Transient expression of the *Arabidopsis thaliana* callose synthase PMR4 increases penetration resistance to powdery mildew in barley

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ABSTRACT

Localized cell wall thickenings, so called papillae, are a common plant defense response to fungal attack at sites of penetration of the plant cell. The major constituent of papillae is callose, a (1,3)-*β*-glucan polymer, which contributes to slowing or blocking the invading fungal hyphae. In the model plant *Arabidopsis thaliana*, we could recently show that the overexpression of *PMR*4 (*POWDERY MILDEW RESITANT 4*), which encodes a stress induced callose synthase, results in complete powdery mildew resistance. To evaluate if these findings are also transferable to monocot crops, we transiently expressed *PMR*4 under control of the 35S promoter in leaves of barley (*Hordeum vulgare*) seedlings, which were subsequently inoculated with the virulent powdery mildew *Blumeria graminis* f. sp. *hordei*. Fusion of the green fluorescent protein (GFP) to PMR4 allowed the identification of successfully transformed barley cells, which showed an increased penetration resistance to *B. graminis* compared to control cells that express only GFP. *PMR*4-*GFP* localized in a similar pattern at the site of attempted fungal penetration as observed in *A. thaliana*, which suggests that similar transport mechanisms of the callose synthase might exist in dicot and monocot plants.

Keywords: Biotic Stress; Callose; Glucan; Fungal Resistance; Plant Defense; Plant Engineering

1. INTRODUCTION

The plant cell wall is one of the first lines of defense against invading pathogens. A strengthening of the cell wall by callose deposition in so called papillae is considered as an important part of the basal plant resistance mechanism against penetration [1]. Callose is a linear polysaccharide, which consists of (1,3)-*β*-linked glucose monomers with some (1,6)-*β*-branches [2]. It is synthesized by membrane-bound callose synthases [3], which might be organized in multiprotein complexes [4] and use UDP-glucose as substrate [5]. In *Arabidopsis thaliana*, 12 callose synthase genes are described [4]. They showed a tissue-specific expression [6], which was also reported from callose synthase genes in wheat [7]. Among the 12 callose synthases in *A. thaliana*, PMR4 (POWDERY MILDEW RESITANT 4, also called GLUCAN SYNTHASE LIKE 5) is responsible for wound- and pathogen-induced callose formation [8,9]. Interestingly, PMR4 did not seem to be required for penetration resistance to powdery mildew in the *A. thaliana mlo*2 (*MILDEW RESISTANCE LOCUS O* 2) mutant [10], whereas an inhibition of callose synthesis in a barley (*Hordeum vulgare*) *mlo* mutant resulted in an increased powdery mildew penetration [11]. Because callose inhibition studies with wheat (*Triticum aestivum*) and oat (*Avena sativa*) show similar effects after callose inhibition [12], it is suggested that callose deposition might have a function in penetration resistance that is more important in monocot than dicot plants. However, we could recently demonstrate that callose deposition can also play a major role in powdery mildew resistance in *A. thaliana*. The overexpression of *PMR*4 in *A. thaliana* led to an early enhanced callose deposition after powdery mildew infection, which conferred complete penetration resistance [3,13]. Based on the scientific and economic importance of powdery mildews and especially *Blumeria graminis* [14], we wanted to evaluate whether modification of callose biosynthesis would also increase penetration resistance to an adapted powdery mildew in barley.

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2. MATERIALS AND METHODS

2.1. Transient Expression

For barley transformation, we used a binary vector that contains the cassette 35*S*::*PMR*4-*GFP* for overexpression of the *A. thaliana* callose synthase PMR4 fused to the green fluorescent protein GFP under control of the constitutive cauliflower mosaic virus promoter 35S [3]. As a control, we generated a binary vector for 35S-controlled GFP overexpression. The *GFP* open reading frame was amplified from pIGPAPA [15] using an *Nco*I-containing 5' and a *Bst* EII-containing 3' primer. The *Nco*I-*Bst* EII-digested *GFP* fragment was cloned into the likewise prepared vector pCAMBIA3301 (Cambia, Australia), which supplied a 35S promoter element upstream to the *GFP* cloning site. The primer sequences are: GFP-fw [5'GCCATGGTGAGCAAGGGCGAG] and GFP-rev [5'GGGTGACCTTACTTGTACAGCTCGTCC]. The two vectors were transformed into the bacterium *Agrobacterium tumefaciens* (strain GV3101). To analyze whether strong powdery mildew resistance could also be induced in crops, we transiently expressed the 35*S*::*PMR*4-*GFP* and 35*S*::*GFP* construct in barley leaves. The respective *A. tumefaciens* suspensions were infiltrated into leaves of 12-day-old barley seedlings (line Algerian-S (CI-16138)) as described for rice [16].

2.2. Barley Leaf Infection and Microscopy

24 h post-infiltration, leaves were inoculated with *B. graminis* f. sp. *hordei* (*Bgh*, race CR3) as described for *A. thaliana* [17] and monitored at 15 h post-inoculation (hpi), when haustorium formation by *Bgh* indicates successful penetration in this susceptible barley line [18]. Barley as well as the powdery mildew *Bgh* were cultivated as described in Stein *et al.* [17]. Leaf samples were mounted between two cover slips in water. Z series were captured with a spinning-disk confocal microscope [19] by using a Leica 63× water-immersion objective. For better visualization of the fungal conidia and hyphae, the samples were stained with propidium iodide. GFP and propidium iodide were excited at 488 nm by using an argon laser. Emission filtering for GFP was done with a 520/50, for propidium iodide with a 570 - 650 nm bandpass filter. 2D projections of Z stacks were produced with ImageJ (Rasband, W.S., US National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2011).

3. RESULTS AND DISCUSSION

Microcopy of infiltrated leaves revealed that the efficiency of *A. tumefaciens*-mediated transient expression was relatively low. At 24 h post-infiltration, only 1 out of 136 cells (±17 cells) showed a GFP signal in 35*S*::*GFP* control leaves, whereas GFP signals were not detectable in leaves infiltrated with the 35*S*::*PMR*4-*GFP* construct before powdery mildew inoculation (**Figure 1(a)**). The leaves were then inoculated with the barley powdery mildew *Bgh*. To increase the coincidence of *GFP*-expression and an infection of the same cell, spore density was set to 65 conidia·mm^{-2}. We determined penetration success by haustoria formation in epidermal cells 15 hpi by using confocal laser-scanning microscopy to additionally identify *PMR*4-*GFP*- and *GFP*-expressing cells, which were infected by *Bgh*. At 15 hpi, GFP-signals also occurred in 35*S*::*PMR*4-*GFP*-infiltrated leaves at sites of attempted fungal penetration (**Figure 1(a)**). The pattern of the GFP-signal was similar to the signal detected in the *A. thaliana* 35*S*::*PMR*4-*GFP* mutants after *G. cichoracearum* infection; a dense core with a surrounding field of lower density [3,13].

Quantification of microscopy images revealed that in untreated barley leaves, 85% of germinated conidia were able to penetrate as determined by haustorium formation, which is in the range of reported penetration rates for *Bgh* on susceptible barley lines [18,20]. Buffer-infiltration reduced penetration success to 70% (**Figure 1(b)**). Counting the overall penetration success in *A. tumefaciens*-infiltrated barley leaves, a difference to buffer-infiltrated leaves was not detectable. Penetration success in cells with distinct *PMR*4-*GFP* presence at the site of *Bgh* penetration was significantly reduced to 36%, whereas simple GFP presence in infected cells did not alter *Bgh* penetration (**Figure 1(b)**).

Our results show that modification of callose biosynthesis can increase resistance to adapted powdery mildew in a monocot plant. The basis for the resistance seems to be similar as recently described for *A. thaliana*, the focal accumulation of the callose synthase at the site of attempted fungal penetration [3]. Interestingly, barley was able to focally accumulate the callose synthase PMR4 from the different, dicot plant species *A. thaliana* at the fungal penetration site. This suggests that transport mechanisms that are involved in stress-induced callose deposition as a defense response to pathogens might be conserved in plants. A stable overexpression of the callose synthase PMR4 would further improve microscopy, which would help to elucidate these regulatory mechanisms. One aim could be to evaluate whether already known regulatory factors of stress-induced callose biosynthesis, like the GTPase ARFA [21], are also involved in the PMR4-based resistance to powdery mildew in barley. In addition, a stable overexpression of the callose synthase would also facilitate the determination of possible alterations in the cell wall composition.

In *A. thaliana*, the overexpression of PMR4 induced changes in the noncellulosic monocarbohydrate composition, especially reflected by the increase in the amount
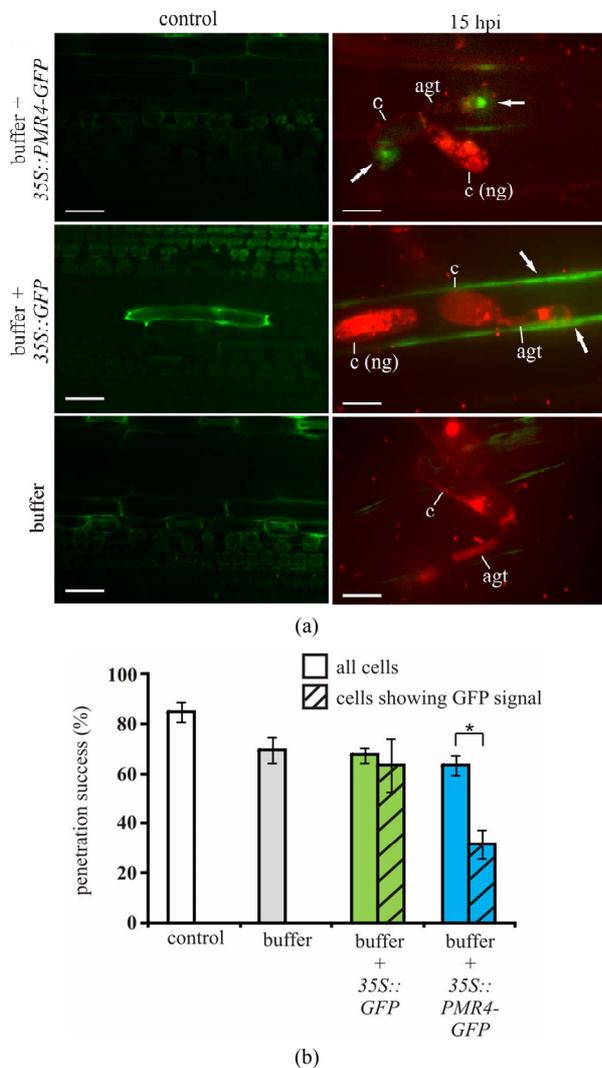
Figure 1. Transient *PMR4-GFP* expression confers elevated resistance to powdery mildew in barley leaves. (a) Z-projected micrographs from confocal laser-scanning microscopy (cLSM) of 12-day-old barley seedling leaves infiltrated with *Agrobacterium* suspensions containing constructs 35S::*PMR4-GFP* and 35S::*GFP* and buffer only. 24 h post-infiltration, leaves were screened for GFP-based fluorescence (left panel, scale bars = 40 µm) and inoculated with *B. graminis* fsp. *hordei*. 15 h post-inoculation (hpi), sites of attempted fungal penetration and additional GFP-based fluorescence (confirming successful expression of *PMR4-GFP* or *GFP*) were identified by cLSM. Powdery mildew conidia were stained with propidium iodide. Arrows indicate GFP fluorescence (right panel, scale bars = 10 µm). agt: appressorial germ tube, c: conidium, ng: not germinated. Micrographs in the right panel are representative for sites used for quantification of penetration success; (b) Quantification of host cell entry determined by haustorium formation per conidium 15 hpi. Penetration success in *Agrobacterium*-infiltrated leaves compared between all cells and those showing a GFP signal (indicator of successful *PMR4-GFP* or *GFP* expression). Non-infiltrated leaves served as control. $^*P < 0.05$, Tukey's test. Error bars represent standard error of the mean, and n = 50 of 4 independent leaves. A repeat experiment gave similar results.

of Glucose [3]. Based on the available barley genome data [22], it would be possible to identify those barley callose synthases that have the highest homology to the stress-induced callose synthase PMR4 from *A. thaliana.* The identified callose synthase genes would be good candidates for an overexpression in barley and *A. thaliana*. This could help to further elucidate whether the observed resistance to powdery mildew in *A. thaliana* and barley is facilitated only by PMR4. The result would support the evaluation of possible molecular breeding strategies for improved powdery mildew resistance in crops, which may only include the callose synthase PMR4 from *A. thaliana* or additional, species-specific callose synthases.

4. ACKNOWLEDGEMENTS

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