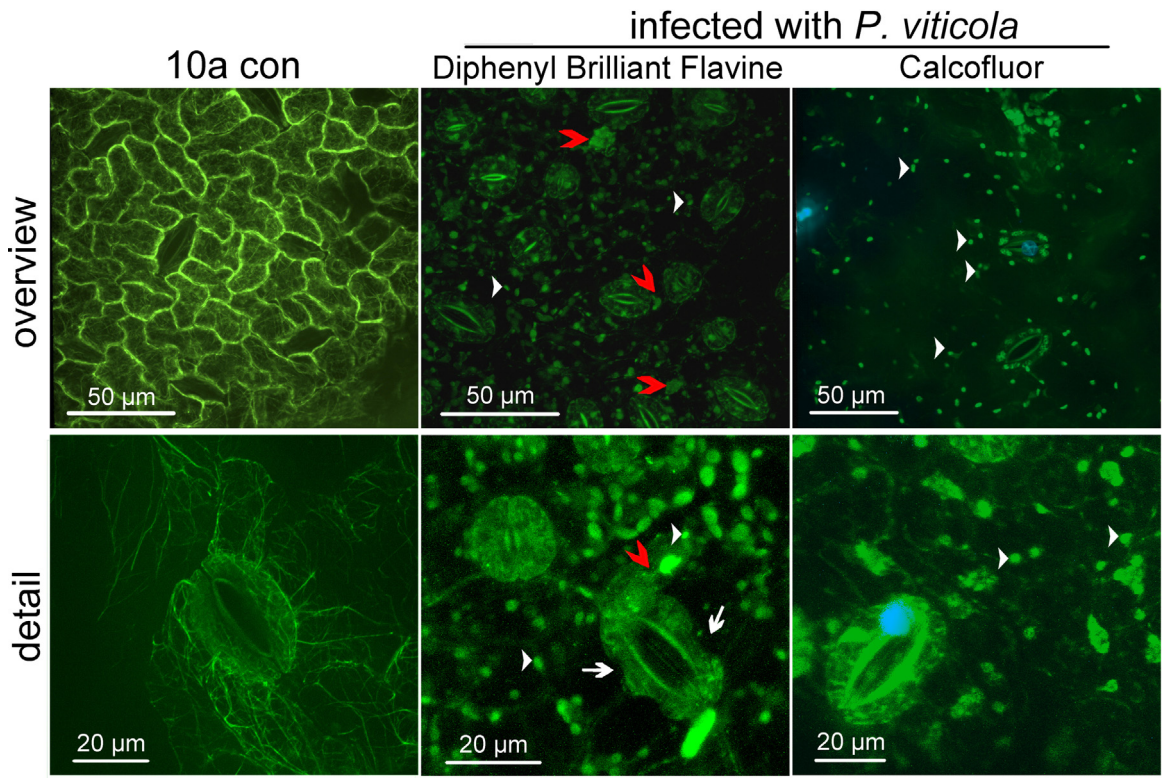

Fig. 4. Response of actin filaments in the lower leaf surface of *V. vinifera* 'Chardonnay' expressing *GFP-AtFABD2* to infection with *P. viticola* visualized by spinning-disk confocal microscopy. Encysted zoospores attached to the stomata were visualized by Calcofluor (blue emission), intracellular hyphae by Diphenyl Brilliant Flavine (green emission, red arrow heads). White arrow heads indicate the perinuclear actin baskets; White arrows indicate plasmolytic guard cells indicative of aberrant AFs organization. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

normally actin-depolymerizing factors would attach resulting in a reduced dynamics of actin. These changes in actin dynamics are minor in the case of the FABD2-marker, but they become detectable through sensitive assays (for details refer to Durst et al., 2013; Zaban et al., 2013). For instance, there is a significant alteration of polar auxin transport observed in *Arabidopsis* lines expressing this marker (Holweg, 2007), and protoplasts from tobacco BY-2 cells expressing this marker show aberrations of cell polarity that can be mimicked by genetic or pharmacological stabilization of actin (Zaban et al., 2013). The specific venation phenotype in line 10a (partial fasciation, steeper angle of the peripheral main veins) is indicative of accelerated polar auxin transport, which would be expected from a slightly elevated stability of actin filaments due to expression of this marker (reviewed in Nick, 2010).

However, despite this slight stabilization, we found that actin organization was completely normal and congruent with the findings reported for the actin marker lines in *Arabidopsis* (Kost et al., 1998; Voigt et al., 2005) and rice (Nick et al., 2009). This was true down to the cellular and tissue-specific details (Fig. 1). The meshwork in epidermal pavement cells, the qualitative difference in actin organization between pavement and guard cells, the progressive alignment of actin with the elongation axis in expanding trichomes as well as the formation of transvacuolar actin cables accompanied by a depletion of cortical actin heralding ensuing cell death in cells of the lateral root cap are all truly reported by this marker and perfectly match the published record on actin organization in other plants such as *Arabidopsis* (Kost et al., 1998; Voigt et al., 2005) and rice (Nick et al., 2009).

Following actin responses to bacterial pathogens in vivo

Since actin remodeling was identified as an early event to the bacterial effector HrpZ in the BY-2 suspension cell system (Guan et al., 2013), we tested whether actin remodeling can also be observed in a real plant with real pathogens using the economically important model plant grapevine. We made use of our transgenic actin-marker grapevine and followed the response of AFs to phytopathogenic Gram-negative bacteria *in vivo*. The outcome of these experiments can basically be condensed into two conclusions: (1) Actin filaments in the guard cells were responsive, whereas AFs in epidermal pavement cells did not respond, even if bacteria were attached. (2) The quality of this actin response in the guard cells was dependent on the type of bacterial pathogen: for inoculation with *E. amylovora*, AFs in the guard cells appeared fragmented, but did not alter their bundling state. In contrast, for inoculation with the two species of *Agrobacterium*, AFs became significantly thinner, but were not fragmented. Why is the actin response confined to the guard cells? There are two possible explanations. Either actin filaments in guard cells are more dynamic compared to those in epidermal pavement cells, or the actin filaments in guard cells are linked to guard-cell specific signaling that is absent in epidermal pavement cells. The hypothesis of actin dynamics in guard cells being elevated over that in pavement cells is not consistent with the outcome of studies where the rate of actin turnover was estimated from fluorescence-recovery after photo bleaching (FRAP) of the fluorescently tagged FABD marker yielding very similar values for the two cell types (Sheahan et al., 2004). This favors the second possibility that guard cell actin is coupled to specific signaling that is absent from pavement cells.

Guard cells can sense their environment and integrate this sensing into complex intracellular signaling (for review see, Kim et al., 2010). This complex signaling network provides numerous targets for pathogen effectors directed to keeping the stomata open in order to facilitate penetration (for instance coronatine and *P. syringae*, Melotto et al., 2006). Stomatal aperture and closure are mediated by ion fluxes driving consequent osmotic fluxes of water.

Actin filaments are necessary for stomatal closure in response to abscisic acid (Kim et al., 1995) and are regulated by abscisic acid triggered signaling involving calcium and kinase/phosphatases as signals (Hwang and Lee, 2001; Eun et al., 2001). Although AFs do not mediate the aperture and closure themselves, they play an important role in the regulation of the underlying signal transduction, for instance by regulation of ion channels (Hwang et al., 1997; Zhang and Fan, 2009) or by regulating the flux membrane material required during volume control of plant cells (Liu et al., 2013).

The attachment of the bacterial pathogens might interfere this guard cell-specific signaling. This interference seems to be specific, because it differs between *E. amylovora* and the two *Agrobacteria* tested in our experiments. In contrast to other bacteria that trigger PTI through binding of a conserved motif on flagellin with the plant receptor FLS2, *Agrobacteria* can escape recognition by FLS2 because they harbor a flagellin lacking this conserved motif (Felix et al., 1999). Additional candidates for the differential actin response include bacterial exopolysaccharides consisting of succinoglycan, cellulose, and cyclic β-(1,2)-glucanes in *Agrobacterium*, but of levan and amylovoran in *E. amylovora* (Denny, 1995; Vrancken et al., 2013). Moreover, *E. amylovora* employs a type-III secretion system, whereas the VirB and VirD4 proteins of *Agrobacterium* constitute a type-IV secretion system (for review McCullen and Binns, 2006).

The underlying mechanisms of stomatal AF reorganization are not known – there exist numerous possibilities such as elongation and shortening, buckling and straightening, bundling and branching (Smertenko et al., 2010). In addition, AFs can form longer filaments by end-joining activity differing from the model of traditional treadmill. To discriminate between these possible mechanisms that can also act in combination is far from trivial, since the configuration of AFs is extremely complex. This obscures any response that is not really drastic – usually observed only in non-physiological situations. To detect the more subtle responses characteristic for the physiological range, quantitative approaches are required. This has been a challenge for the complex actin cytoskeleton, but has been facilitated by semiautomatic algorithms for quantitative image analysis (Higaki et al., 2010).

Using this strategy, we found that the two species of *Agrobacterium* caused a significant debundling of AFs, whereas the fragmentation of AFs for inoculation with *E. amylovora* was not accompanied by changes of bundling (Fig. 3). From these data, two (speculative) working models can be proposed for the molecular mechanism. *E. amylovora* might trigger the severing of actin bundles, but this is not followed by the next step of natural actin remodeling, the annealing of fragments to new actin cables such that actin is disrupted and stomatal closure suppressed. Annealing is a process involving phospholipase D-dependent inhibition of actin capping proteins (Li et al., 2012), which might be a target for pathogen effectors. In contrast, the two *Agrobacteria* species trigger a detachment of actin bundles into finer strands, which is more likely caused by changes in the activity of nucleation regulators such as Arp2/3 and ADFs. Nevertheless, the reduction in bundling should lead to the same outcome, suppression of stomatal closure. However, one should bear in mind that *Agrobacteria* do not use this route of infection in nature – they enter through the root system, in contrast to *E. amylovora* that infects the upper parts of the plants, and enters the plant *via* open stomata (reviewed by Melotto et al., 2008), but is not a natural pathogen of grapevine. These bacteria were used mainly for operational reasons – chemical elicitation by the proteinacious elicitors HrpN (Qiao et al., 2010) and HrpZ (Guan et al., 2013) that could efficiently trigger defense-related actin responses in suspension cells turned out to be difficult in leaves, likely due to the cuticle impeding penetration of the elicitors. We therefore resorted to the natural source of these elicitors, the phytopathogenic bacterium *E. amylovora*. To test whether the observed response requires activation flagellin triggered signaling, we inoculated, in parallel, with *A. tumefaciens* (as non-host pathogen), and *A. vitis* (as host pathogen, but normally not infecting through the leaves), because these pathogens can evade flagellin-triggered immunity.

Actin-targeted signaling by guard cells in response to Plasmopara viticola

If the specificity of actin responses in the guard cells is caused by specific modulation of pathogen effectors, different pathogens might trigger different actin responses. With respect to practical relevance, Downy Mildew (*P. viticola*), as cognate oomycete pathogen of grapevine and as eukaryotic organism not related with phytopathogenic bacteria, was chosen. Based on the newly generated marker line we were able to study, for the first time, the response of AFs to infection with *P. viticola in vivo*. Similar to the case of *E. amylovora*, we observed disruption of AFs in guard cells, suggesting a common molecular mechanism. There was, however, a decisive difference: upon infection with *P. viticola*, AFs also responded in the epidermal pavement cells by forming a perinuclear actin basket in epidermal pavement cells, although they had not directly been in contact with the zoospores.

Such perinuclear actin baskets are normally observed in vacuolated plant cells preparing for mitosis (for review, see Nick, 2012). Together with radial arrays of cytoplasmic microtubules and plant-specific minus-end directed kinesins containing a calponin homology domain (KCH), this perinuclear actin basket controls the positioning of the premitotic nucleus (Klotz and Nick, 2012). In epidermal cells, these perinuclear baskets are not observed under physiological conditions. However, they can be induced by elimination of cortical AFs by mild treatment with cytochalasin D, indicating that two populations of actin-nucleation sites, in cell cortex *versus* nuclear envelope, compete for a limited pool of assembly competent G-actin (Wang and Nick, 1998). In a rice mutant, *Yin-Yang*, where the responsiveness of cell growth to auxin is elevated (Waller et al., 2002), such baskets can be induced by incubation with auxin (Wang and Nick, 1998). The effect of auxin upon actin is mediated by actin-depolymerization factors (Durst et al., 2013), *i.e.* by the same group of actin-associated proteins already shown to initiate ETI in case of *P. syringae* (Tian et al., 2009). The nature of the signal conveyed from the attacked guard cells to the neighboring pavement cells remains to be elucidated, however. It is not even clear whether this signal originates from the pathogen or from the guard cell, where the pathogen has attached. *P. viticola* is an obligate biotrophic pathogen invading its highly vacuolated victim cells by haustoria, which means that the pathogen has to overcome conspicuous counterforces (in the range of 5–10 bars), due to the difference of osmotic potential between cytoplasm and apoplast at the site, where the cell wall has been penetrated. It is highly likely that the pathogen reprograms the actin cytoskeleton of the host, as it has already been shown for the reprogramming of root hairs into an infection thread during rhizobial symbiosis or the generation of arbuscular mycorrhiza (Miller et al., 1999). The sequestering of actin into the perinuclear basket might represent an efficient strategy of the host cell to prevent the *pathogen-triggered* reprogramming of actin. On the other hand, 'Chardonnay' is susceptible to Downy Mildew. It did not coevolve with this pathogen and therefore, unlike its North American relatives, fails to trigger hypersensitive cell death in response to infection with *P. viticola*. Remodeling of AFs culminating in massive bundling of actin cables is an essential element of programmed cell death in plants (for review, see Smertenko and Franklin-Tong, 2011). The sequestering of actin into the perinuclear basket might therefore also represent an efficient strategy of the pathogen to prevent the *host-triggered* reprogramming of actin.

Outlook: toward an actin guard cell gatekeeper model

Guard cells represent the vulnerable point of the otherwise well protected leaf surface and therefore are used as major entrance route for many phytopathogenic microorganisms. Stomatal closure is under control of complex signaling driving ion fluxes across the plasma membrane, and actin is directly involved in the regulation of major ion channels. On the other hand, actin remodeling represents a pivotal signal for the induction of hypersensitive cell death, one of the most effective means to block the spread of an invading pathogen. This makes guard cell actin a prime target for microbial effectors on the one hand; on the other actin is an important player to execute efficient plant defense. Our data are consistent with a model under which guard cell actin acts as a gatekeeper that is under antagonistic evolutionary pressure from both host and pathogen. From the host perspective, actin functionality ensures closing stomata, which is a very efficient way to block pathogen attack. From the pathogen perspective, impaired actin functionality allows the keeping of stomata open, which facilitates colonization of the host. A second level of warfare is targeted on the signaling role of actin in defense. Actin remodeling in the surrounding host cells would trigger programmed cell death and block infection, and suppression of this actin response would allow biotrophic pathogens to quietly exploit the infected host cells. A prediction derived from this actin guard cell gatekeeper model would be signaling from the guard cells to the neighboring cells. This has been detected for infection with the cognate pathogen *P. viticola* (in contrast to the non-host pathogens). This is a working model, and by its nature it is still speculative, of course. However, it sets the path for future work: The signal conveyed from guard cells to neighboring cells has to be characterized with respect to timing, mode of transport and quality as precondition to identify this signal. To understand the biological function of this signal, it is important to compare the susceptible interaction (present in 'Chardonnay') with the situation in a resistant host. We are therefore launching attempts to obtain actin marker lines in Downy Mildew resistant genotypes of North American origin (*V. rupestris* and *V. riparia*).

Conflict of interests

The authors declare that there is no conflict of interests whatsoever.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2014.03.019.

References

Bertani G. Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. J Bacteriol 1951;62:293–300.

Bestwick CS, Bennett MH, Mansfield JW. Hrp mutant of *Pseudomonas syringae* pv *phaseolicola* induces cell wall alterations but not membrane damage leading to the hypersensitive reaction in lettuce. Plant Physiol 1995;108:503–16.

Chen H, Nelson RS, Sherwood JL. Enhanced recovery of transformants of *Agrobacterium tumefaciens* after freeze-thaw transformation and drug selection. Biotechniques 1994;16:664–70.

Colby SM, Meredith CP. Kanamycin sensitivity of cultured tissues of *Vitis*. Plant Cell Rep 1990;9:237–40.

Cárdenas L, Vidali L, Domínguez J, Pérez H, Sánchez F, Hepler PK, et al. Rearrangement of actin microfilaments in plant root hairs responding to *Rhizobium etli* nodulation signals. Plant Physiol 1998;116:871–7.

Day B, Henty JL, Porter KJ, Staiger CJ. The pathogen–actin connection: a platform for defense signaling in plants. Annu Rev Phytopathol 2011;49:483–506.

De Lumley H. La stratigraphie du remplissage de la Grotte du Vallonnet. L'Anthropologie 1988;92:407–28.

Denny TP. Involvement of bacterial polysaccharides in plant pathogenesis. Annu Rev Phytopathol 1995;33:173–97.

Díez-Navajas AM, Greif C, Poutaraud A, Merdinoglu D. Two simplified fluorescent staining techniques to observe infection structures of the oomycete *Plasmopara viticola* in grapevine leaf tissues. Micron 2007;36:680–3.

Doyle JJ, Doyle JL. A rapid DNA isolation procedure from small quantities of fresh leaf tissues. Phytochem Bull 1987;19:11–5.

Duncan DB. Multiple range and multiple *F* test. Biometrics 1955;11:1–42.

Durst S, Nick P, Maisch J. Actin-depolymerizing factor 2 is involved in auxin dependent patterning. J Plant Physiol 2013;170:1057–66.

Eun SO, Lee Y. Actin filaments of guard cells are reorganized in response to light and abscisic acid. Plant Physiol 1997;115:1491–8.

Eun SO, Bae SH, Lee Y. Cortical actin filaments in guard cells respond differently to abscisic acid in wild type and *abi1-1* mutant *Arabidopsis*. Planta 2001;212:466–9.

Fan CH, Pu N, Wang XP, Wang YJ, Fang L, Xu WR, et al. *Agrobacterium*-mediated genetic transformation of grapevine (*Vitis vinifera* L.) with a novel stilbene synthase gene from Chinese wild *Vitis pseudoreticulata*. Plant Cell Tiss Org 2008;92:197–206.

Felix G, Duran JD, Volko S, Boller T. Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. Plant J 1999;18:265–76.

Gambino G, Ruffa P, Vallania R, Gribaudo I. Somatic embryogenesis from whole flowers, anthers and ovaries of grapevine (*Vitis* spp.). Plant Cell Tiss Org 2007;90:79–83.

Gessler C, Pertot I, Perazzolli M. *Plasmopara viticola*: a review of knowledge on downy mildew of grapevine and effective disease management. Phytopathol Mediterr 2011;50:3–44.

Gianessi L, Williams A. Fungicides have protected European wine grapes for 150 years. International pesticide benefits case study, No. 19. Washington, DC: CropLife Foundation; 2011.

Guan X, Buchholz G, Nick P. The cytoskeleton is disrupted by the bacterial effector HrpZ, but not by the bacterial PAMP flg22, in tobacco BY-2 cells. J Exp Bot 2013;64:1805–16.

Hammer Ø, Harper DAT, Ryan PD. PAST: Paleontological statistics software package for education and data analysis. Palaeontol Electron 2001;4:9.

Higaki T, Kutsuna N, Sano T, Kondo N, Hasezawa S. Quantification and cluster analysis of actin cytoskeletal structures in plant cells: role of actin bundling in stomatal movement during diurnal cycles in *Arabidopsis* guard cells. Plant J 2010;61:156–65.

Holweg CL. Living markers for actin block myosin-dependent motility of plant organelles and auxin. Cell Motil Cytoskel 2007;64:69–81.

Hwang JU, Lee Y. Abscisic acid-induced actin reorganization in guard cells of dayflower is mediated by cytosolic calcium levels and by protein kinase and protein phosphatase activities. Plant Physiol 2001;125:2120–8.

Hwang JU, Suh S, Yi H, Kim J, Lee Y. Actin filaments modulate both stomatal opening and inward K^+-channel activities in guard cells of *Vicia faba* L. Plant Physiol 1997;115:334–42.

Jaillon O, Aury JM, Noel B, Policriti A, Clepet C, Casagrande A, et al. The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. Nature 2007;449:463–8.

Jones JD, Dangl JL. The plant immune system. Nature 2006;444:323–9.

Ketelaar T, Anthony RG, Hussey PJ. Green fluorescent protein-mTalin cause defects in actin organization and cell expansion in *Arabidopsis* and inhibits actin depolymerizing factor's actin depolymerizing activity *in vitro*. Plant Physiol 2004;136:3990–8.

Kim M, Hepler PK, Eun SO, Ha KS, Lee Y. Actin filaments in mature guard cells are radially distributed and involved in stomatal movement. Plant Physiol 1995;109:1077–84.

Kim TH, Böhmer M, Hu H, Nishimura N, Schroeder JI. Guard cell signal transduction network: advances in understanding abscisic acid, CO_2, and Ca^{2+} signaling. Annu Rev Plant Biol 2010;61:561–91.

Kirchheimer F. Beiträge zur näheren Kenntnis von Vitaceen-Samenformen tertiären Alters. Planta 1938;28:582–98.

Klotz J, Nick P. A novel actin-microtubule cross-linking kinesin, NtKCH, functions in cell expansion and division. New Phytol 2012;193:576–89.

Kobayashi Y, Kobayashi I. Microwounding is a pivotal factor for the induction of actin-dependent penetration resistance against fungal attack. Planta 2013;237:1187–98.

Kost B, Spielhofer P, Chua N. A GFP-mouse talin fusion protein labels plant actin filaments *in vivo* and visualizes the actin cytoskeleton in growing pollen tubes. Plant J 1998;16:393–401.

Lee J, Klüsener B, Tsiamis G, Stevens C, Neyt C, Tampakakii AP, et al. HrpZPsph from the plant pathogen *Pseudomonas syringae pv. phaseolicola* binds to lipid bilayers and forms an ion-conducting pore *in vitro*. Proc Natl Acad Sci USA 2001;98:289–94.

Li J, Henty-Ridilla JL, Huang S, Wang X, Blanchoin L, Staiger CJ. Capping protein modulates the dynamic behavior of actin filaments in response to phosphatidic acid in *Arabidopsis*. Plant Cell 2012;24:3742–54.
Liu Q, Qiao F, Ismail A, Chang X, Nick P. The plant cytoskeleton controls regulatory volume increase. Biochim Biophys Acta 2013., http://dx.doi.org/10.1016/j.bbamem.2013.04.027.
Maisch J, Fišerová J, Fischer L, Nick P. Tobacco Arp3 is localized to actin-nucleating sites *in vivo*. J Exp Bot 2009;60:603–14.
Mathur J, Radhamony R, Sinclair AM, Donoso A, Dunn N, Roach E, et al. mEosFP-based green-to-red photoconvertible subcellular probes for plants. Plant Physiol 2010;154:1573–87.
Martinelli L, Bragagna P, Poletti V, Scienza A. Somatic embryogenesis from leaf- and petiole- derived callus of *Vitis rupestris*. Plant Cell Rep 1993;12:207–10.
McCullen CA, Binns AN. *Agrobacterium tumefaciens* and plant cell interactions and activities required for interkingdom macromolecular transfer. Annu Rev Cell Dev Biol 2006;22:101–27.
Melotto M, Underwood W, He SY. Role of stomata in plant innate immunity and foliar bacterial diseases. Annu Rev Phytopathol 2008;46:101–22.
Melotto M, Underwood W, Koczan J, Nomura K, He SY. Plant stomata function in innate immunity against bacterial invasion. Cell 2006;126:969–80.
Miguel C, Marum L. An epigenetic view of plant cells cultured *in vitro*: somaclonal variation and beyond. J Exp Bot 2011;62:3713–25.
Miller DD, de Ruijter NCA, Bisseling T, Emons AMC. The role of actin in root hair morphogenesis: studies with lipochito-oligosaccharide as a growth stimulator and cytochalasin as an actin perturbing drug. Plant J 1999;17:141–54.
Moroldo M, Paillard S, Marconi R, Fabrice L, Canaguier A, Cruaud C, et al. A physical map of the heterozygous grapevine 'Cabernet Sauvignon' allows mapping candidate genes for disease resistance. BMC Plant Biol 2008;8:66–80.
Mullins MA, Tang FCA, Facciotti D. *Agrobacterium*-mediated transformation of grapevines: transgenic plants of *Vitis rupestris* Scheele and buds of *Vitis vinifera* L. Nat Biotechnol 1990;18:1041–5.
Nick P. Probing the actin-auxin oscillator. Plant Signal Behav 2010;5:4–9.
Nick P. Microtubules and the tax payer. Protoplasma 2012;249(Suppl. 2):S81–94.
Nick P, Han M, An G. Auxin stimulates its own transport by actin reorganization. Plant Physiol 2009;151:155–67.
Nitsch JP, Nitsch C. Haploid plants from pollen grains. Science 1969;163:85–7.
Opalski KS, Schultheiss H, Kogel KH, Huckelhoven R. The receptor-like MLO protein and the RAC/ROP family G-protein RACB modulate actin reorganization in barley attacked by the biotrophic Powdery Mildew fungus *Blumeria graminis* f. sp. *hordei*. Plant J 2005;41:291–303.
Qiao F, Chang X, Nick P. The cytoskeleton enhances gene expression in the response to the Harpin elicitor in grapevine. J Exp Bot 2010;61:4021–31.
Ren H, Gibbon BC, Ashworth SL, Sherman DM, Yuan M, Staiger CJ. Actin purified from maize pollen functions in living plant cells. Plant Cell 1997;9:1445–57.
Robatzek S, Chinchilla D, Boller T. Ligand-induced endocytosis of the pattern recognition receptor FLS2 in *Arabidopsis*. Genes Dev 2006;20:537–42.
Sano T, Higaki T, Oda Y, Hayashi T, Hasezawa S. Appearance of actin microfilament 'twin peaks' in mitosis and their function in cell plate formation, as visualized in tobacco BY-2 cells expressing GFP-fimbrin. Plant J 2005;44:595–605.
Schellenbaum P, Mohler V, Wenzel G, Walter B. Variation in DNA methylation patterns of grapevine somaclones (*Vitis vinifera* L.). BMC Plant Biol 2008;8:78–88.
Schmelzer E. Cell polarization, a crucial process in fungal defence. Trends Plant Sci 2002;7:411–5.
Sheahan MB, Staiger CJ, Rose RJ, McCurdy DW. A green fluorescent protein fusion to actin-binding domain 2 of *Arabidopsis* fimbrin highlights new features of a dynamic actin cytoskeleton in live plant cells. Plant Physiol 2004;136:3968–78.
Smertenko AP, Deeks MJ, Hussey PJ. Strategies of actin reorganisation in plant cells. J Cell Sci 2010;123:3019–28.
Smertenko A, Franklin-Tong VE. Organisation and regulation of the cytoskeleton in plant programmed cell death. Cell Death Differ 2011;18:1263–70.
Tian M, Chaudhry F, Ruzicka DR, Meagher RB, Staiger CJ, Day B. *Arabidopsis* actin depolymerizing factor AtADF4 mediates defense signal transduction triggered by the *Pseudomonas syringae* effector AvrPphB. Plant Physiol 2009;150:815–24.
Voigt B, Timmers ACJ, Šamaj J, Müller J, Baluška F, Menzel D. GFP-FABD2 fusion construct allows *in vivo* visualization of the dynamic actin cytoskeleton in all cells of *Arabidopsis* seedlings. Eur J Cell Biol 2005;84:595–608.
Vrancken K, Holtappels M, Schoofs H, Deckers T, Valcke R. Pathogenicity and infection strategies of the fire blight pathogen *Erwinia amylovora* in Rosaceae: state of the art. Microbiology 2013;159:823–32.
Waller F, Furuya M, Nick P. Expression of *OsARF1* an auxin response factor from rice (*Oryza sativa* L.) correlates positively with auxin-regulated differential growth. Plant Mol Biol 2002;50:415–25.
Wang QY, Nick P. The auxin response of actin is altered in the rice mutant Yin-Yang. Protoplasma 1998;204:22–33.
Wang W, Wen Y, Berkey R, Xiao S. Specific targeting of the *Arabidopsis* resistance proteinRPW8.2 to the interfacial membrane encasing the fungal haustorium renders broad-spectrum resistance to powdery mildew. Plant Cell 2009;21:2898–913.
Yang Z, Fu Y. ROP/RAC GTPase signaling. Curr Opin Plant Biol 2007;10:490–4.
Zaban B, Maisch J, Nick P. Dynamic actin controls polarity induction de novo in protoplasts. J Integr Plant Biol 2013;55:142–59.
Zhang W, Fan LM. Actin dynamics regulates voltage-dependent calcium-permeable channels of the *Vicia faba* guard cell plasma membrane. J Integr Plant Biol 2009;51:912–21.