Alternative Cell Death Mechanisms Determine Epidermal Resistance in Incompatible Barley-*Ustilago* Interactions

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Programmed cell death is a key feature of epidermal plant immunity, which is particularly effective against biotrophic microbes that depend on living host tissue. The covered smut fungus *Ustilago hordei* establishes a compatible biotrophic interaction with its host plant barley. The maize smut *U. maydis* triggers a nonhost response in barley, which results in epidermal cell death. Similarly, *Ustilago* mutants being deleted for *pep1*, a gene encoding a secreted effector, are blocked upon host penetration. We studied the epidermal responses of barley to incompatible *Ustilago* strains. Molecular and cellular analyses were used to test the impact of Bax inhibitor-1 (BI-1), a suppressor of programmed cell death, on the barley nonhost resistance to *U. maydis* as well as *Ustilago* $\Delta pep1$ mutants. Overexpression of BI-1 resulted in partial break of barley nonhost resistance to *U. maydis*. By contrast, the epidermal cell death response triggered by *pep1* deletion mutants was not impaired by BI-1. Hypersensitive-response-like cell death caused by *U. maydis* wild-type infection showed features of necrotic cell death, while $\Delta pep1$ mutant-induced host responses involved hallmarks of autophagy. Therefore, we propose that the mechanisms of epidermal cell death in response to different types of incompatible pathogens depend on spatial and temporal appearance of cell-death-triggering stimuli.

Biotrophic plant pathogens depend on an effective suppression of host immune responses to establish a compatible interaction. This particularly implies the avoidance of host cell death reactions, which typically result from the recognition of avirulence factors through specific resistance (R) proteins (Jones and Dangl 2006). Such cell death reactions are part of the hypersensitive response (HR), which is associated with a strong burst of reactive oxygen species (ROS) and shares similarities with apoptotic cell death processes (Greenberg and Yao 2004; Heath 2000; Lord and Gunawardena 2012). In addition,

unspecific defenses triggered by the perception of pathogen associated molecular patterns (PAMPs) can also induce plant cell death when not being suppressed by the pathogen (Thomma et al. 2011). Thus, the induction of programmed cell death (PCD) is a crucial component of plant immunity. In mammals, a clear distinction can be made between apoptotic (type I) and autophagic (type II) cell death, determined by the interplay of the anti-apoptotic factor Bcl-2 and the autophagic induced Beclin-1 (Maiuri et al. 2007). In plants, however, this differentiation is vague because PCD can display hallmarks of both processes (van Doorn 2011; van Doorn et al. 2011). Although still under controversial debate, it is largely accepted that apoptotic cell death is not conserved in plants. PCD in plants mainly differentiates between necrotic cell death, which is characterized by an early rupture of the plasma membrane, protoplast shrinkage, and swelling of mitochondria; and vascular cell death, which includes accumulation of autophagosomes and other lytic vacuoles (van Doorn et al. 2011). A mixed situation can be found in HR-associated cell death, which usually shows characteristics of necrotic cell death but also involves hallmarks of vacuolar cell death such as tonoplast rupture and the requirement of vacuolar processing enzyme (VPE) activity (van Doorn et al. 2011).

The role of autophagy in pathogen-triggered plant cell death is also controversially discussed. There is evidence for autophagy to promote cell survival and restrict spread of cell death in HRs in *Nicotiana benthamiana* (Liu et al. 2005). However, a pro-death role of autophagy was observed in *Arabidopsis thaliana* during the HR cell death in an R-gene-triggered immune response (Hofius et al. 2009, 2011).

A well-studied factor in PCD is Bax inhibitor-1 (BI-1). BI-1 has been identified as an endoplasmic reticulum (ER)-resident protein that represents an ancient suppressor of PCD, being functionally and structurally conserved within eukaryotes (Hückelhoven 2004; Watanabe and Lam 2009). A connection of BI-1 and autophagy has recently been described in mice, where BI-1 was found to act as a stress integrator that modulates autophagy levels (Castillo et al. 2011). In line with this, Sano and associates (2012) recently showed that BI-1-deficient mice show reduced autophagy levels.

In maize and barley, it was found that the PCD-inhibiting function of BI-1 is required for full susceptibility to the biotrophic fungal pathogens *Ustilago maydis* and *Blumeria graminis* f. sp. *hordei*, respectively (Eichmann et al. 2010; van der Linde et al. 2011). Complementary to this, overexpression of

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BI-1 in barley leads to an increased susceptibility to the non-adapted biotroph *B. graminis* f. sp. *tritici* but causes partial resistance to the necrotroph *Fusarium graminearum* (Babaeizad et al. 2009; Eichmann et al. 2004).

It is an accepted model that development of the plant immune system and microbial virulence factors are the product of an evolutionary arms race that decides on disease resistance or susceptibility (Jones and Dangl 2006). Key players in this contention are effector proteins, which are produced by pathogens to interfere with the plant immune system. In this regard, one can distinguish “core effectors” that suppress PAMP-triggered immunity (PTI) and, therefore, are basal factors of microbial virulence; and more specialized effectors that interfere with specific defense signaling processes and, thereby, also inhibit the suppression of R-protein-mediated resistance (effector-triggered immunity [ETI]) (Doehlemann and Hemetsberger 2013; Jones and Dangl 2006). A core effector with an essential function for fungal virulence is Pep1. This protein was identified in *U. maydis*, which establishes a biotrophic interaction with its host plant maize and develops intracellular hyphae upon direct penetration of the host epidermis (Doehlemann et al. 2009). Deletion mutants for *pep1* are blocked in penetration of the first attacked epidermal cell and elicit various plant defenses, particularly the accumulation of ROS and, finally, cell death. Recently, it was found that Pep1 inhibits apoplastic peroxidases and, thereby, suppresses the apoplastic, PAMP-triggered oxidative burst response which identifies Pep1 as a basal suppressor of plant immunity (Hemetsberger et al. 2012). Also, in the barley covered smut pathogen *U. hordei*, Pep1 is required for epidermal penetration (Doehlemann et al. 2009). Similar to *U. maydis* on maize, *U. hordei* establishes a biotrophic interaction with barley. However, whereas *U. maydis* forms plant tumors locally at infection sites, *U. hordei* colonizes the plant almost symptom free until seed are replaced by fungal spores in the inflorescences (Laurie et al. 2012). Both related pathogens are

well adapted to their host plants, and *U. maydis* does not cause disease in barley.

In this study, we describe the cellular responses of barley to the compatible biotroph *U. hordei* in comparison with the non-host responses to *U. maydis* as well as the *U. hordei* and *U. maydis pep1* deletion ($\Delta pep1$) mutants, respectively. The findings presented in this study suggest that, in different types of incompatible interactions, differential epidermal cell death responses are activated.

RESULTS

Ustilago-triggered epidermal immune responses in barley.

For a quantitative analysis of the infection process, compatible *U. hordei* wild-type strains were inoculated on leaves of barley seedlings (‘Golden Promise’) 10 days postsowing. In addition, three incompatible interactions were analyzed: *U. maydis* wild type as well as the *U. hordei* and *U. maydis* $\Delta pep1$ mutants. The $\Delta pep1$ mutants have previously been described to be defective in epidermal penetration, while inducing an epidermal cell death response (Doehlemann et al. 2009). To monitor pathogen differentiation, penetration efficiency, and viability of the infected host cells, a combination of stains for life-cell imaging was applied for confocal microscopy. Appressoria formation was quantified using Calcofluor staining of fungal filaments on the leaf surface. In addition, penetration efficiency was quantified at 30 h postinoculation (hpi) after treating the infected leaves with the dyes Calcofluor and fluorescein diacetate (FDA) to stain fungal structures on the leaf surface and, additionally, show the penetrated hypha within the epidermal cell by visualization of the invaginated plant cytoplasm (Fig. 1; Supplementary Fig. S1).

At 24 hpi, filaments of the *U. hordei* and *U. maydis* wild-type strains, as well as the *U. hordei* and *U. maydis* $\Delta pep1$ mutant

Fig. 1. Formation of infection structures and barley epidermal penetration by *Ustilago maydis* and the *U. hordei* wild type (WT) and $\Delta pep1$ mutant strains. **A**, Quantification of appressoria formation 20 h postinoculation (hpi) on barley leaves. **B**, Quantification of penetration efficiency of filaments that formed appressoria, 30 hpi. **C**, Cell viability of penetrated epidermal cells, 48 hpi. **D** to **G**, Confocal images of representative epidermal penetration events of **D**, *U. hordei* wild type; **E**, *U. hordei* $\Delta pep1$; **F**, *U. maydis* wild type; and **G**, *U. maydis* $\Delta pep1$. Images were taken 30 hpi. Green = fluorescein diacetate stain, white = Calcofluor-stained fungal hyphae, red = FM4-64-stain. Bars = 20 μ m. Open arrowheads = penetration attempts of $\Delta pep1$ strains. White arrowheads = intracellular hypha of *U. hordei* and *U. maydis* WT strains. All experiments were performed in at least three biological replicates. Error bars indicate standard deviation. Asterisks indicate significance $P < 0.0005$, calculated by an unpaired *t* test.

strains, had formed appressoria to similar rates on the barley leaves (Fig. 1A). In case of the *U. hordei* wild type, 74% $\pm$ 14% of appressoria led to a successful epidermal penetration and, in 97% $\pm$ 1%, the invaded host cells were viable 48 h after infection (Fig. 1B and C). This reflects the compatibility of *U. hordei* to its host plant barley (Fig. 1B to D). In contrast, only 13% $\pm$ 4% of *U. maydis* wild-type appressoria successfully penetrated an epidermal cell. Similar rates were observed for both *U. hordei* and *U. maydis* $\Delta pep1$ mutants (Fig. 1B). However, cytological analysis showed that the $\Delta pep1$ hyphae of both *U. hordei* and *U. maydis* were blocked immediately upon penetration, showed multiple penetration attempts, and did not show subsequent intracellular growth (Fig. 1E and G). This observation was in line with previous reports on the *U. maydis* $\Delta pep1$ –maize interaction (Doehlemann et al. 2009; Hemetsberger et al. 2012). Similarly, *U. maydis* wild-type hyphae were blocked within the first penetrated epidermis cell (Fig. 1F). However, multiple penetration attempts, as found for $\Delta pep1$ hyphae, were not observed and intracellular growth of *U. maydis* hyphae was blocked within the cell lumen rather than immediately at the cell wall (Fig. 1E and F). Nevertheless, penetration efficiency of the *U. maydis* wild type was not higher than that of $\Delta pep1$ mutants, indicating an effective epidermal resistance to both wild-type and mutant strains. Penetration attempts by either the *U. maydis* wild type or the *U. hordei* and *U. maydis* $\Delta pep1$ mutants caused cell death in most cases (Fig. 1C). Only 15% $\pm$ 2% of the *U. maydis*-infected cells were viable after 48 h, and 26% $\pm$ 5% (33% $\pm$ 5%) of the *U. maydis* (*U. hordei*) $\Delta pep1$ -mutant-infected cells were viable (Fig. 1C). In summary, these observations show that both *U. maydis* as well as the $\Delta pep1$ mutants of *U. hordei* and *U. maydis* are blocked in epidermal penetration and fail to establish a biotrophic interaction with barley.

Overexpression of barley BI-1 rescues epidermal resistance to the *U. maydis* wild type but not $\Delta pep1$ mutants.

In a previous study, we concluded that disruption of *pep1* leads to a “non-host-like” situation (Doehlemann et al. 2009). Indeed, the inability of $\Delta pep1$ mutants to suppress the peroxidase-driven oxidative burst (Hemetsberger et al. 2012) results in an epidermal immune response of barley that shares similarities with the nonhost interaction to *U. maydis* (Fig. 1). Because the observed epidermal defense response suggested an involvement of PCD, the role of PCD was studied by using barley lines that constitutively overexpress green fluorescent protein (GFP)-tagged BI-1 (Babaeizad et al. 2009). Surprisingly, overexpression of BI-1 did not have any detectable impact on $\Delta pep1$ mutant infections. In three independent BI-1-overexpressing barley lines, the penetration efficiency of both the *U. maydis* $\Delta pep1$ and *U. hordei* $\Delta pep1$ mutants was not

altered (Fig. 2A) (i.e., overexpression of BI-1 did not affect the epidermal resistance of barley to these fungal strains). By contrast, the *U. maydis* wild type showed significantly increased penetration efficiencies on the BI-1 plants. At 30 h after infection, approximately 34% $\pm$ 6% of *U. maydis* wild-type appressoria resulted in a successful penetration, compared with only 13% $\pm$ 4% in barley control plants (Fig. 2A). This enhanced penetration efficiency was also associated with a significant suppression of H₂O₂ formation and cell death in the penetrated

Fig. 2. Influence of Bax inhibitor-1 (BI-1) on epidermal resistance of barley to *Ustilago hordei* and *U. maydis* wild type (WT) and respective $\Delta pep1$ mutants. **A**, Quantification of penetration efficiency, 30 h postinoculation (hpi). E14L, E4L3, and E15L7 are independent transgenic lines overexpressing BI-1 (Babaeizad et al. 2009). **B**, Visualization and quantification of H₂O₂ accumulation 48 hpi using diaminobenzidine (DAB) stain. Blue = Calcofluor-stained fungal hyphae. Brown = DAB precipitation. Arrowheads = *U. maydis* penetration attempt. **C**, Visualization and quantification of barley cell death, 48 hpi. Green = *U. maydis* hyphae stained by WGA-Alexa Fluor 488. Blue = Epidermal cell stained by trypan blue. Arrowheads = *U. maydis* penetration attempt. Bars = 20 μ m. BI-1 oe = BI-1 overexpressing line E14L1 (see A); WT = wild-type barley control. All experiments were performed in at least three biological replicates. Error bars indicate standard deviation. Asterisks indicate significance $P < 0.005$, calculated by an unpaired t test.

epidermis cells (Fig. 2B and C). In wild-type barley plants 48 h after infection, approximately 70% of all *U. maydis*-attacked cells showed an accumulation of H₂O₂, indicated by diaminobenzidine (DAB) precipitation. At the same time, approximately 75% of epidermal cells underwent cell death, indicated by trypan blue staining (Fig. 2B and C). In the BI-1-overexpressing barley plants, only 12% ± 8% cells showing DAB precipitation and 17% ± 9% were stained by trypan blue, respectively (Fig. 2B and C). In line with this, BI-1-overexpressing plants contained intracellular *U. maydis* wild-type cells that showed hyphal branching, which was not the case in wild-type plants (Supplementary Fig. S2). Taken together, the overexpression of BI-1 significantly suppresses epidermal nonhost resistance to *U. maydis*, whereas the responses induced by the *Δpep1* mutants appeared to be not affected by BI-1. Based on these observations, we hypothesized that the cell death response triggered by *Δpep1* infections differs from the reaction which is triggered by the *U. maydis* wild type.

Barley responses

to *U. maydis* wild-type and *Δpep1* infections.

Barley leaves infected by the *U. maydis* wild type showed different macroscopic symptoms when compared with *Δpep1* mutant infections. The *U. maydis* wild type caused large clusters of chlorotic cells and necrotic lesions in the infected leave areas, whereas *Δpep1* mutant strains induced only locally restricted chlorosis and no spreading necrotic lesions 6 days after inoculation (Supplementary Fig. S3). In line with this, *U. maydis*-infected plants showed a significant retardation in growth, whereas leaf length of *Δpep1*-mutant-infected plants was comparable with that of *U. hordei* wild-type infections.

To get a more detailed insight into the different barley responses to the *U. maydis* wild type and the *Δpep1* mutants, transcriptional responses of the defense- or HR-associated marker genes pathogenesis-related (*PR*)-1 and *PR*-10 and the class-III *WRKY*-22 transcription factor were tested. Quantitative real-time polymerase chain reaction (qRT-PCR) showed almost no induction of these genes in the compatible *U. hordei* infection at 24 and 48 hpi (Fig. 3A to C). By contrast, *U. maydis* infection caused a strong transcriptional activation of all three genes. At 24 hpi, *PR*-1 (35-fold), *PR*-10 (64-fold), and *WRKY*-22 (39-fold) showed a significant induction. This induction was even more pronounced at 48 hpi, when all marker genes showed a >100-fold induction compared with mock controls (Fig. 3A to C; Supplementary Table S3). In contrast to this, in *Δpep1* infections, only a moderate induction was observed for the *PR* genes and *WRKY*-22 at 24 hpi whereas, at 48 hpi, expression levels of the three marker genes were not altered or even lower compared with 24 hpi (Fig. 3A to C).

Another typical plant response to nonadapted pathogens is the oxidative burst. To test this reaction in the different *Ustilago* spp. interactions, H₂O₂ concentrations in apoplastic fluids of barley leaves were quantified at the infection sites using a xylenol-orange-based readout (Bindschedler et al. 2001, 2006). At 12 hpi, when filaments are growing on the leaf surface but not yet started epidermal penetration, no significant elevation of H₂O₂ was detectable in any of the *Ustilago* infections (Fig. 3D). At the 24-h time point, which represents the penetration phase, both the *U. maydis* *Δpep1* and *U. hordei* *Δpep1* strains caused a strong H₂O₂ accumulation. In contrast, *U. maydis* wild-type-infected samples did not show elevated H₂O₂ levels at this stage (Fig. 3D). This situation, however, changed at 48

Fig. 3. Barley defense responses to *Ustilago maydis* and *U. hordei* wild type (WT) and respective *Δpep1* mutant strains. **A–C**, Quantitative real-time polymerase chain reaction of barley defense genes in response to *U. hordei* and *U. maydis* wild type (WT) and *Δpep1* mutant infections. **A**, *PR*-1; **B**, *PR*-10; and **C**, *WRKY*-22 relative to mock-treated samples. **D**, Quantification of H₂O₂ in apoplastic fluid of *Ustilago*-infected barley leaves in relation to mock-treated leaves (dashed line). All experiments were performed in at least three biological replicates. Error bars indicate standard error of the mean. Asterisks indicate significance $P < 0.005$, calculated by an unpaired t test.

h. At that point, the *U. maydis* wild-type-infected leaves displayed a strong oxidative burst, which exceeded the H₂O₂ contents of $\Delta pep1$ samples at 24 hpi. The H₂O₂ levels in $\Delta pep1$ infections were comparable with those of *U. hordei* wild-type infections at this time point, indicating that the triggered oxidative burst response had already declined at 48 hpi (Fig. 3D).

Epidermal cell death responses

to *U. maydis* wild-type and $\Delta pep1$ mutant strains.

BI-1 has previously been shown to be involved in suppression of plant PCD and HR-associated cell death (Eichmann et al. 2006; Kawai-Yamada et al. 2001; Wang et al. 2012). The observed partial breakdown of nonhost resistance of BI-1 overexpressing barley to *U. maydis* suggests an involvement of necrotic or HR-like cell death in this interaction. Therefore, we used fluorescence-based enzymatic assays to monitor caspase-like activities as well as VPE activity in barley leaves. DEVDase activity (caspase-3-like) during plant cell death was reported in various cases (del Pozo and Lam 1998; Fernández et al. 2012; Han et al. 2012; Rodríguez-Serrano et al. 2012; Zhang et al. 2009). VPE and caspase-1-like activities were previously shown to be required for hypersensitive cell death in plants in response to viral infection and mycotoxin treatment (Hatsugai et al. 2004; Kuroyanagi et al. 2005; van Doorn et al. 2011). We used N-Acetyl-Asp-Glu-Val-Asp-4-methyl-coumaryl-7-amide (Ac-DEVD-MCA) as a selective substrate for caspase-3-like activity, to be incubated with crude leaf extracts of infected plants. At 12 hpi (prepenetration), no differences in substrate cleavage were detected in all tested *Ustilago* infections compared with mock treated samples. At 30 hpi, (post-penetration), the infection by the *U. maydis* wild type resulted in a significant induction of caspase-3-specific substrate cleavage (Fig. 4). In contrast, neither *U. hordei* wild-type nor the $\Delta pep1$ strains led to a significant induction of DEVDase activity (Fig. 4). Treating the samples with the specific caspase-3 inhibitor N-Acetyl-Asp-Glu-Val-Asp-1-aldehyde (Ac-DEVD-CHO) reduced the activity in all samples to a similar background level, confirming specificity of the observed DEVDase activity (Fig. 4). Similarly as for DEVDase, we tested substrates Ac-YVAD-MCA for caspase-1-like activity and Ac-ESEN-MCA for VPE activity. These activities also were induced only by *U. maydis* wild-type infection but not by $\Delta pep1$ mutants. In both cases, a significant induction of activity was found at the post-penetration stage 30 hpi (Supplementary Fig. S4) although, for both caspase-1-like and VPE, the level of induction was weaker compared with DEVDase (Fig. 4).

The specific induction of caspase-like and VPE activities in *U. maydis* wild-type-infected leaves as well as the finding that $\Delta pep1$ infections are independent from BI-1 suggest the execution of different types of PCD. Because autophagy was reported to play an important role in innate immunity and PCD in plants, we hypothesized that this process could be involved in cell death reactions to $\Delta pep1$ infections. Therefore, we visualized the induction of autophagy upon *Ustilago* infections by staining infected leaf areas with monodansylcadaverine (MDC). This basic, permeable dye has been shown to localize to acidic compartments and, therefore, stains autophagosomes (Contento et al. 2005; Kabbage et al. 2013). NaCl-induced plasmolysis was used as a positive control to trigger autophagy in barley epidermis cells. Although mock-treated cells did not show MDC-staining (Fig. 5A), plasmolytic epidermal cells that served as positive control showed MDC-stained particles (Fig. 5B). Next, the MDC stain was applied to *Ustilago* spp.-infected leaves during epidermal penetration (30 hpi). Typically, neither *U. hordei* nor *U. maydis* wild-type-infected cells showed MDC-stained particles (Fig. 5C and D). Conversely, epidermal cells infected by the *U. hordei* and *U. maydis* $\Delta pep1$

mutants contained MDC-stained particles (Fig. 5E and F) and these structures were found motile in the infected cells (Fig. 5G to I). Moreover, induction of autophagy was exclusively observed in infected cells and did not spread to surrounding tissue (Fig. 5J). Quantification revealed MDC-stained particles in $5\% \pm 2\%$ of *U. hordei* wild-type-infected cells. A similar picture was observed after *U. maydis* wild-type infection, where $8\% \pm 3\%$ of infected cells were positive for MDC (Fig. 5K). For both the *U. hordei* and *U. maydis* $\Delta pep1$ mutants, MDC signals were detected in more than 80% of attacked barley cells (Fig. 5K). Because specificity of MDC staining is questioned in recent literature, LysoTracker red was used as an alternative assay to probe autophagy-related acidification of vacuolar or lysosomal structures. In line with the observations made for MDC, LysoTracker-red-stained structures accumulated in $\Delta pep1$ -infected cells, whereas both *U. hordei* and *U. maydis* wild-type infections showed only background signal (Fig. 6). The co-staining of LysoTracker red with FDA also showed loss of protoplast viability in *U. maydis* wild-type infection already during the penetration phase at 30 hpi (Fig. 6B).

In addition to life-cell imaging, the cellular responses to *Ustilago* infections were analyzed by transmission electron microscopy (TEM), which is considered to be the prime analytical method to diagnose plant cell death (van Doorn et al. 2011). In the TEM experiments, cerium chloride (CeCl₃) was used to visualize H₂O₂. *U. hordei* wild-type hyphae were found to grow inside barley cells without any obvious accumulation of H₂O₂ and no ultrastructural changes of the host cell were evident (Fig. 7). By contrast, accumulation of H₂O₂ could be visualized in *U. maydis* wild-type-infected barley cells. In particular, H₂O₂ was found to accumulate in the mitochondria but was also detected in plant cytoplasm and cell walls (Fig. 7). A quantification of subcellular H₂O₂ accumulation in response to fungal infection showed increased H₂O₂ levels in all incompatible interactions compared with the *U. hordei* wild type. However, whereas, in *U. maydis* $\Delta pep1$ and *U. hordei* $\Delta pep1$, H₂O₂ was only induced in plant and fungal cell walls, *U. maydis* wild-type infection caused a strong accumulation of H₂O₂ in plant cytoplasm and mitochondria (Fig. 8).

Furthermore, *U. maydis*-penetrated cells showed tonoplast rupture, blebbing of the plasma membrane, and swollen mitochondria with dilated cristae (Fig. 7). In barley cells penetrated

Fig. 4. Caspase-3-like activity in *Ustilago*-infected barley leaves 12 and 30 h postinoculation (hpi). Caspase-3-like (DEVDase) activity was measured using the specific fluorogenic substrate Ac-DEVD-MCA relative to mock-treated samples. As inhibitor of caspase-3-specific activity, Ac-DEVD-CHO was used. All experiments were performed in at least three biological replicates. Error bars indicate standard error of the mean. Asterisks indicate significance $P < 0.05$, calculated by an unpaired *t* test.

by either *U. maydis* $\Delta pep1$ or *U. hordei* $\Delta pep1$ hyphae, H_2O_2 accumulation was restricted to cell walls and was not observed in cytoplasm and mitochondria (Fig. 7). Strikingly, $\Delta pep1$ -infected cells clearly showed autophagosomes in both cytoplasm and vacuole, which was true for both *U. maydis* $\Delta pep1$ and *U. hordei* $\Delta pep1$ (Fig. 7).

Fig. 5. Life-cell imaging of autophagosomes in barley epidermis cells in response to *Ustilago* infection using monodansylcadaverine (MDC) stain. **A**, Mock-treated barley epidermal cell. **B**, Barley epidermal cell showing NaCl-triggered plasmolysis. **C** to **F**, Barley epidermis cells infected by **C**, *Ustilago hordei* wild type (WT); **D**, *U. maydis* WT; **E**, *U. hordei* $\Delta pep1$; and **F**, *U. maydis* $\Delta pep1$. **G** and **H**, Images showing inset marked in **E** with moving MDC-stained particles (arrowhead). **I**, Overlay of an image series (**G** and **H**) following movement of an MDC-stained particle (bracket). **A** to **G**, Images were taken at 30 h postinoculation (hpi). **J**, *U. hordei* $\Delta pep1$ infected epidermal cell (arrow) and neighboring cell without autophagy staining at 48 hpi. Bars = 20 μm . **K**, Quantification of autophagy in infected epidermal cells 30 hpi. White arrowheads = MDC-stained particles. Red arrowheads = infectious hyphae. All experiments were performed in at least three biological replicates. Error bars indicate standard deviation. Asterisks indicate significance $P < 0.0005$, calculated by an unpaired t test.

Together, these data provide evidence for distinct cellular responses of barley to infection by *U. maydis* wild-type- and $\Delta pep1$ -mutant-infected cells. Although nonhost reactions to *U. maydis* trigger BI-1-controlled HR-like cell death, responses to $\Delta pep1$ infections show typical hallmarks of autophagy, are independent from BI-1, and also lack further features of necrotic death such as mitochondrial disintegration and induction of caspase-like activity. Therefore, autophagy in response to $\Delta pep1$ infections appears to be an alternative mechanism to the HR that is triggered by the *U. maydis* wild type, which shows several hallmarks of necrotic cell death.

DISCUSSION

In this study, we analyzed the epidermal defense responses of barley during incompatible interactions with the biotrophic smut fungi *U. maydis* and *U. hordei*. Prior to host penetration, the maize pathogen *U. maydis* forms infection structures at the same level as the adapted barley pathogen *U. hordei*, indicating that surface cues of barley leaves fully trigger the patho-

Fig. 6. LysoTracker-red staining of barley epidermis cells in response to *Ustilago* infections 30 h postinoculation. **A**, *Ustilago hordei* wild-type infection and **B**, *U. maydis* wild-type infection. Asterisks mark the non-fluorescein diacetate (FDA)-stained cell that underwent cell death. **C**, Infection with *U. hordei* $\Delta pep1$. **D** and **E**, Infection with *U. maydis* $\Delta pep1$. White = Calcofluor-stained fungal hyphae and green = FDA-stained plant cytoplasm and plant nuclei. In mesophyll cells, green autofluorescence gets visible (**A**, **C**, and **E**). Red = LysoTracker-red-stained lysosomes or vacuoles. White arrowheads = *Ustilago* penetration attempts.

genic differentiation of *U. maydis*. The same observation was made for both *U. hordei* and *U. maydis* mutants that carry deletions for the *pep1* gene. However, the *U. maydis* wild type as well as the $\Delta pep1$ mutants were blocked upon epidermal penetration and, in most cases, the infected cells underwent cell death, although *U. maydis* wild-type-triggered cell death

responses were more severe compared with $\Delta pep1$ infections. The *U. maydis* wild type caused large necrotic areas and stunted leaf growth whereas responses to $\Delta pep1$ mutants remained restricted to infection sites. Interestingly, the deletion of *pep1* in *U. maydis* led to a phenotype indistinguishable to that of the *U. hordei* $\Delta pep1$ mutants. This suggests that the

Fig. 7. Transmission electron microscopy of *Ustilago* infected barley cells with visualization of H_2O_2 using cerium chloride ($CeCl_3$). H_2O_2 accumulation is visualized by $CeCl_3$ staining (black arrowheads) in cell walls (CW), the cytosol (CCP), and mitochondria (M) in *Ustilago maydis* wild-type (WT)-infected cells. Tonoplast rupture (open chevrons) could also be observed in penetrated cells. Inset: swollen mitochondria with dilated cristae. Bars = 1 μm . *U. hordei* WT penetrated cells show a dense cytosol with chloroplasts (Ch) and vacuoles (V) with intact tonoplast. $CeCl_3$ staining was absent or very low. Inset: enlarged biotrophic interface. Bars = 1 μm . Cells penetrated by $\Delta pep1$ mutant strains show autophagosomes (gray arrowheads) and H_2O_2 accumulation (black arrowheads) in CW. Insets: enlarged autophagosomes. Bars = 1 μm . EPC = epidermal cell, PC = plant cytosol, BIZ = biotrophic interaction zone, FCW = fungal cell wall, FCP = fungal cytoplasm, H = hypha, degC = degraded cytosol, CM = cytoplasm membrane, and degCh = degraded chloroplast.

more dramatic cell death response toward the *U. maydis* wild type actually depends on the presence of Pep1. Activity of this effector is required for an activation of PCD that can be suppressed by BI-1 (i.e., in the absence of Pep1, a BI-1 independent cell death pathway is triggered in barley epidermal cells).

The Pep1 effector is a secreted peroxidase-inhibitor, which is required for epidermal penetration and the suppression of the PAMP-triggered oxidative burst (Hemetsberger et al. 2012). The *U. maydis* $\Delta pep1$ penetration defect was complemented by either scavenging extracellular ROS or silencing of the Pep1-targeted oxidative-burst maize peroxidase POX12 (Hemetsberger et al. 2012). Given the defense-suppressing function of Pep1, it may appear contradictory that nonhost responses to *U. maydis* $\Delta pep1$ mutants are less severe compared with wild-type infections. An explanation comes from the cell-death mechanisms that are activated in the different interactions. Although the HR-like cell death in the nonhost resistance of barley to the *U. maydis* wild type was suppressed by BI-1, its expression did not have any effect on the $\Delta pep1$ mutant infections. First, this indicates that nonhost resistance to *U. maydis* involves necrotic cell death, which is in line with several previous findings (Eichmann et al. 2004, 2006). Second, this strongly suggests that cell death induced by the $\Delta pep1$ mutants does not implicate necrotic cell death. The same conclusion can be drawn from the quantification of VPE and caspase-like activities. Although caspases themselves are not present in plants, metacaspases have been identified, which show homologies to the caspases of apoptosis (Uren et al. 2000) and are able to induce PCD in yeast (Watanabe and Lam 2005). Additionally, accumulating evidence supports the existence of caspase-like activity in plants and its functional involvement in various types of plant PCD (Bonneau et al. 2008; He et al. 2008; Lam 2005; Qiang et al. 2012; Reape et al. 2008; Sanmartín et al. 2005; Woltering 2004; Woltering et al. 2002; Zhang et al. 2009). *U. maydis* wild-type infection significantly induced caspase-3-like (and, to a weaker extent, caspase-1-like and VPE) activity at 30 hpi whereas, in *U. maydis* $\Delta pep1$ mutant infections, these activities were comparable with *U. hordei* infections.

Fig. 8. Compartment-specific accumulation of H_2O_2 . The subcellular accumulation of H_2O_2 was quantified on transmission electron micrographs by determining the area of cerium chloride ($CeCl_3$) staining in the different cell compartments. Graphs show means with standard errors which document the percentage of areas covered by $CeCl_3$ precipitates in the individual cell compartments; $n > 40$ for the individual cell compartments. Different lowercase letters indicate significant differences ($P < 0.05$) between the individual cell compartments whereas uppercase letters indicate significant differences between the total amounts of $CeCl_3$ staining for all analyzed cell compartments taken together. Data were analyzed with the Kruskal-Wallis test followed by post-hoc comparison according to Conover (1999).

Instead of hallmarks for necrotic cell death, $\Delta pep1$ mutant infections were associated with an induction of autophagy. Strong accumulation of MDC-positive vesicles was observed in $\Delta pep1$ -infected cells but not in *U. maydis* wild-type infections. Similarly, Lysotracker-red stain was specifically found in $\Delta pep1$ -infected epidermal cells. However, because MDC is not strictly selective for autophagosomes and might also stain other acidic compartments such as early endosomes (Klionsky et al. 2008), confirmation for the induction of autophagy came from the TEM data. Autophagosomes got visible in $\Delta pep1$ mutant-infected barley cells but were observed in neither *U. hordei* nor *U. maydis* wild-type-challenged cells. Together, this provides substantial evidence for a specific induction of autophagy in $\Delta pep1$ infections. However, a significant transcriptional induction of autophagy-related *atg* genes (Bassham et al. 2006) was not observed by using qRT-PCR (not shown). A likely explanation for this negative result is the small number of cells that actually perform autophagy in response to an *Ustilago* infection. Only the epidermal cells that were infected by $\Delta pep1$ mutant hyphae executed autophagy, whereas the surrounding tissue did not show any detectable responses. This situation very likely leads to a dilution of transcriptional changes in whole-leaf RNA samples. In addition, the restricted cell death reaction in $\Delta pep1$ -infected leaves also explains the weaker macroscopic symptoms compared with *U. maydis* wild-type infections, where spreading cell death and retarded growth were observed. At the same time, this phenotype supports the suggested role of autophagy in limiting the spread of pathogen-induced PCD (Liu et al. 2005; Yoshimoto et al. 2009) because the expanding cell death in the *U. maydis* wild-type-infected leaves is restricted in $\Delta pep1$ infections.

At this point, one should state that the presented data provide support for an activation of autophagy during epidermal resistance to $\Delta pep1$ mutant strains. What cannot be doubtlessly concluded thus far is whether these autophagic processes actually cause the observed cell death. In order to elucidate a functional role of the autophagy pathway in cell death, Shen and Codogno (2011) proposed that cell death by autophagy could occur independent of necrotic cell death, which is true in the situation described in this study. In addition, autophagic cell death should also be associated with an increase of autophagic flux and, consequently, suppression of autophagy is able to prevent cell death (Shen and Codogno 2011). In other systems, this was tested by inhibitors such as 3-methyladenine. This approach, however, is not possible in the *Ustilago* system, because infections occur to primordia within the leaf whorl, which cannot be infiltrated by inhibitor solutions prior to fungal infection. Therefore, future experiments will require stable knockdown barley lines for autophagy components, such as Atg8, to directly confirm the functional connection of autophagy and cell death in $\Delta pep1$ infections.

Lacking evidence of autophagy induction during *U. maydis* wild-type infections does not implement an autophagy independent from HR. In *A. thaliana*, a role of autophagy during the immunity-associated hypersensitive cell death was shown recently (Kwon et al. 2013). In light of the present study, one could summarize that, depending on the trigger, an HR either could be executed primarily through necrotic cell death, because it shows up in *U. maydis* wild-type infections, or it could be characterized by autophagy instead, as in $\Delta pep1$ infections. For the necrotrophic fungal pathogen *Sclerotinia sclerotiorum*, a recent report discovered an important role of the interplay between autophagy and necrotic cell death for successful infection (Kabbage et al. 2013). *S. sclerotiorum* induces necrotic cell death to kill host tissue but it also requires the ROS-scavenging reductant oxalic acid as a virulence factor to successfully establish infection (Williams et al. 2011). Kabbage and

associates (2013) concluded that oxalic acid functions as suppressor of autophagy, because this needs to be suppressed by *S. sclerotiorum* to establish infection; whereas, in the onset of the interaction, this necrotrophic fungus promotes necrotic cell death to cause disease. This observation, although being made in a quite unrelated system (dicot host versus monocot host and necrotrophic pathogen versus biotrophic pathogen) shows remarkable parallels to the situation we found in the present study. First, these complementary independent findings strengthen the hypothesis that necrotic cell death and vacuolar cell death, including autophagy, are the two major pathways in pathogen responses. Second, these two cell-death mechanisms seem to act alternatively (i.e., depending on the trigger, either autophagy or necrotic cell death is activated). This view may also bring together the partially contradictory reports considering autophagy as either “pro-survival” or “pro-death” (Hofius et al. 2009, 2011; Liu et al. 2005). With regard to its impact on necrotic cell death, autophagy can be considered as a pro-survival mechanism because, when activated, necrotic responses are attenuated. However, during PTI, autophagy functions as an efficient, local response to restrict epidermal penetration and avoid spreading cell death. This is what we observed in *Δpep1* infections.

The decision as to whether autophagy is triggered likely depends on i) the compartment and ii) the time point when ROS accumulation occurs. The *Δpep1*-infected cells show H₂O₂ stain mostly in the cell wall, while *U. maydis* wild-type-infected cells show strongly increased H₂O₂ levels in the cytoplasm and the mitochondria, which appeared swollen and showed dilated cristae. The latter is similar to the HR-type cell death triggered in barley ETI to *B. graminis* f. sp. *hordei* (An et al. 2006). In *Δpep1* infections, the early, PAMP-triggered apoplastic oxidative burst is not suppressed because the peroxidase-inhibiting Pep1 effector is lacking. This leads to a massive accumulation of extracellular H₂O₂ (Hemetsberger et al. 2012) which may act as a trigger of autophagy. This is in line with the elevated H₂O₂ levels in *Δpep1* infections at 24 hpi, which represents the penetration phase of *Ustilago* spp. At 48 h after *Δpep1* infection, H₂O₂ levels were decreased almost to control levels and barley cells around infection sites were unaffected, indicating that no spreading cell death occurred. In contrast, in *U. maydis* wild-type infections, an oxidative burst was observed only after epidermal penetration (48 hpi). Here, the early PAMP-triggered burst is suppressed by Pep1, which is functionally conserved among *U. maydis* and *U. hordei* (Doehlemann et al. 2009). However, after penetration of the epidermal cell wall, predominantly necrotic cell death is induced, coinciding with a massive induction of *PR-1*, *PR-10*, and *WRKY-22* and an oxidative burst response that exceeds the H₂O₂ levels induced upon *Δpep1*-infections. This nonhost response is likely triggered by *U. maydis* effectors being not adapted to barley and, therefore, recognized in barley to cause ETI. Remarkably, the *U. maydis Δpep1* mutants do not cause this response. It behaves indistinguishably from *U. hordei Δpep1* and induces an early oxidative burst and autophagy but no indications for necrotic cell death. This substantiates the specific induction of the HR-like cell death in the presence of Pep1, where PAMP-induced responses are suppressed. Generally, a block of plant pathogens that fails to suppress the initial PAMP-triggered, extracellular oxidative burst is associated with epidermal autophagy, which remains restricted to the challenged cells and, therefore, causes only limited damage to the plant. This is also reflected by the minor macroscopic symptoms of barley plants treated with *Δpep1* mutants compared with *U. maydis* wild-type infections. A nonadapted pathogen that deploys core effectors such as Pep1 can suppress basal defense and thereby trigger an HR-like ETI response that

shows hallmarks of BI-1-dependent, necrotic cell death. Compared with PTI-triggered cell death, this response is more costly to the plants, which is indicated by macroscopically visible necrosis and stunted growth.

MATERIALS AND METHODS

Strains and growth conditions.

All *Ustilago* strains used in this study (Supplementary Table S1) were grown in 0.4% yeast extract, 0.4% peptone, and 2% sucrose, incubating the *U. hordei* strains at 22°C and *U. maydis* strains at 28°C, with shaking at 200 rounds min⁻¹. For plant infections, cultures were grown to an optical density at 600 nm (OD₆₀₀) of 0.7 to 1.0. Cells were centrifuged at 1,000 × g for 10 min, resuspended in 0.1% Tween20 to an OD₆₀₀ of 3, and used for infection of 10-day-old barley seedlings. Plant infections were performed comparable with the established maize infection method as described earlier (Molina and Kahmann 2007). For experiments, barley plants (*Hordeum vulgare* L. Golden Promise and Golden Promise expressing GFP-HvBI-1 under control of promoter CaMV35S) (Babaeizad et al. 2009) were grown in phytochambers at 22°C during the light period (30,000 lux, 16 h) and at 18°C during the dark period (8 h), with 60% relative humidity.

Enzymatic caspase-like activity assay.

For the preparation of total leaf extracts, 2- to 4-cm sections of the infected leaves were excised 1 cm below the injection site at the respective time points, frozen in liquid nitrogen, and ground to a powder with a cold mortar and pestle. The powder was collected and resuspended in two volumes of cold extraction buffer (10 mM sodium phosphate buffer [pH 7.3], 150 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol, 20 μM E-64, and Aprotinin at 10 mg/ml), followed by three rounds of centrifugation for 10 min at 16,000 × g and 4°C to remove cell debris. Supernatants were then filtered through a 35-μm nylon filter (BD Biosciences, Heidelberg, Germany) and samples were maintained at 4°C. Protein content was measured according to Bradford (1976) and samples were adjusted to a protein concentration of 1 mg/ml, immediately proceeding with proteolytic activity measurements.

Proteolytic activity was measured using the fluorimetric mammalian caspase-3 substrate Ac-DEVD-MCA (dissolved in dimethyl sulfoxide [DMSO]) (Peptanova, Sandhausen, Germany), which leads to release of fluorescence at 460 nm when cleaved by caspase-3 activity (Enari et al. 1996; Nicholson et al. 1995; Thornberry et al. 1997; Zimmerman et al. 1976). Then, 10 μl of 100 μM substrate was incubated with 80 μl of leaf extract (1 mg/ml) and added to a final reaction volume of 100 μl with 10 μl of DMSO. To analyze the effect of a specific caspase-3 inhibitor on substrate cleavage, 80 μl of soluble extract was incubated with 10 μl of the reversible inhibitor peptide Ac-DEVD-CHO (dissolved in DMSO) for 10 min at room temperature, prior to substrate addition. Measurements of caspase-1-like activity and VPE were performed following the same protocol used for caspase-3-like activity. For caspase-1-like activity, substrate Ac-YVAD-MCA was used and, for VPE activity-measurements, we applied substrate Ac-ESEN-MCA. All substrates were supplied by Peptanova. Fluorescence was monitored every 10 min over a time course of 1 h using a fluorometer (Safire; Tecan, Crailsheim, Germany) and slope values were calculated. Background measurements were taken in the respective buffers and subtracted from sample values.

Determination of ROS in apoplastic fluid.

For the preparation of apoplastic fluid, 2- to 4-cm sections of the infected leaves were excised 1 cm below the injection

site at the respective time points, followed by evacuation under water in a vacuum chamber for 3 × 15 min at 100 mbar. The evacuated leaf sections were then stacked into packs of 10 to 20 and squeezed into the barrel of a 5-ml syringe so that the cut edges of the leaves faced the ends of the barrel. The barrel was then put into a 15-ml falcon tube with the needle hub facing downward and spun for 20 min at 2,500 × g and 4°C. Afterward, the extracted apoplastic fluid was collected from the falcon tube and immediately used for the colorimetric visualization of ROS.

H₂O₂ production was quantified with a xylenol-orange-based readout according to Bindschedler and associates (2001, 2006) by incubating 10 µl of apoplastic fluid with 90 µl of xylenol orange solution. Absorption measurements of the assay solution were undertaken in a Tecan Safire plate reader (Infinite M 200 Pro; Tecan) every 5 min over a time course of 1 h, and slope values were calculated. Background measurements were taken in the respective buffers and subtracted from sample values.

RNA preparation, cDNA synthesis, and qRT-PCR.

For expression analyses of HR-associated marker genes, 2- to 4-cm sections of the infected leaves were excised 1 cm below the injection site at the respective time points, frozen in liquid nitrogen, and ground to a powder with a cold mortar and pestle. The powder was collected and total RNA was extracted with TRIzol (Invitrogen, Karlsruhe, Germany) and purified using an RNeasy kit (Qiagen, Hilden, Germany), as described by the manufacturer. After extraction, the first-strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany) was used to reverse transcribe 1 mg of total RNA with oligo(dT) primers. The qRT-PCR analysis was performed using an iCycler (Bio-Rad, Munich, Germany) in combination with the iQ SYBR Green Supermix (Bio-Rad). Primers for quantification of barley gene transcription levels are shown in Supplementary Table S2. Barley glyceraldehyde dehydrogenase (gapdh) was used as reference gene for normalization. Cycling conditions were as follows: 2 min at 95°C, followed by 45 cycles of 30 s at 95°C, 30 s at 61°C, and 30 s at 72°C. After each PCR, the threshold cycle above background was calculated using Bio-Rad iCycler software. Gene expression levels were then calculated relative to gapdh expression levels. Relative quantification of gene expression was carried out using a relative expression software tool (REST; Qiagen), applying an improved $\Delta\Delta$ analysis (Pfaffl et al. 2002).

Staining procedures and microscopy.

Autophagic vesicles were stained with MDC (Sigma-Aldrich, Deisenhofen, Germany) by incubating corresponding leaf sections with 100 µM MDC final concentration in 1× phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, and 12 mM phosphate buffer, pH 7.4). Lysotracker red (Life Technologies, Darmstadt, Germany) for visualization of acidified vesicles was applied at a final concentration of 0.5 µM in 1× PBS. Fungal hyphae were stained with WGA-AF 488 (Molecular Probes, Karlsruhe, Germany) by incubation in WGA-AF 488 at 10 µg/ml and 0.02% Tween20 in 1× PBS. Plant membranes were visualized using FM4-64 (Molecular Probes, Karlsruhe, Germany) (0.5 µg/ml in 1× PBS) or propidium-iodide (Sigma-Aldrich) (1 µg/ml in 1× PBS). FDA (Sigma-Aldrich) (0.5 µg/ml in 1× PBS) was used to stain the cytoplasm of plant cells. For all staining methods, leaf sections were infiltrated with the corresponding staining solution in a vacuum chamber for 3 × 15 min at 100 mbar. Plasmolysis was induced by incubating the corresponding leaf sections in 1 M NaCl for 10 min. Visualization of H₂O₂ by DAB was performed as previously described by Molina and Kahmann (2007). For the analysis of cell via-

bility, infected barley leaves were stained with trypan blue according to a modified protocol (Keogh et al. 1980). Leaves were submerged in ethanolic lactophenol-trypan blue staining solution and heated to 99°C for 8 min. Subsequently, the staining solution was replaced by a saturated chloral hydrate solution (2.5 g/ml) which was changed several times until the leaves were cleared. Leaves were stored in 50% (vol/vol) glycerol. To stain fungal material on leaf surfaces, excised leaf segments were incubated in Calcofluor Fluorescent Brightener 28 (Sigma-Aldrich) (100 µg/ml in 0.2 M Tris/HCl, pH 8.0) for 30 s.

Confocal images were recorded on a TCS-SP5 confocal microscope (Leica, Bensheim, Germany) using an excitation at 488 nm and detection at 490 to 540 nm for WGA-AF 488 and FDA. Autofluorescence of cell wall material and Calcofluor-white-stained fungal hyphae were excited at 405 nm and detected at 415 to 460 nm. For MDC-stained vesicles, excitation at 405 nm and detection at 490 to 520 nm was used. Lysotracker red was excited at 563 nm and detected at 570 to 600 nm. FM4-64-stained plant membranes were visualized by excitation at 561 nm and detection at 570 to 640 nm. Trypan-blue-stained cells as well as DAB precipitates were recorded using a Leica DFC500 fluorescence camera that was installed at the TCS-SP5 confocal microscope.

Sample preparation for TEM was performed according to Hemetsberger and associates (2012). Small samples of leaves (1 mm²) were incubated with a 5-mM CeCl₃ solution dissolved in 50 mM 3-(N-morpholino)propanesulfonic acid buffer for 1 h at pH 6.5. Subsequently, samples were fixed in a mixture of 2.5% paraformaldehyde and 2.5% glutaraldehyde dissolved in buffer at pH 7.2 for 90 min, rinsed in buffer (4 × 10 min), and postfixed with 1% osmium tetroxide dissolved in buffer (pH 7.2). Dehydration was performed with increasing concentrations of acetone (50, 70, 90, and 100%) for 20 min for each step. Acetone was then exchanged with propyleneoxide and samples were infiltrated with increasing concentrations of Agar 100 epoxy resin (30, 60, and 100%). Samples were polymerized at 60°C for 48 h. Ultrathin sections (80 nm) were cut with a Reichert Ultracut S ultramicrotome and poststained for 5 min with a 2% lead citrate solution and for 15 min with 1% aqueous uranyl acetate before they were observed with a Philips CM10 TEM.

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