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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.