Disruption of the *Bcchs3a* Chitin Synthase Gene in *Botrytis cinerea* Is Responsible for Altered Adhesion and Overstimulation of Host Plant Immunity

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The fungal cell wall is a dynamic structure that protects the cell from different environmental stresses suggesting that wall synthesizing enzymes are of great importance for fungal virulence. Previously, we reported the isolation and characterization of a mutant in class III chitin synthase, *Bcchs3a*, in the phytopathogenic fungus *Botrytis cinerea*. We demonstrated that virulence of this mutant is severely impaired. Here, we describe the virulence phenotype of the cell-wall mutant *Bcchs3a* on the model plant *Arabidopsis thaliana* and analyze its virulence properties, using a variety of *A. thaliana* mutants. We found that mutant *Bcchs3a* is virulent on *pad2* and *pad3* mutant leaves defective in camalexin. Mutant *Bcchs3a* was not more susceptible towards camalexin than the wild-type strain but induced phytoalexin accumulation at the infection site on Col-0 plants. Moreover, this increase in camalexin was correlated with overexpression of the *PAD3* gene observed as early as 18 h postinoculation. The infection process of the mutant mycelium was always delayed by 48 h, even on *pad3* plants, probably because of lack of mycelium adhesion. No loss in virulence was found when *Bcchs3a* conidia were used as the inoculum source. Collectively, these data led us to assign a critical role to the BcCHS3a chitin synthase isoform, both in fungal virulence and plant defense response.

Botrytis cinerea Pers. FR (perfect stage: *Botryotinia fuckeliana* (de Bary) Whetzel) is the causal agent of gray mold diseases affecting a broad range of plant species, resulting in considerable economic losses. This necrotrophic pathogen has different modes of attack with diverse inocula sources, such as mycelia or conidia (Choquer et al. 2007). Until now, the control of *Botrytis* diseases relied on the frequent use of fungicides, leading to the development of resistant strains (Leroux et al. 2002). Effective control of this pathogen is dependent on the development of new strategies.

The fungal cell wall provides both protective and virulence functions towards the host (Latgé 2007). For example, this

complex barrier protects the cell from changes in osmotic pressure imposed by the environment and, at the same time, must retain adequate plasticity to allow cell growth. In addition, the wall mediates the fungal cell adherence to the host and serves as a signaling center to activate signal transduction pathways within the host cell. Changes in cell-wall structure lead to severe defects in fungal morphology and consequently, impact fungal aggressiveness (Garcera-Teruel et al. 2004; Liu et al. 2004; Madrid et al. 2003; Munro et al. 2001; Sanz et al. 2005; Soulié et al. 2006). These vital roles of the fungal cell wall suggested it as a source for new targets of antifungal agents (Bowman and Free 2006; Latgé 2007).

Chitin, a long linear homopolymer of β -1,4-linked *N*-acetylglucosamine, is one of the essential components of the fungal wall. This molecule is biosynthesized by a family of membrane proteins, the chitin synthases (CHS). Blast analysis of all amino acid sequences of CHS allowed the identification of seven classes of fungal CHS in filamentous fungi; classes I, II, and IV are common to yeasts and filamentous fungi, while classes III, V, VI, and VII are present only in the latter (Bowen et al. 1992; Choquer et al. 2004; Roncero 2002; Ruiz-Herrera et al. 2001). Systematic mutagenesis of each CHS gene (*chs*) has been undertaken in several fungal species, including *Neurospora crassa* on four genes (Beth-Din and Yarden 2000; Din and Yarden 1994; Din et al. 1996; Yarden and Yanofsky 1991), *Fusarium oxysporum* and *Aspergillus nidulans* on six genes (Borgia et al. 1996; Culp et al. 2000; Horiuchi et al. 1999; Madrid et al. 2003; Martin-Udiroz et al. 2004, 2008; Motoyama et al. 1994; Specht et al. 1996), *Aspergillus fumigatus* and *Wangiella dermatitidis* on seven genes (Aufauvre-Brown et al. 1997; Liu et al. 2004; Mellado et al. 1996, 2003; Wang et al. 1999, 2001; Zheng et al. 2006), and *Ustilago maydis* on eight genes (Garcera Teruel et al. 2004; Gold and Kronstad 1994; Xoconostle-Cazares et al. 1996, 1997). The single deletion of a class I or II gene did not lead to a marked phenotype in the latter, except the class I mutant in *N. crassa*, in which unincleate conidia were unable to germinate (Beth-Din and Yarden 2000). CHS classes III, V, VI, and VII are mold specific, suggesting they might play an important role in hyphal growth. Our previous work showed that *B. cinerea* has at least eight CHS genes (Choquer et al. 2004). Several mutants, including class I *Bcchs1* (Soulié et al. 2003), class III *Bcchs3a* (Soulié et al. 2006), class IV *Bcchs4*, class VI *Bcchs6*, and class VII *Bcchs7*, were constructed by reverse genetics. Mutation in the *Bcchs6* gene was revealed to be lethal (M.-C. Soulié, *unpub-*

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lished data). These mutants are interesting for cell-wall studies in particular, because the cell-wall composition of *B. cinerea* wild type has been described (Cantu et al. 2009). Interesting phenotypic traits of mutant *Bcchs3a* have been reported in other class III mutants of filamentous fungi, including unchanged growth rate in liquid medium. Drastic reduced growth on solid medium with highly branched mycelia was observed, suggesting a preferential role of class III CHS in apex extension and hyphal organization. On the other hand, some characteristics are specific to the *B. cinerea* *Bcchs3a* mutant, i.e., the presence of a thick extracellular matrix (ECM) around the hyphal tips and a reduced virulence on *V. vinifera* and *A. thaliana* leaves. Microscopic in planta examinations showed empty hyphal structures resembling ghost cells. Soulié and associates (2006) suggested that this mutant could be more susceptible to plant-defense molecules than the wild-type strain Bd90, due to changes in the cell-wall structure resulting from lack of BcCHS3a protein.

Plant defense against invading pathogens is governed by a complex web of signaling pathways leading to activation of subsets of defense effectors. Three major signaling pathways are mediated by phytohormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (Glazebrook 2005). The JA- and ET-mediated pathways were shown to establish a basic level of defense against necrotroph pathogens like *B. cinerea* (Diaz et al. 2002; Ferrari et al. 2003; Glazebrook 2005; Thomma et al. 1999). Soulié and associates (2006) found that the BcCHS3a protein is not necessary to protect the pathogen from defense controlled via JA and ET. However, the *Bcchs3a* mutant could be more sensitive to other defense mechanisms known to be triggered by pathogens (Hammond-Kosack and Jones 1996), such as reinforcement of cell walls, production of antimicrobial compounds (phytoalexins, phenolic compounds) or the oxidative burst. The role of the phytoalexin camalexin in resistance of *A. thaliana* to *B. cinerea* has been well studied (Denby

et al. 2004; Ferrari et al. 2003; Kliebenstein et al. 2005). A relationship between in vitro camalexin susceptibility and virulence of the fungus on ecotype Col-0 has been established by Kliebenstein and associates (2005), demonstrating that camalexin tolerance of *B. cinerea* was isolate-dependant. Two *Arabidopsis* mutants, *pad2* and *pad3*, isolated on the basis of reduced accumulation of camalexin after inoculation with bacterial pathogens (Glazebrook and Ausubel 1994), showed higher susceptibility to *B. cinerea* (Chassot et al. 2008; Ferrari et al. 2003; van Baarlen et al. 2007).

In this work, we further searched for the reasons why *Bcchs3a* disruption alters *B. cinerea* virulence. We then conducted a detailed analysis of the *B. cinerea* mutant virulence compared with wild-type Bd 90 on a set of *A. thaliana* mutants defective in various defense responses. Our results support the proposal that lack of a functional *Bcchs3a* CHS gene in *B. cinerea* is responsible for altered adhesion and overstimulation of host plant immunity.

RESULTS

Mutant *Bcchs3a* is greatly impaired in lesion formation on *Arabidopsis* ecotype Col-0.

The *Bcchs3a* mutant defective in class III CHS has reduced virulence on host plants (Soulié et al. 2006), suggesting an increased susceptibility of this cell-wall mutant towards plant defense molecules or its incapacity to elude the defense mechanisms of the host. To investigate this hypothesis, we first compared the lesion development of wild-type strain Bd90 with that of the *Bcchs3a* mutant on leaves of *A. thaliana* ecotype Col-0 (Fig. 1A). We applied young mycelium plugs on detached leaves and daily recorded the number of leaves showing spreading lesions. A typical lesion of *B. cinerea* on *Arabidopsis* leaves (Fig. 1A) consists of a maceration spreading around the mycelium plug. The histogram shown in Figure 1A shows the symp-

Fig. 1. Disease development of wild-type strain Bd90 and mutant *Bcchs3a* on *Arabidopsis thaliana* leaves. **A**, Col-0 and **B**, *Pad3* leaves were inoculated with mycelium plugs, and disease development was followed for up to 6 days postinoculation (dpi). Spreading lesions (3 dpi) of wild-type strain Bd90 are shown on the left and mutant *Bcchs3a*-inoculated leaves without spreading lesions are shown on the right. The graphs show the percentages of leaves with spreading lesions up to 6 dpi ($n = 30$). A lesion was considered spreading when the lesion size reached 0.4 cm^2 . Bars represent the means of two experiments ($\pm$ standard deviation). Spreading lesions are shown for wild-type strain Bd90 (3 dpi) and mutant *Bcchs3a* (6 dpi).

tom evolution as a function of time. For the wild-type strain, 70% of inoculated Col-0 leaves displayed spreading lesions after 2 days postinoculation (dpi), reaching almost 100% after 4 days. In contrast, mutant *Bcchs3a* generated spreading lesions on less than 5% of Col-0 leaves after 2 dpi. After 4 days, this percentage reached 35% and did not increase further, even after 6 dpi. Therefore, in 65% of leaves inoculated with the *Bcchs3a* mutant, no spreading lesions occurred. Instead, necrotic spots were visible underneath the mycelium plug (Figs. 1A and 4). We also compared the size of lesions caused by both fungal strains. We measured lesion size daily and calculated the lesion growth rate ($\text{cm}^2 \text{h}^{-1}$) on Col-0 leaves. The histogram in Figure 2 shows that the growth rate is not statistically different between Bd90 ($0.03 \text{ cm}^2 \text{h}^{-1}$) and *Bcchs3a* ($0.034 \text{ cm}^2 \text{h}^{-1}$). Thus, the mutant *Bcchs3a* generates a drastically reduced number of spreading lesions on Col-0 leaves. However, if lesion formation occurs, the mutant spreads throughout the plant to the same extent as Bd90.

Mutant *Bcchs3a* is virulent on *pad3* leaves of *Arabidopsis*.

B. cinerea is known to be confronted by high amounts of reactive oxygen species (ROS) at the infection site, and it has been shown that the pathogen is well adapted to the oxidizing environment in the host tissue (Mayer et al. 2001; Schouten et al. 2002). A defect in the cell wall of *B. cinerea* leading to increased sensitivity to active oxygen species would, therefore, be deleterious for the pathogen. In addition, plant secondary metabolites like flavonols and, in particular phytoalexins (camalexin), influence lesion size in infected tissue (Denby et al. 2004; Ferrari et al. 2003; Kliebenstein et al. 2005) and may be more efficient towards a fungal cell-wall mutant. We thus tested the sensitivity of *A. thaliana* mutants altered in oxidative burst (*AtrbohDF*), flavonols (*Atfah1-7tt5*), or camalexin production (*pad3* and *pad2*) to wild-type strain Bd90 and mutant *Bcchs3a*. The susceptibility of *AtrbohDF* and *Atfah1-7tt5* mutant leaves to infection was the same as on Col-0 for both fungal strains (data not shown). With the *pad3* mutant (Fig. 1B), we found that, 2 dpi with Bd90 strain, 90% of the leaves displayed spreading lesions. Mutant *Bcchs3a* also caused 90% of spreading lesions but only after 4 dpi, indicating a 2-day delay in the infection process. Mutant *Bcchs3a* showed the same maceration phenotype as Bd90 (Fig. 1B). A similar phenotype was found on *A. thaliana* mutant *pad2* (data not shown). We

Fig. 2. Lesion growth rate of wild-type strain Bd90 and mutant *Bcchs3a* on Col-0 and *pad3* *Arabidopsis* leaves. The lesion size on each diseased leaf was measured over several days and the lesion growth rate on each leaf was measured ($\text{cm}^2 \text{h}^{-1}$). For strain Bd90, 28 diseased Col-0 and 29 diseased *pad3* leaves were examined; for mutant *Bcchs3a* 11, diseased Col-0 and 24 diseased *pad3* leaves were examined. Bars represent the mean of the lesion growth rates and different letters indicate significantly unequal rates (Kruskal-Wallis test, $P = 0.05$). The experiment was conducted twice and similar results were obtained.

calculated the lesion growth rate in *pad3* leaves for both strains (Fig. 2). The lesions of Bd90 strain spread significantly faster ($0.046 \text{ cm}^2 \text{h}^{-1}$) than those of mutant *Bcchs3a* ($0.028 \text{ cm}^2 \text{h}^{-1}$) (Kruskal-Wallis test, $P = 0.05$). A comparison of these results with those obtained on Col-0 leaves indicated that Bd90 lesions spread significantly faster on *pad3* leaves (Kruskal-Wallis test, $P = 0.05$). This was not the case for the lesions caused by the *Bcchs3a* mutant. These results suggest that wild-type Bd90 is a camalexin-susceptible isolate. In leaves defective in production of camalexin, the mutant *Bcchs3a* is rescued in the capacity to cause spreading lesions and the wild-type strain has increased virulence.

In vitro susceptibility of the mutant *Bcchs3a* towards camalexin.

Our results suggest that the presence of camalexin in *Arabidopsis* Col-0 leaves hinders the formation of spreading lesions by mutant *Bcchs3a*. Camalexin could be more toxic to the cell-wall mutant *Bcchs3a* than to wild-type strain Bd90, arresting

Fig. 3. In vitro growth of wild-type strain Bd90 and mutant *Bcchs3a* in the presence of different camalexin concentrations in liquid culture. **A**, Inhibition of growth was followed by measurement of the absorbance at 405 nm in the presence of 0, 5, 10, and 25 $\mu\text{g ml}^{-1}$ of camalexin per milliliter. Growth inhibition is indicated at 4 days postinoculation. Values in the absence of camalexin are indicated by 100%. Different letters indicate significantly different growth rates from one another at $P = 0.05$. **B** and **C**, Presence of camalexin in incubation mixtures of wild-type strain Bd90 and mutant *Bcchs3a*. Microtitre-plate incubation mixtures (30 μl) were separated by silica thin-layer chromatography, and camalexin was visualized by its blue fluorescence under an ultraviolet lamp. Incubation mixtures are shown after 4 (B) and 7 days (C) of incubation. Lanes 1 and 14 show purified blue fluorescent camalexin ($R_f = 0.43$). Incubation mixtures of wild-type strain Bd90 are shown in lanes 2 to 5, of mutant *Bcchs3a* in lanes 6 to 9, and incubation mixtures without fungi in lanes 10 to 13. Camalexin concentrations were 0 $\mu\text{g ml}^{-1}$ (lanes 2, 6, and 10), 5 $\mu\text{g ml}^{-1}$ (lanes 3, 7, and 11), 10 $\mu\text{g ml}^{-1}$ (lanes 4, 8, and 12), and 25 $\mu\text{g ml}^{-1}$ (lanes 5, 9, and 13). A second spot of yellow fluorescence is visible at $R_f = 0.62$.

mutant growth before the onset of lesion spreading. To test this hypothesis, we measured fungal growth in vitro in the presence of increased camalexin (a gift from J. Glazebrook) concentrations corresponding to 0, 5, 10, 25, and 50 $\mu\text{g ml}^{-1}$ in liquid Czapek glucose medium (incubation mixture) in a microtiter plate. Growth was recorded at 4 dpi by measuring absorbance at 405 nm, and growth inhibition in the presence of camalexin is illustrated in Figure 3A. No significant inhibition occurred at 5 $\mu\text{g ml}^{-1}$ for either strain. A significant inhibition of mycelium growth was found for both strains at 10 $\mu\text{g ml}^{-1}$ ($\Delta\text{absorbance} = -0.20$; standard error [SE] = 0.05 for Bd90 and *Bcchs3a*; $P < 0.01$) without difference between the strains. At 25 $\mu\text{g ml}^{-1}$, growth reduction was significant for both strains but significantly higher for wild-type strain Bd90 ($\Delta\text{absorbance} = -0.45$; SE = 0.05; $P < 0.01$) than for mutant *Bcchs3a* ($\Delta\text{absorbance} = -0.25$; SE = 0.05; $P < 0.01$). At camalexin concentrations of 50 $\mu\text{g ml}^{-1}$, growth of both strains was totally inhibited (data not shown). These results confirm that wild-type strain Bd90 is susceptible to camalexin in vitro. In order to know if the fungus was killed in the presence of camalexin, we inoculated malt agar plates with the fungus present in the incubation mixtures (8 dpi). At all camalexin concentrations tested, both *B. cinerea* strains recovered normal growth on the malt medium. Altogether these results demonstrate that mutant *Bcchs3a* is not more susceptible towards camalexin than wild-type strain Bd90. It is even slightly more resistant at elevated concentrations of this compound. In addition, the effect of camalexin on *B. cinerea* is likely fungistatic rather than fungitoxic up to 25 $\mu\text{g ml}^{-1}$.

We then compared the capacity of mutant *Bcchs3a* and wild-type strain Bd90 to detoxify camalexin. For this purpose, we used the microtiter plate growth assay described above in combination with thin-layer chromatography, followed by camalexin visualization by fluorescence. Samples of the fungal incubation mixtures (Bd90 or *Bcchs3a*) containing different camalexin concentrations (0, 5, 10, and 25 $\mu\text{g ml}^{-1}$) were examined 4 and 7 dpi in this way. In Figure 3B and C, we can observe that camalexin ($R_f = 0.43$) disappeared over time in the presence of the fungal strains. Moreover, a new fluorescent compound with a higher R_f value ($R_f = 0.62$) appeared in both fungal incubation mixtures 7 dpi. The appearance of this compound was linked to fungal growth, and the fluorescence intensity was strongest at 10 $\mu\text{g ml}^{-1}$ camalexin, when fungal growth was partially inhibited and camalexin in the incubation mixture disappeared. Thus, there was a correlation between camalexin disappearance and appearance of the yellow fluorescent spot. For all observations, no obvious difference was found between the two strains Bd90 and *Bcchs3a*.

Camalexin accumulation is correlated to a decrease in *Bcchs3a* mutant virulence.

To test whether camalexin accumulated to higher amounts at *Bcchs3a* infection site in comparison to Bd90, we measured camalexin concentrations in Col-0 leaves inoculated with mycelium plugs of the two strains. Camalexin was determined in leaf disks collected after 3 dpi. For wild-type strain Bd90, we quantified camalexin in an extract of the healthy area adjacent to macerated tissue (+2 mm) (Fig. 4). According to Kliebenstein and associates (2005), a dramatic increase in camalexin content takes place in the healthy region immediately surrounding the lesion. For mutant *Bcchs3a*, camalexin was quantified in extracts from leaf disks sampled directly below the mycelium plug containing necrotic spots (Fig. 4). We took into consideration only the area surrounding these necrotic spots (Fig. 4, outlined in red). Camalexin content after inoculation with Bd90 was 1 ng mm^{-2} . This amount did not stop fungal growth.

Similar amounts were found for the wild-type B0510 isolate used by Chassot and associates (2008) and Stefanato and associates (2009). Camalexin content after *Bcchs3a* infection was 19 ng per square millimeter, while in mock-inoculated leaves, the concentration reached only 0.04 ng mm^{-2} . These values are all significantly different from one other (Mann-Whitney test, $P = 0.05$). Thus, there were higher amounts of camalexin at infection sites of *Bcchs3a* compared with those of wild-type strain Bd90. This result could be due to overexpression of camalexin biosynthesis genes. The cytochrome P450 cyp71B15 enzyme encoded by the *PAD3* gene (Zhou et al. 1999) catalyzes the final step in camalexin biosynthesis downstream from indole-3 acetaldoxime, which is synthesized from tryptophan (Schuhegger et al. 2006). Thus, expression of the *PAD3* gene can mirror the accumulation of camalexin. *PAD3* gene expression was monitored using real-time reverse transcription-polymerase chain reaction (RT-PCR) in *Bcchs3a*- and Bd90-infected leaves of *A. thaliana* ecotype Col-0. This study was performed 18 and 48 h after inoculation (hpi), with mycelium plugs or conidia suspensions on detached leaves. *PAD3* gene expression in infected tissues was expressed per nanogram of *B. cinerea* cDNA (Fig. 5). We observed an induction of the *PAD3* gene in leaves infected with mycelium plugs whatever the fungal strain or the incubation time. *PAD3* gene-expression levels in conidia-infected tissues were very low (Fig. 5). At 18 hpi, the expression levels were two to four times higher in the tissues infected with the *Bcchs3a* mutant than with wild-type Bd90. On the other hand, we observed a decrease in *PAD3* gene expression for both strains 48 hpi. Thus, it is likely that the increased camalexin accumulation observed in *Bcchs3a* mycelium-infected tissues is due to enhanced transcriptional activation of the *PAD3* gene involved in camalexin biosynthesis.

Fig. 4. Camalexin accumulation at wild-type strain Bd90 and *Bcchs3a* infection sites in ecotype Col-0 *Arabidopsis*. Cut leaves were inoculated on the upper side with mycelium plugs, and symptoms are shown 3 days postinoculation; closeups of symptoms are shown on the right. For Bd90, only the tissue adjacent to the maceration was used for camalexin extraction (area surrounded in red). For *Bcchs3a* and mock inoculation, the whole leaf disk (black circle) was used for phytoalexin extraction. Camalexin concentrations are expressed in nanograms per square millimeter of surface area encircled in red on the closeups (33 mm^2 for Bd90 and 8.8 mm^2 for *Bcchs3a*). Different letters indicate significantly unequal values (Mann-Whitney test, $P = 0.05$). The experiment was conducted four times and similar results were obtained. Arrowheads indicate mycelium plugs.

The mycelium of *Bcchs3a* mutant is impaired in adhesion.

Virulence tests performed on *pad3* leaves showed that *Bcchs3a* mutant is able to form spreading lesions in the absence of camalexin. Nevertheless, the lesions appeared 2 days later compared with those of Bd90 (Fig. 1B). This delay might be due to a weak adhesion of *Bcchs3a* mycelium plugs to the plant surface. To investigate this hypothesis we inoculated Col-0 and *pad3* leaves with Bd90 and *Bcchs3a* mycelium plugs, turned over the leaves 0 and 6 hpi, and counted the detached mycelium plugs. Out of 20 mycelium plugs tested for each timepoint and each strain, we found, on Col-0 leaves, six (seven on *pad3* leaves) Bd90 plugs detached at 0 hpi and 0 plugs (three on *pad3* leaves) at 6 hpi. On the contrary, for *Bcchs3a* mutant, 16 mycelium plugs fell off the Col-0 and *pad3* leaves at 0 hpi and 15 plugs (17 on *pad3* leaves) at 6 hpi. These results clearly show a better adhesion of wild-type mycelium to the leaf surface than for *Bcchs3a* mutant. Poor adhesion of mycelium might lead to delayed penetration and lesion formation. We then used a penetration test on onion epidermis strips (Gourgues et al. 2004) inoculated with wild-type strain Bd90 and mutant *Bcchs3a* mycelium plugs. Observations were performed after 15 and 24 h of incubation (Fig. 6). At 15 hpi, the wild-type strain had formed long hyphae growing on the epidermis surface before penetrating. At this timepoint, many infectious hyphae were observed inside the tissue (Fig. 6A). On the contrary, *Bcchs3a* mutant had formed only a few hyphae growing on the surface without penetration (Fig. 6B). At 24 hpi, *Bcchs3a* mycelia had formed some complex structures with agglomerated hyphae (Fig. 6C) on the host surface, and we observed infectious hyphae inside the tissues (Fig. 6D). Thus, we conclude that *Bcchs3a* mycelium adheres poorly to

the plant surface and that its penetration is delayed. These observations could explain the delay in initiation of lesion formation observed on *pad3* leaves.

The *Bcchs3a* mutant is virulent when inoculated via conidia suspensions.

As all virulence tests in this study were performed with mycelium plugs as inoculum, we determined the expression level of *Bcchs3a* gene in Bd90 mycelium. For this purpose, Bd90 mycelium was grown in vitro for 3 days on cellophane sheets applied on malt extract (ME) solid medium, the actively growing hyphae were harvested, and RNA was extracted. We chose a 3-day-old mycelium in accordance with the age of the mycelium used as inoculum. The expression of *Bcchs3a* gene was analyzed by real-time RT-PCR. We observed a strong expression of this gene (21 ± 9 arbitrary units at 3 dpi), consistent with an essential role for BcCHS3a at early stages of infection following mycelium inoculation. In nature, however, *B. cinerea* has a variety of modes of attack, such as mycelia or conidia (Choquer et al. 2007), and we further investigated the expression of *Bcchs3a* gene in conidia, frequently used as inoculum in virulence assays (Williamson et al. 2007). Conidia suspensions (10^9 conidia mL^{-1}) were spread on a cellophane sheet in petri dishes and, after 5, 10, 18, and 72 h of incubation, total RNA was extracted. The expression of *Bcchs3a* gene was followed by real-time RT-PCR (Fig. 7). During conidia germination, we observed only a weak accumulation of *Bcchs3a* transcripts after 10 h of incubation. A strong increase in *Bcchs3a* gene expression was apparent after 18 h of incubation that was maintained until 72 h. These results indicate that *Bcchs3a* is probably not expressed in germinated conidia before and during tissue penetration, which occurs as early as 10 hpi (Soulié et al. 2006). Consequently, the enzyme BcCHS3a should not be required at early stages of infection if conidia suspensions are inoculated. Thus,

Fig. 5. *PAD3* mRNA accumulation in leaves of *Arabidopsis thaliana* ecotype Col-0 inoculated with mycelium or conidia suspension of Bd90 wild-type strain or *Bcchs3a* mutant. Expression levels of *PAD3* gene were determined at 18 and 48 h postinoculation and were quantified by real-time polymerase chain reaction. Transcript levels were normalized to the plant reference EF1, and *Botrytis cinerea* was quantified in infected tissue with the fungal reference gene actin. *PAD3* transcript levels were then quantified in arbitrary units (a.u.) per ng of *B. cinerea* biomass present in infected leaves. Data are the means of triplicates ($\pm$ standard deviation), and the experiment was repeated twice with similar results.

Fig. 6. Onion penetration assay with A, Bd90 wild-type strain and B, C, and D, *Bcchs3a* mutant. Inverted mycelium plugs (6 mm in diameter) of the two strains were applied on the hydrophobic side of onion epidermis and were maintained at 21°C. After incubation for 15 (A and B) and 24 h (C and D), fungal cells were stained with cotton blue. Infection structure formation (stained in blue) and in planta growth (not stained) were observed. More than 50 infection sites were analyzed. External hyphae are indicated by eh, internal hyphae by ih, and the onion epidermis cell wall by cwo. Photographs were taken at 150 $\times$ magnification.

we conducted virulence tests using conidia suspensions of Bd90 or *Bcchs3a* mutant on *A. thaliana* ecotype Col-0 as well as bean and grapevine leaves. Results showed that, whatever the fungal strain or the host plant, all the inoculated leaves displayed typical symptoms of maceration (data not shown). We also analyzed camalexin accumulation in *A. thaliana* leaves inoculated with Bd90 or *Bcchs3a* conidia. The same amounts of camalexin were obtained for both strains (1 ng per square millimeter of leaf surface). These results indicate that BCCHS3a plays an essential role only during mycelium attack and that different mechanisms are involved in the *B. cinerea* conidia or mycelium infection process.

The ECM of *Bcchs3a* mutant.

In our previous work on the characterization of *Bcchs3a* mutant, microscopic analysis revealed the presence of a thick ECM around hyphae (Soulié et al. 2006), and the use of specific antibodies allowed us to conclude that the ECM did not contain any β 1-3 glucan, 1-4 glucan, or chitin. In this study, we analyzed the ECM composition in neutral hexoses of two strains, Bd90 wild type and mutant *Bcchs3a* (Table 1). The components known to be predominant in the ECM are reinforced up to 30% in mutant *Bcchs3a*; mannose and galactose contents are enhanced by 58 and 48%, respectively, compared with wild-type strain Bd90.

We also investigated whether the thick ECM observed around *Bcchs3a* hyphae was present in germinating conidia. In our previous work, we found that the *Bcchs3a* ECM appeared out of focus when stained with calcofluor white. Using this fluorescent staining that binds to nascent microfibrils of chitin, no morphological difference was observed between the wild-type strain Bd90 and *Bcchs3a* mutant conidia (Fig. 8). This

means that this thick *Bcchs3a* ECM is present on mycelium but absent on conidial germlings.

DISCUSSION

The fungal cell wall is a dynamic structure that maintains cell shape and integrity in the face of environmental stress. In pathogenic fungi, the cell wall is the first element that interacts with the host surface and mediates the adhesion of cells to one another and the host. Moreover, the fungal cell wall is a reservoir of molecules involved in host recognition, suggesting that changes in fungal wall structure can be perceived by plant cells at early stages of infection. CHS enzymes participate in the biosynthesis of the fungal cell wall. Their role during vegetative development is well documented, but little is known about their function during pathogenic growth in planta (Madrid et al. 2003; Martin-Urdiroz et al. 2008; Werner et al. 2007). In a previous study, we showed that a class III CHS, BcCHS3a, is involved in hyphal tip elongation of *B. cinerea* and is required in the virulence of the fungus on its major host plant, grapevine, as well as on the model plant *Arabidopsis thaliana*. In the present work, we demonstrated that the absence of a functional *Bcchs3a* gene in the mycelium causes a drastic effect on accumulation of camalexin, a phytoalexin known to be produced by *A. thaliana* in response to avirulent pathogens and to contribute to resistance to *B. cinerea* (Denby et al. 2004; Ferrari et al. 2003; Kliebenstein et al. 2005).

Accumulation of camalexin in response to the *Bcchs3a* mutant is likely due to an increased production of this compound and not to a lack of detoxification. The enhanced camalexin amount at *Bcchs3a* mutant infection sites (20 times more than the wild-type strain) is probably the consequence of an overinduction of *PAD3* gene involved in camalexin biosynthesis. Recent papers published by Ferrari and associates (2007) and Chassot and associates (2008) have shown that the *PAD3* gene was induced by *B. cinerea* attack. In this paper, we show that both strains, wild-type strain Bd90 and mutant *Bcchs3a*, metabolize camalexin, whereby a new fluorescent compound appears in the incubation mixture. Stefanato and associates (2009) have demonstrated a camalexin detoxification mechanism in *B. cinerea* involving the ABC transporter

Fig. 7. Expression of the *Bcchs3a* gene during conidia germination and mycelium growth of wild-type strain Bd90. Expression levels of the *Bcchs3a* gene were quantified after 5, 10, and 18 h of incubation for conidia germlings and after 72 h for conidia and mycelium, using quantitative reverse transcription-polymerase chain reaction and were normalized to the transcript level of the *B. cinerea* reference gene actin. Data are expressed as mean normalized expression in arbitrary units (a.u.) and are the means of triplicates ($\pm$ standard deviation). The experiment was repeated twice with similar results.

Table 1. Extracellular matrix composition in neutral hexoses of Bd 90 wild-type and mutant *Bcchs3a* mycelium

Strains	Neutral hexoses ^a	Mannose ^b	Galactose ^b	Glucose ^b
Bd90 wild type	2.94 $\pm$ 0.86	0.94 $\pm$ 0.01	0.37 $\pm$ 0.09	1
Mutant <i>Bcchs3a</i>	4.30 $\pm$ 0.60	2.21 $\pm$ 0.19	0.70 $\pm$ 0.05	1

^a In milligrams per gram of fresh weight.

^b Molar ratio.

Fig. 8. Microscopy analysis of germling conidia from Bd 90 wild-type strain and *Bcchs3a* mutant. Conidia were incubated for 7 h in potato dextrose broth diluted by half at 21°C and were observed after Calcofluor white staining. Photographs were taken at 500 $\times$ magnification.

BcatrB, which functions in the efflux of fungitoxic compounds. Other phytopathogenic fungi were able to degrade and detoxify camalexin (Pedras and Ahiahonu 2004), such as *Rhizoctonia solani* a pathogen of *Camelina sativa* (Pedras and Kahn 1997, 2000) and *Sclerotinia sclerotiorum*, which causes stem-rot disease in a vast range of plants (Pedras and Ahiahonu 2002). According to these authors, the resulting biotransformation products were significantly less inhibitory than camalexin itself. In this study, we showed for the first time that *B. cinerea* is most likely able to deactivate camalexin via a still-uncharacterized metabolic process in addition to exporting camalexin via an ABC transporter (Stefanato et al. 2009).

We did not find an increased susceptibility of the *Bcchs3a* mutant to camalexin *in vitro*, which could have explained its loss in virulence. The cell-wall mutant is even slightly more resistant towards elevated camalexin concentrations. In our previous paper, we revealed the ECM around *Bcchs3a* mutant hyphae is considerably more important compared with that of wild-type strain Bd90 (Soulié et al. 2006). This ECM in *B. cinerea*, first described by Doss and associates (1995), could protect the *Bcchs3a* mutant against antimicrobial compounds. Another filamentous fungus, the human pathogen *Aspergillus fumigatus*, also produces a matrix, under aerial conditions, that cohesively bonds hyphae into a contiguous sheath (Beauvais et al. 2007). These authors have shown that *Aspergillus fumigatus* aerial colonies were more resistant to polyenes like amphotericin B and nystatin than colonies grown in liquid culture. The ECM observed in *Bcchs3a* mutant could reduce permeability and penetration of drugs like camalexin into the fungal cell.

The increase in camalexin production could be due to the recognition by the host of a new pathogen-derived elicitor. The overexpression of the *PAD3* gene is well observed at 18 hpi. However, in a standard test on onion epidermis, penetration of the *Bcchs3a* mycelium occurs only 24 hpi. Therefore, *PAD3* gene induction most likely takes place before fungal entry in host tissue and could continue during early penetration stages, the mutant hyphae generating phytoalexin accumulation, necrosis, and pathogen restriction. Brummer and Steven (2009) have shown that components, covalently cross-linked in a network of the fungal cell wall, constituted ligands (pathogen-associated molecular patterns [PAMPs]) recognized by the host, inducing defense reactions. Cell wall and glucoprotein components of yeast or the CBEL (cellulose-binding elicitor lectin) from the cell wall of *Phytophthora parasitica nicotianae* have been reported to exhibit elicitor activity (Bleichert et al. 1995; Gaulin et al. 2002, 2006; Hahn and Albersheim 1978; Suzuki et al. 2005). The CHS mutant *Bcchs3a* presents a change in its cell wall with 39% less chitin content (Soulié et al. 2006), which could lead to a modification of the ECM composition (Doss 1999). One possible hypothesis is that a molecule in the altered ECM could play an important role as an elicitor (PAMP) inducing the *pad3* gene. The fact that germinated conidia of mutant *Bcchs3a* without this ECM are virulent, reinforces this hypothesis. Preliminary studies on *Bcchs3a* and Bd90 ECM showed an interesting increase of galactose and mannose contents in the mutant. Mannose-rich glycans have been shown to induce plant defense. Indeed, Basse and associates (1992) have found an elicitor activity of a pure yeast glycoprotein in tomato cells; the elicitor activity resulting essentially from the glycan part composed of mannosyl residues. Glycoproteins, commonly secreted by fungi, often generate signals in the host plant. For the *Bcchs3a* mutant of *B. cinerea*, the increase in mannose levels in the ECM could therefore play an elicitor role in *A. thaliana* leaves, leading to accumulation of *PAD3* gene transcripts. In the case of human infection by pathogenic fungi, mannose-binding lectins (MBL) are cir-

culating collectins and are recognized as important factors in nonspecific innate immune responses to pathogens (Brummer and Stevens 2009). These molecules bind glycopeptides containing mannose present at the surface of pathogenic micro-organisms and form complexes that activate the complement cascade and facilitate phagocytosis. MBL deficiency was associated with severe and repeated infections in infants and immunocompromised hosts, showing clearly an important role of MBL in host resistance. For *Bcchs3a* mutant, we thus suggest that mannose present in the ECM could play an important role as elicitor. This PAMP could be recognized by a carbohydrate-binding protein receptor like MBL, inducing defense responses such as phytoalexin production. The presence of a new *PAD3*-inducing elicitor in the altered ECM of the mutant *Bcchs3a* as a consequence of mycelium cell-wall alterations seems to be a plausible hypothesis. Now we need to investigate the presence of such a PAMP in the ECM of the mutant and determine its exact nature. An alternative hypothesis could be that this PAMP is already present in the Bd90 ECM but in a small quantity or masked by components due to BcCHS3a CHS activity.

Interestingly, in some cases (Fig. 1A), the mutant *Bcchs3a* escaped plant defenses and produced spreading lesions with the same lesion growth rate as wild-type strain Bd90. Preliminary results showed that camalexin accumulation at the edge of these spreading lesions corresponded to concentrations found for Bd90. This could indicate that mutant *Bcchs3a* does not provoke enhanced *PAD3* expression once deeply advanced into the plant tissues. Dynamic changes of cell-wall composition during plant infection have been shown for *Magnaporthe grisea* (Fujikawa et al. 2009) and a range of other plant pathogens (El Gueddari et al. 2002). In *Botrytis cinerea*, similar cell-wall changes could occur during infection, leading to the loss of the ECM in *Bcchs3a* mutant and the lack of overelicitation.

The accumulation of camalexin generated a decrease in virulence of the *Bcchs3a* mutant, but we found that it is not the sole cause of this defect. *Bcchs3a* mycelium adheres poorly to the plant surface, and this could explain why the infection process of mutant *Bcchs3a* is always delayed (48 h), even on *pad3* leaves, compared with that of the wild-type strain. Little is known about genes involved in mycelium and hyphae adhesion to plant surfaces. Hydrophobins, unusual surface-active proteins secreted by fungi, are known to form an amphiphilic monolayer around hyphae, which mediates fungal attachment to hydrophobic surfaces (Wösten et al. 1994). However, we did not find any difference in the expression patterns of hydrophobin genes between the wild-type strain and the mutant *Bcchs3a* (data not shown). Poor mycelium adhesion could be due to the presence of the above-mentioned ECM in the *Bcchs3a* mutant. *Aspergillus fumigatus* possesses a well-defined ECM (Latgé et al. 1994). In this fungus, galactofuranose (Galf) is a major molecule found particularly in cell-wall polysaccharides and ECM (Latgé 2009). A mutant lacking the *afUGM1* gene encoding the UDP-galactopyranose mutase (last step in the Galf pathway) has been constructed by Lamarre and associates (2009). Interestingly, the major phenotype of the mutant was a modification of the cell-wall surface resulting in an increase in adhesion to different inert surfaces. In contrast, changing the cell-wall surface of *B. cinerea* CHS mutant *Bcchs3a* generated the loss of correct mycelium adhesion.

In this study, we established that CHS BcCHS3a of *B. cinerea* plays an important role at early stage of infection performed with mycelium inoculum. In nature, *B. cinerea* has a variety of modes of attack, conidia being a primary source of inoculum. Mycelium surviving within infected dead host tissues can also be a primary inoculum source, and infected wind-blown petals containing mycelium can be regarded as dispersal propagules

in some cases (Williamson et al. 2007). Depending on the inoculum source, the infection process is not the same. Plants inoculated with mycelium showed hyphae growing on the plant surface heavily ramified into cell aggregates named 'infection cushions' before penetration (Choquer et al. 2007; Kunz et al. 2006). Plants inoculated with conidia showed frequent germ-tube penetration without previous ramification and appressorium-like structures (Fourie and Holz 1995; Tenberge 2004). We found that *Bcchs3a* gene is not or is little expressed in conidia inoculum (from 8 h postgermination). In addition, at this stage of development, we did not detect any ECM on germling conidia of *Bcchs3a* mutant. Virulence tests on *A. thaliana* ecotype Col-0 conducted with conidia suspension of Bd90 and *Bcchs3a* revealed the same typical symptoms for the two strains. Thus, we conclude that CHS BcCHS3a is not involved at early conidia infection stages. These results show the importance of inoculum source during the infection process.

In conclusion, the use of both the fungal CHS mutant and the *Arabidopsis pad3* mutant allowed us to evaluate the role of BcCHS3a during the infection process. The class III CHS is important for proper mycelium adhesion to the plant surface and rapid host-tissue penetration. Moreover, it plays a crucial role in protecting *B. cinerea* from early detection by the host defense system, either in masking PAMPs on the fungal cell surface or in avoiding mycelium cell-wall alterations leading to early recognition. Lack of the BcCHS3a enzyme allows the host plant to accumulate sufficiently high camalexin concentrations at the infection sites to arrest fungal growth before lesion formation.

MATERIALS AND METHODS

Plant maintenance.

Arabidopsis thaliana seed from ecotype Col-0 were obtained from the Institut National de la Recherche Agronomique (INRA) Versailles collection. The mutants *AtrbohDF*, *Atfah1-7tt5*, *pad2*, and *pad3* (*pad3-1*; Zhou et al. 1999) were provided by the Nottingham Arabidopsis Stock Center. All plants, *A. thaliana*, *Phaseolus vulgaris* (cv. Contender), and *Vitis vinifera* (cv. Chardonnay), were grown in nonsterile soil with a photoperiod of 16 h at 19°C (23°C for *V. vinifera*) and 70% of relative humidity.

Culture of *B. cinerea* and infection method.

The wild-type *B. cinerea* Bd90, collected in 1986 from grapevine in Bordeaux (Reignault et al. 1994), and a resulting mutant CHS of class III, *Bcchs3a* (Soulié et al. 2006), were grown on potato dextrose agar at 21°C with a 12-h photoperiod. After 10 days, each strain produced a dense carpet of conidia. For spore infections, conidial suspensions (10^5 conidia per milliliter) were realized in Difco potato dextrose broth (PDB) diluted by half. Spore suspension (10- μ l droplets) were deposited on either *A. thaliana* leaves of 6-week-old plants, *Phaseolus vulgaris* cotyledon leaves of 10-day-old plants, or on young *Vitis vinifera* leaves. Camalexin determination and virulence tests were realized 4 dpi. RNA extractions were realized 18 and 48 hpi. For mycelium plug infections, each strain was grown on 1.5% ME agar medium. One mycelium plug (3 mm in diameter), taken from the actively growing edge, was inverted onto the upper surface of one excised leaf. Infected leaves were kept in a petri dish under high humidity and were incubated with a photoperiod of 12 h at 21°C. For each strain, 30 leaves were inoculated, and mean lesion surfaces were determined daily, using OptiLab/Pro-F 2.6.3. Growth rates were determined ($\text{cm}^2 \text{h}^{-1}$) and were analyzed using the Kruskal-Wallis test. Camalexin was determined 3 dpi and RNA extractions realized after 18 and 48 hpi.

Microscopy.

For the assay of onion epidermis penetration, we adapted the procedure of Chida and Sisler (1987). Mycelium plugs (6 mm in diameter) were applied on the hydrophobic side of onion epidermis and were kept in a humid chamber for 10, 15, and 24 h at 21°C. After staining with cotton blue, samples were examined under a light microscope.

For visualization of the ECM of germling conidia, suspensions were realized in PDB diluted by half. Suspensions (1 ml) were spread on a cellophane sheet in Petri dishes and were incubated 7 h at 21°C. Samples were stained with Calcofluor white (0.01%) for 30 min, were rinsed in H_2O , and were mounted in a medium for fluorescence observation (Vectashield; Vector Laboratories, Burlingame, CA, U.S.A.) on microscope slides. Observations were performed under UV light with a Zeiss Axiophot microscope (Oberkochen, Germany).

RNA extraction and real-time PCR.

In planta. For mycelium infection, total RNA was extracted from 30 mg of fresh weight corresponding to five foliar disks of *A. thaliana* Col-0 (8 mm in diameter), cut out under the mycelium plug. For spore infection, six spore droplets were applied on the leaves, which were washed after incubation, and total RNA was extracted from 100 mg of fresh weight. Infected tissues were ground in liquid nitrogen and were extracted using Trizol reagent (Invitrogen, Carlsbad, CA, U.S.A.). RT was performed using an oligo-dT20 for a primer and Superscript II RNaseH-reverse transcriptase (Invitrogen). Real-time quantitative PCR analysis was performed using a 7300 system (Applied Biosystems, Foster City, CA, U.S.A.). A 1:5 dilution of cDNA (5 μ l) was amplified in a 15- μ l reaction mix containing Power SYBR green PCR master mix (Applied Biosystems) and 0.5 μ M of each primer. The primers used were fwpad3 (5'-CCATCATCGGAACTTACACCA-3'), revpad3 (5'-GGCGAGATTACGGCGATTC-3'), fwact (5'-CGCCATTGCTCGTGTGAC-3'), revact (5'-TCGCGACTGGTGGAGAAAGT-3'), fwEF1 (5'-CGTCACCCTAGCCGTTTAC-3') and revEF1 (5'-CCCATGGTTAGAGACTGTCAAACA-3'). Gene expression values were normalized to expression of the *A. thaliana* gene EF1 or *B. cinerea* gene actin. For *PAD3* expression, serial dilutions of 100 ng to 0.032 ng of pure cDNA of *B. cinerea* were amplified with the act1/act2 primer couple, which is specific to *B. cinerea* actin. Cycle thresholds obtained were used to trace a calibration curve, which allowed quantification of fungal cDNA in infected tissues (Gachon and Saindrenan 2004).

In vitro. A dense (10^9 conidia ml^{-1}) conidial suspension (5 ml each) was spread on a cellophane sheets in petri dishes and, after incubation for 5, 10, 18, and 72 h, total RNA was extracted with the same method described above. The primers used were fwBcchs3a (5'-GAAACGTATGCGAACGAT-3'), revBcchs3a (5'-AGGAGTGTGCGTTTGTG-3') and fwact/revact cited above. Gene expression values were normalized to the expression of the *B. cinerea* gene actin.

In vitro effect of camalexin on the growth of *B. cinerea*.

In order to measure fungal growth, we applied a bioassay according to Ludwig and Boller (1990). Antifungal activity of camalexin was estimated following turbidity of fungal cultures growing in microtiter plate wells. Fungal spores were germinated in liquid Czapek culture medium (containing per liter: 2.5 g NaNO_3 , 0.5 g KCl, 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g K_2HPO_4 , and 10 mg Na molybdate; the medium was supplemented with 1.5% glucose) for 3 h. Germ-lings (5×10^3 spores ml^{-1} final concentration) were then applied in wells of a microtiter plate (Techno Plastic Products, Trasadingen, Switzerland) in the presence of different cama-

lexin concentrations: 0, 5, 10, 25, or 50 $\mu\text{g ml}^{-1}$ (total incubation volume of 90 μl). Growth was determined by measuring absorbance at 405 nm every 12 h. Results of two independent tests, each including 12 replicates per condition, were analyzed in detail for the values obtained at 4 dpi. A multivariate linear regression, in which the continuous dependent variable was the absorbance, was used, and it included camalexin concentrations, strain, and interaction terms between concentrations and strain. The statistical tests were two-tailed, and the value of α was set to 5%.

Camalexin extraction and determination.

For each extraction, six leaf disks (total surface, 120 mm^2) were combined. For wild-type strain Bd90, leaf disks were sampled in the region immediately surrounding the lesion (+2 mm) and only healthy tissue was harvested for extraction (total surface, 33 mm^2). For mutant *Bcchs3a*, whole disks immediately under the mycelium plug were used (Fig. 4). Mock inoculation consisted of malt-agar plugs without mycelium, and the leaf disks under the malt-agar plug were used for extraction. Camalexin extractions were performed 3 dpi. Means of 20 extractions for Bd90 and six extractions for *Bcchs3a* and mock inoculation were calculated and analyzed using the Mann-Whitney test. Extraction and purification was conducted as described by Glazebrook and Ausubel (1994) using methanol and chloroform extraction and silica thin-layer chromatography. Camalexin was visualized by its blue fluorescence under an ultraviolet lamp (365 nm). The silica containing camalexin was scraped off the plate and extracted into 1 ml of methanol. The emission at 385 nm after excitation at 315 nm was measured with a Hitachi F2000 fluorometer (Hitachi Ltd, Tokyo), and the camalexin concentration was calculated by comparison with a standard curve obtained by using purified camalexin provided by J. Glazebrook (University of Minnesota, Saint Paul, MN, U.S.A.).

In order to follow camalexin degradation during in vitro growth of *B. cinerea* in the microtiter plate bioassay, 30 μl of filtrate were separated on silica thin-layer chromatography plates. Camalexin was then visualized as described above.

Extraction and composition of the ECM.

Mycelium grown on cellophane sheets for 4 days was scraped off and was shaken in 150 mM NaCl. After 5 min of centrifugation at $4,000 \times g$, the supernatant was poured in dialysis bags (molecular weight cut off = 3,500) (Spectrum Medical Industries, Carl Roth GmbH, Karlsruhe, Germany) and was dialyzed against water for 24 h. Samples were then lyophilized.

Total neutral hexoses of ECM were analyzed and quantified by the phenol-sulfuric acid procedure, using glucose as standard (Dubois et al. 1956). Hexoses were identified by gas liquid chromatography as their alditol acetates obtained after hydrolysis (4 N trifluoroacetic acid, 100°C for 4 h), according to Sawardeker and associates (1967). GLC was performed on a Perichrom 2100 instrument with a flame ionization detector using a BP-1 capillary column (30 m $\times$ 0.32 mm id) (Scientific Instrument Services, Inc., Ringoes, NJ, U.S.A.), under the following conditions: gas vector and pressure, helium 0.7 bar; temperature program, 120 to 180°C at 2°C per min and 180 to 240°C at 4°C per min.

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