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Morphological and Molecular Analyses of Host and Nonhost Interactions Involving Barley and Wheat and the Covered Smut Pathogen *Ustilago hordei*

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Ustilago hordei **interactions on coleoptiles of barley host cultivars Odessa (compatible), Hannchen (incompatible, carrying the *Ruh1* resistance gene), and on nonhost Neepawa wheat were studied using light and fluorescent microscopy. Autofluorescence, mainly caused by callose accumulation, was more rapidly expressed in nonhost wheat at 30 to 72 h compared with the incompatible reaction between 72 and 144 h. Microarray results demonstrated that more than half of the 893 differentially regulated genes were observed in Neepawa; of these genes, 45% fell into the defense- and stress-related classes in Neepawa compared with 25 and 37% in Odessa and Hannchen, respectively. Their expression coincided with the early morphological defense responses observed and were associated with the jasmonic acid and ethylene (JA/ET) signaling pathway. Expression patterns in Odessa and Hannchen were similar, involving fewer genes and coinciding with later morphological defense responses of these varieties. Although no visible hypersensitive response was apparent in Hannchen or Neepawa, specific upregulation of hypersensitivity-related proteins was observed, such as beta-VPE at 48 h. Expression levels of the *callose synthase* gene were closely associated with callose accumulation. Differential responses in defense-gene expression among disease reaction types included upregulation of *PR-1.1b* and downregulation of a nonspecific lipid transfer protein in the incompatible and compatible interactions, respectively. Transcript levels of *EDS1* and *PAD4*, involved in both basal resistance and *R*-mediated resistance to avirulent pathogens, were up-regulated during both nonhost and *Ruh1*-mediated resistance. Application of methyl-jasmonate, salicylic acid and ET to leaves revealed that only *PR1.1b* is strongly up-regulated by all three compounds, while the majority of the defense-related genes are only slightly up-regulated by these signaling compounds.**

A plant species is considered a nonhost if it is immune to all biotypes of a particular parasite (Abramovitch et al. 2006; Heath 2000; Thordal-Christensen 2003). The terms nonadapted pathogens and nonhost resistance (basal or innate immunity) refer to the pathogen and host components of this incompatible interaction, respectively. At a molecular level, pathogen components (pathogen or microbe-associated molecular patterns, PAMPs or MAMPs) are recognized by the host as nonself to initiate PAMP-triggered immunity (PTI) (Jones and Dangl 2006). Conversely, if the plant species is susceptible to a particular pathogen but members of that species are resistant to one or more of the pathogen biotypes, the resistance is known as host resistance. Genetically, these interactions are controlled by resistance (*R*) genes that interact with pathogen avirulence (*Avr*) genes (Abramovitch et al. 2006; Belkhadir et al. 2004; Bent and Mackey 2007; Chisholm et al. 2006; Jones and Dangl 2006). In molecular terms, *R* gene products, often from the nucleotide binding-leucine-rich repeat (NBS-LRR) receptor class, interact either directly or indirectly with effectors delivered into hosts to elicit effector-triggered immunity (ETI) (Jones and Dangl 2006). Nonhost resistance is considered the most broadly effective and durable form of resistance (Heath 2000; Mysore and Ryu 2004), while *R* genes tend to lose their effectiveness, a phenomenon that has been attributed to the highly specific interaction between NBS-LRR cognate receptors and the corresponding pathogen effectors, coupled with the high incidence of mutations among *Avr* genes (Rep 2005).

Host and nonhost resistance appear to be two branches of the same plant immune system that interact with various classes of pathogen factors or virulence effectors to generate the characteristic outcomes of the incompatible host-parasite interaction (Abramovitch et al. 2006; Bent and Mackey 2007; Chisholm et al. 2006; Jones and Dangl 2006). Models for plant disease immunity responses integrating host and nonhost resistance responses into a series of chronologically defined but interrelated stages, have been proposed in *Arapbidopsis* (Chisholm et al. 2006; Bent and Mackey 2007; Jones and Dangl 2006).

One of the earliest known plant defense responses, following the perception of pathogen effectors with avirulence function, is the oxidative burst accompanied by the accumulation of H_2O_2 (Heath 2000; Lamb and Dixon 1997; Shetty et al. 2008; Thordal-Christensen et al. 1997). Other subsequent host responses include the hypersensitive response (HR), upregulation of phenylalanine ammonium lyase (PAL), a key enzyme in plant defense, and synthesis of a wide range of antimicrobial compounds, including pathogenesis-related (PR)-proteins and phytoalexins (Veronese et al. 2003). Additionally, host plants can reinforce cell walls by depositing cell wall–strengthening materials such as lignin and callose (Maor and Shirasu 2005). Callose synthase is a key enzyme in the biosynthesis pathway of callose, a (1-3)-β-D-glucan, and frequently accumulates in response to mechanical damage or pathogen infection and may provide a physical barrier to penetration by pathogens

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(Kudlicka and Brown 1997). However, contradictory roles for callose in plant defense response have been proposed, and its clear contribution to plant defense remains to be established (Maor and Shirasu 2005; Nishimura et al. 2003).

Mysore and Ryu (2004) have further divided nonhost resistance into type I resistance, whereby the pathogen is unable to overcome the preformed defenses, antimicrobial substances, or reinforcement of cell walls, or type II resistance, which involves the HR at penetration sites. The nature of the HR may be similar in both nonhost and *R* gene–associated HR responses (Abramovitch et al. 2006; Cai et al. 2007; Mysore and Ryu 2004). Enhanced disease susceptibility 1 (*EDS1*) along with its counterpart phytoalexin deficient 4 (*PAD4*) genes are key post-invasion plant defense regulators involved in basal resistance to obligate parasites (Lipka et al. 2008; Wiermer et al. 2005). These genes are also involved in a subset of *R* gene–mediated resistance pathways especially in conjunction with toll-interleukin-1 receptor (TIR)-type NBS-LRR *R* genes (Feys et al. 2001; Wiermer et al. 2005). TIR NBS-LRR *R* genes have been identified in dicot species and in some monocots such as rice but not in wheat (Bai et al. 2002).

The cereal smuts and bunts are ubiquitous plant pathogens on cereals and remain serious constraints to yield and quality worldwide (Goates 1996). For many of the surface-borne smuts and bunts, resting teliospores are dispersed by wind or mechanical threshing onto healthy seeds (Fischer and Holton 1957). Germination of the teliospores generally coincides with seed germination and the fungus usually undergoes meiosis and plasmogamy to reestablish the binucleate condition before penetrating the developing coleoptile (Fischer and Holton 1957; Thomas 1991). Subsequent spread of the mycelium occurs both inter- and intracellularly (Fischer and Holton 1957; Hu et al. 2002, 2003). In a compatible interaction, a successful infection results when the fungus is able to spread inwards from the coleoptile to establish itself in the region directly below the apical growing point of the developing culm, following the intercellular passage through several embryonic leaves, a process that takes three to five weeks after seeding (Fischer and Holton 1957), although macroscopic symptoms (i.e., presence of the infected heads) are only visible when the plant is approaching maturity (Fischer and Holton 1957).

A gene-for-gene interaction exists between *Avr* genes of many smuts and bunts and the many individual host resistance *R* genes (Sidhu and Person 1972; Goates 1996). Compatible and *R* gene–incompatible reactions of smuts and bunts on their host plants have been described (Gaudet et al. 2007; Hu et al. 2003; Kozar 1969; Woolman 1930). For surface-borne smuts and bunts of wheat and barley, the initial germination and infection processes are similar in both susceptible and *R* gene–resistant varieties, but fungal growth remains restricted to the coleoptile in the resistant interaction, whereas the fungus attains the apical meristems in the susceptible interaction (Kozar 1969; Woolman 1930). Additionally, in the incompatible interaction, callose accumulation in a zone surrounding the site of penetration is associated with *R* gene–resistance expression and termination of fungal growth (Gaudet et al. 2007; Hu et al. 2003). No evidence of a HR has been observed in wheat infected by bunt caused by *Tilletia tritici* (Bjerk.) G. Wint. in Rabenh. and *Tilletia laevis* Kühn in Rabenh. (Gaudet et al. 2007; Woolman 1930), whereas evidence of a localized necrosis involving two to three cells has been reported in the incompatible interaction involving barley and the covered smut pathogen *Ustilago hordei* (Pers.) Lagerh. two days following inoculation (Hu et al. 2003). The function of callose deposition during pathogen attack has not been unequivocally demonstrated, but it has been suggested that callose deposition acts as a physical barrier to impede microbial penetration and progression. The accumulation of callose has been associated with a micro-HR following infection of pepper leaves with an avirulent strain of *Xanthomonas campestris* pv. *vesicatoria* (Lee and Hwang 2005) and with the HR in host and nonhost interactions involving *Phytophthora infestans* (Vleeshouwers et al. 2000).

Wheat is a nonhost for *U. hordei* (Fischer 1953), but on barley, its natural host, incompatibility is governed through various *R* genes interacting with six known *avr* genes in various races (Linning et al. 2004; Sidhu and Person 1971). The *avr* gene *UhAvr1* interacts with the *Ruh1* in the barley cultivar Hannchen (Hu et al. 2003; Sidhu and Person 1972). The cytological details of nonhost incompatible interactions have been described for a number of host-parasite interactions (Heath 2000; Krzymowska et al. 2007; Lipka et al. 2008) but have not yet been revealed for any of the cereal smut fungi.

The objective of this study is to describe the interaction between *U. hordei* and wheat, a nonhost, and compare it with the incompatible interaction involving the *Ruh1*/*UhAvr1* gene pair in Hannchen and a compatible interaction in the nonisogenic barley variety Odessa. A timecourse study of infection was conducted in which fluorescent microscopy, 3,3′-diaminobenzidine (DAB) staining for reactive oxygen species, wheat microarrays, and quantitative real-time polymerase chain reaction (qPCR) using selected defense-related genes were employed to study these pathogen interactions on the coleoptiles of wheat and barley.

RESULTS

Host reactions in *U. hordei*–inoculated wheat and barley.

Successful infection with *U. hordei* can only be evaluated at the end of the life cycle of the plant (i.e., in the grain head) (Fischer and Holton 1957). Therefore, residual plants for all inoculation studies were reared to maturity to determine the percent infection of smutted heads and ensure that successful infection had occurred. Infection levels among inoculated Odessa barley plants were consistently high in all experiments, ranging from 30 to 40%. Infection levels for incompatible interactions in Hannchen barley and Neepawa wheat were 0%. The first signs of *U. hordei* teliospore germination on barley and wheat coleoptiles were observed at 8 h after inoculation (hai), ranging between 8 to 12% of the teliospore load, became widespread at 28 hai, showing between 50 to 60% of the spores germinating, and reached maximum levels at 48 hai, ranging from 90 to 95% spore germination. All stages of germination and subsequent fungal development were observed on wheat and barley coleoptiles in both petri dishes and in soilless potting mixture (Figs. 1A to D). A basidium that extended from the germinating teliospore either produced a tetrad of four sporidia that subsequently continued to bud to form large numbers of new sporidia (Fig. 1A) or directly formed clamp connections on the basidium (Fig. 1B) to develop the dikaryon. Sporidial fusions (Fig. 1C) were frequently observed along the entire length and surface of the developing coleoptiles. Dikaryotization in the basidium or formation of sporidia on the coleoptile were equally common. Following sporidial pairing or formation of clamp connections on the basidium, infection hyphae developed over the coleoptile surface (Figs. 1B to D and 2A to C), growing by moving the fungal cytoplasm into the extending fungal cell walls and leaving the empty ghost cells behind. Prior to penetration of the coleoptile, no differences in fungal germination, development, or host reactions were observed among the wheat and barley cultivars.

In the compatible interaction involving Odessa barley, penetration was often preceded by a swelling of the tip of the infection hyphae to form an appressorium-like structure (Fig. 2A and B). Ingress into the coleoptile was rapid, with penetration

occurring intra- or intercellulary at the junctions between two or more cells (Fig. 2A) between 30 and 144 hai. When penetration occurred intracellularly, the formation of a disk-shaped hypha immediately following penetration was observed inside some of the cells. Movement of the cytoplasm from exterior fungal cells to the growing inter- and intra-cellular hyphae coincided with the appearance of ghost hyphae (Fig. 2C) and a transient cotton blue-stained fungal cytoplasm on the surface of the coleoptile that represented the infection court (Fig. 2D). Spread of the fungus within the coleoptile was rapid and occurred mostly intercellularly via unbranched hyphae that traversed several cell layers at 48 hai (Fig. 2E and F). The first visible sign of host reaction was diffuse autofluorescence of cells in the vicinity of infection sites and was regularly observed from 72 to 168 hai in inoculated treatments in Odessa (Fig. 2E), and its occurrence coincided with the intercellular spread of the fungus. Small deposits of callose surrounding the penetration site (Fig. 2F) and at the junctions of cells adjacent to hyphae were observed late in the infection process, from 96 to 168 hai.

In the *Ruh1* gene–mediated incompatible interaction involving Hannchen, prepenetration and early penetration events were very similar to those observed in Odessa. Penetration was rapid after 30 hai, with the fungus traversing several cell layers at 48 hai (Fig. 3A and B). Also, obvious host reactions including autofluorescence were not observed during penetration (Fig. 3A). Small deposits of callose appeared as a thin sheath around the remnant fungal cell walls (ghost cells) at the cell junctions (Fig. 3B) and around penetration sites at 72 hai (Fig. 3C), 24 h earlier than observed in Odessa. Between 96 and 144 hai, clear anatomical differences in the host reaction were observed between the incompatible interaction in Hannchen and the compatible interaction in Odessa. In Hannchen, the continuing defense response consisted of accumulation of callose, extending away from the penetration site and along the remnant fungal cell wall at 72 to 96 hai (Fig. 3C and D). From 120 to 168 hai, extensive accumulation of callose surrounding the penetration site and on the walls of adjacent mesophyll cells was observed (Fig. 3E). Clear evidence of dissolution of the middle lamella between epidermal cells during penetration was evident following accumulation of callose around the penetration site. Growth arrest of the active fungal hyphae in the interior of the coleoptile was accompanied by extensive accumulation of callose around the hyphae at 120 and 144 hai (Fig. 3F). Additionally, there was no visible evidence that the fungus had progressed beyond the interior of the coleoptile into the primary leaves after 168 hai. No evidence of cell collapse, necrosis, or cell death indicative of an HR was observed.

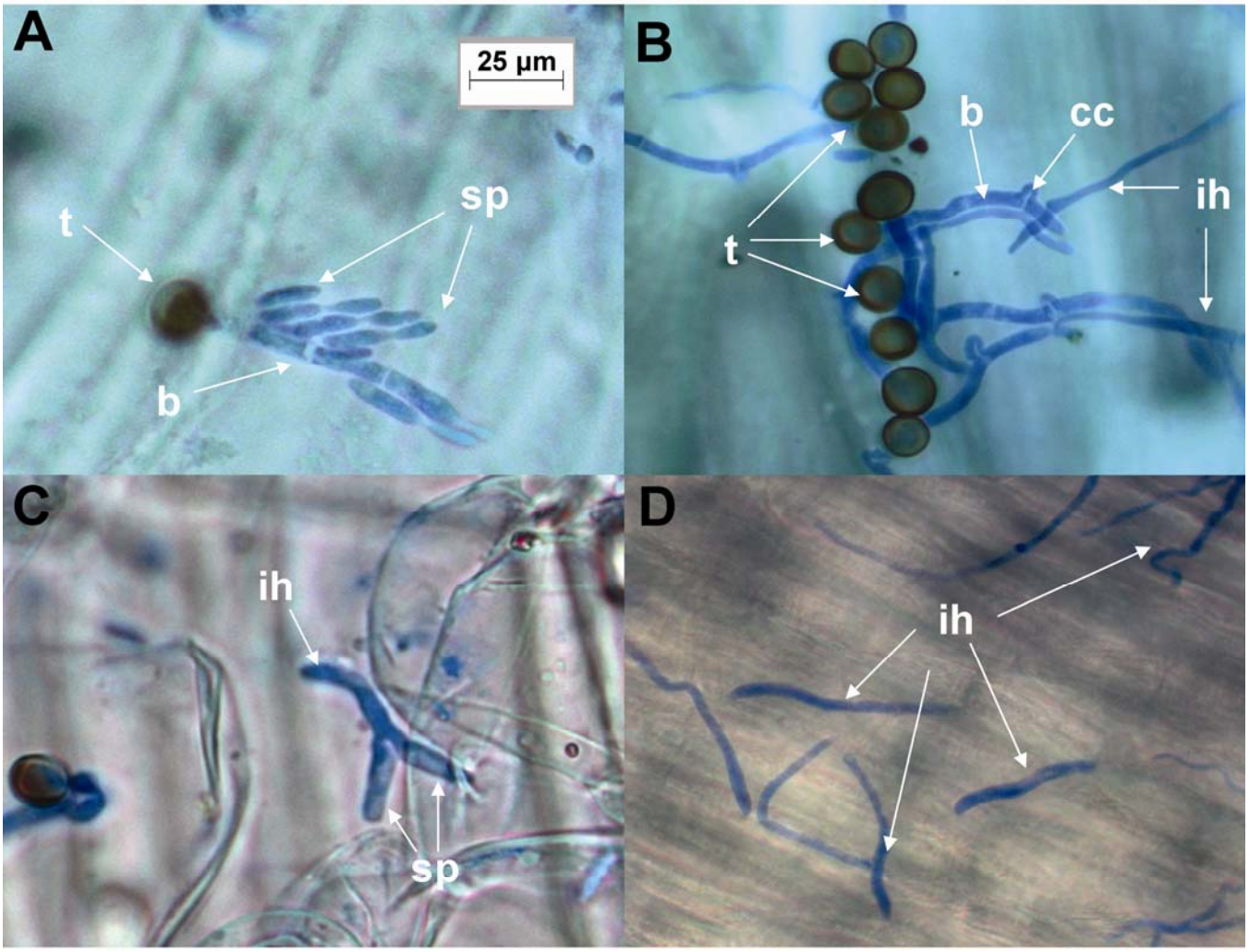

Fig. 1. Interaction involving isolate Uh4857 of *Ustilago hordei* on barley and wheat. Germinating teliospores on the coleoptiles of **A,** Hannchen barley, **B,** Neepawa wheat, and **C** and **D,** Odessa barley. Seedlings were inoculated with teliospores and were incubated at 20°C for 18 (A and B) and 24 h (C and D). **A,** Teliospore (t) germinating to produce a basidium (b) primary sporidia (sp), and infection hyphae (ih). **B,** Teliospores germinating to produce clamp connections (cc) and infection hypha (ih) directly from the basidium (b). **C,** Two sporidia (sp) fuse to form a single dikaryotic infection hypha (ih). **D,** Numerous infection hyphae n the surface of the coleoptile.

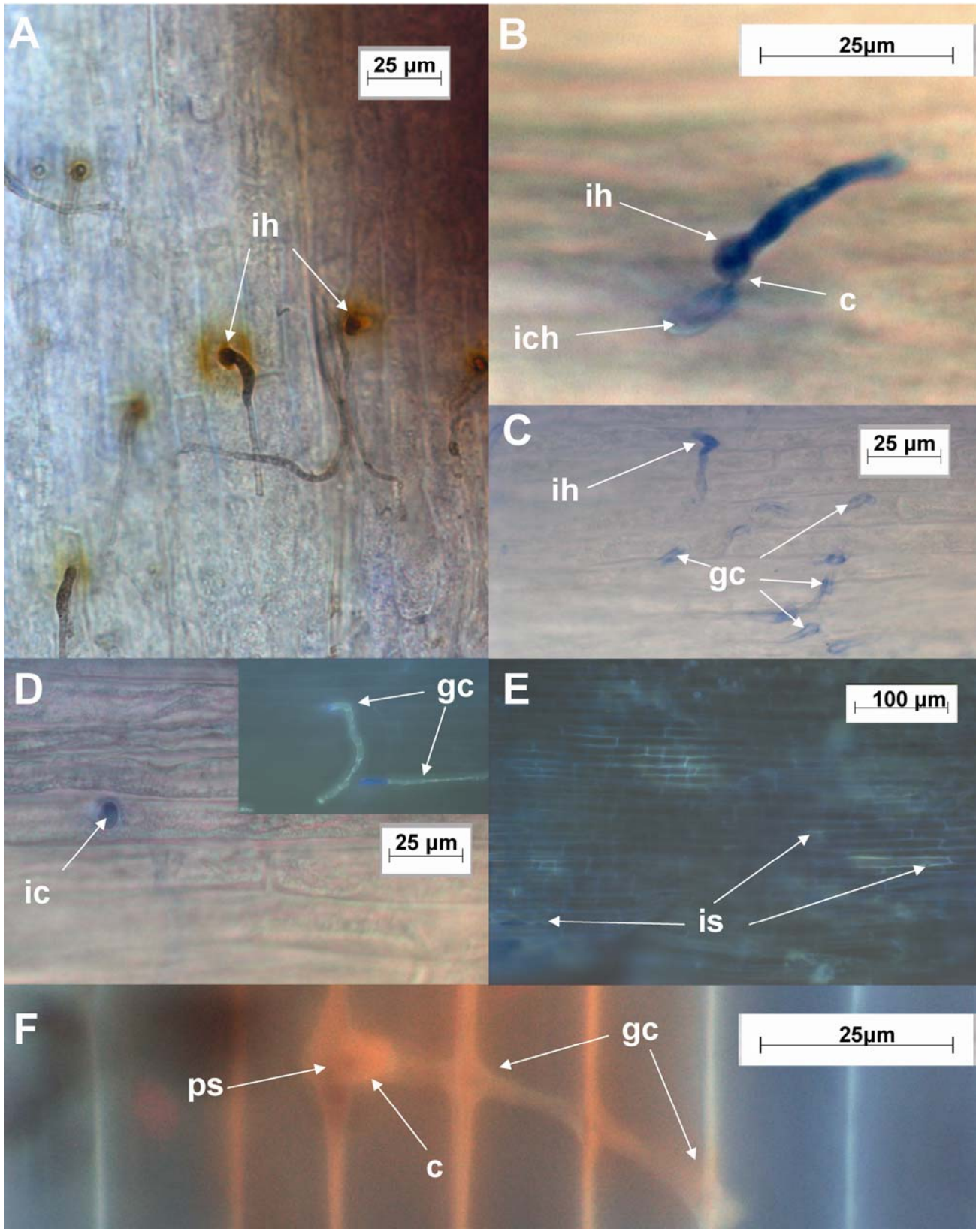

Fig. 2. Compatible interaction involving isolate Uh4857 of *Ustilago hordei* on surface cells of Odessa barley coleoptiles. Seedlings were inoculated with teliospores and incubated at 20°C for 30 to 144 h. **A,** Infection hyphae (ih) penetrating mesophyl cells of the coleoptile at 30 hai. Brown discoloration denotes area of oxidative burst. **B,** Penetration and intercellular development of the hypha. Infection hypha (ih) with appressorium, constriction (c) at the site of penetration and development of a flattened disk-shaped intracellular hypha (ich). **C,** Hyphal ghost cells (gc) remaining on coleoptile surface following infection and single remaining infection hypha (ih) containing cytoplasm, 72 h after inoculation (hai). **D,** Infection court (ic) showing residual cytoplasm (blue) belonging to infection hypha and corresponding fluorescent image (insert) showing remnant ghost cells (gc) of infection hyphae on the exterior surface of the coleoptile. **E,** Diffuse fluorescence associated with successful penetration and spread. Remnant infection sites (is) associated with fluorescence, 72 hai. **F,** Remnant fungal cell traversing the mesophyll cells of the coleoptile that originated from a single penetration site (ps), 144 hai. Note small zone of callose (c) deposition surrounding the fungal cell-wall remnant adjacent to penetration site. A and E, 3,3′-diaminobenzidine stained.

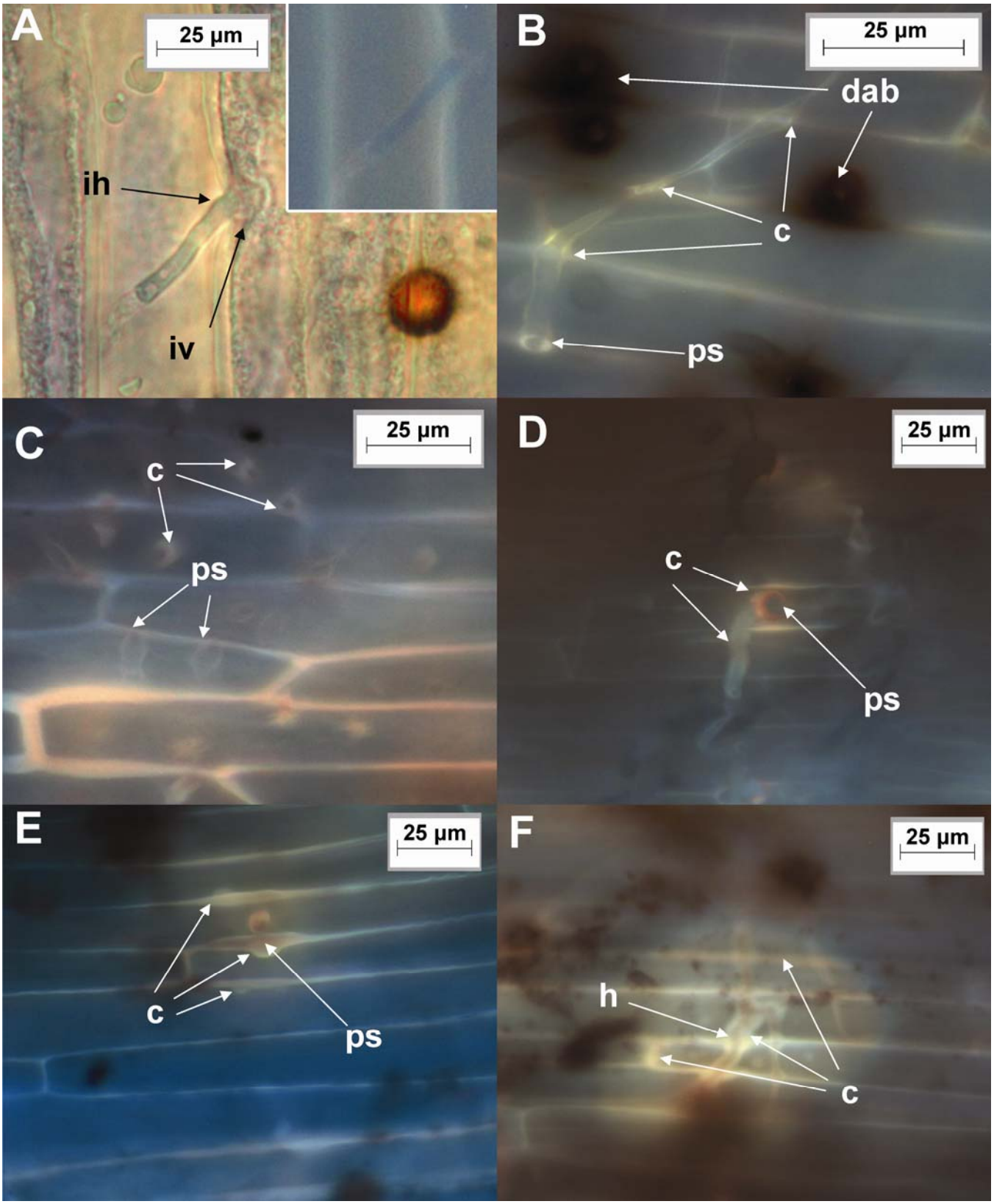

Fig. 3. Incompatible host interaction involving isolate Uh4857 of *Ustilago hordei* on surface cells of coleoptiles of Hannchen barley, which possesses *Ruh1*. Seedlings were inoculated with teliospores and were incubated at 20°C for 28 to 168 h. **A,** Infection hypha (ih) penetrating intracellulary at 28 h after inoculation (hai) and associated invagination (iv) of host cell cytoplasm. Corresponding fluorescent micrograph (insert) showing no specific fluorescence. **B** through **E,** 3,3′-Diaminobenzidine (DAB) staining. **B,** Remnant infection hypha at the penetration site (ps) and traversing intercellulary after 48 hai. Note small accumulations of fluorescent callose (c) at the penetration sites and at cell junctions. Brown spots (dab) reflect DAB staining. **C,** Limited callose accumulating around several penetration sites at 72 hai. In other penetration sites, callose has not yet accumulated. **D,** Callose surrounding the penetration site and extending partially down the remnant hyphal tube (arrow) after 96 hai. **E,** Callose coalescing around penetration site and on adjacent cell walls after 96 hai. **F,** Callose accumulation surrounding hyphae (h) and coating the cell walls of cells in the interior of the coleoptile after 144 hai.

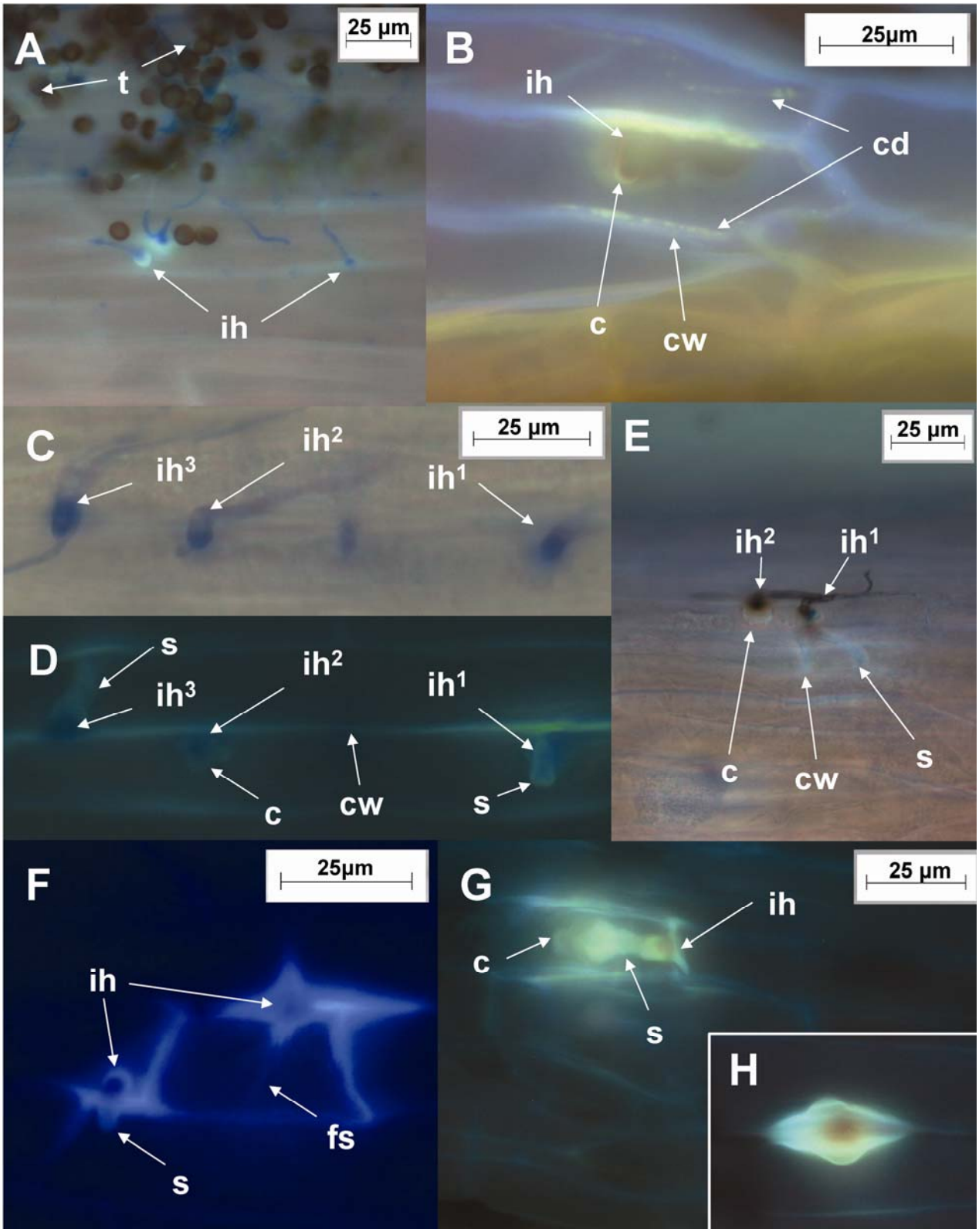

Fig. 4. Incompatible nonhost interaction involving isolate Uh4857 of *Ustilago hordei* on coleoptiles of Neepawa wheat. Seedlings were inoculated with teliospores and were incubated at 20°C for 30 to 144 h. **A,** Germinating teliospores (t) and infection hyphae (ih) with fluorescence 30 h after inoculation (hai). **B,** Beginning of defense reaction as small fluorescing callose droplets (cd) accumulating in cell walls (cw) and coalescing into a callose (c) plug around the penetrating infection hypha (ih) after 48 hai. **C** and **D,** Corresponding light and fluorescent images, respectively, of infection hyphae after 72 hai. Note that ih^1 and ih^2 are co-penetrating the same cell whereas ih^3 is penetrating a different cell; ih^1 and ih^3 penetrate deep beyond the cell wall (cw) into the cell and are surrounded by sheaths (s) while ih^2 has callose deposits that appear to terminate ingress. Also note fluorescing cell wall (cw). **E,** Micrograph represents a Z-stack of corresponding light and fluorescent images. Co-penetration of a single cell by two infection hyphae after 72 hai. Note the ih^1 penetrates through the first cell into the underlying cell, whereas ih^2 ingress is apparently terminated by a callose plug. Fluorescence associated with the cell wall (cw) and callose sheath around hyphae. **F,** Formation of a sheath involves extension of fibrous strands (fs) originating from the cell wall around infection hypha after 96 hai. **G,** Longitudinal view of the final stages of callose deposition to form a sheath (s) around a penetrating infection hypha 96 hai. **H,** (inset) Remnant fluorescence associated with callose plug around the infection site after 144 hai.

In the nonhost incompatible interaction involving Neepawa wheat, signs of a defense response were also evident during penetration at 30 hai. Two types of reactions were observed. The first was a stronger reaction that consisted of the rapid accumulation of a callose plug distal to and around the attempted penetration site, accompanied by bright fluorescence (Fig. 4A and B). The callose plug appeared to result from the accumulation and fusion of callose droplets deposited along the cell walls adjacent to the penetration site observed from 30 to 48 hai (Fig. 4B). Secondly, a weaker reaction occurred that consisted of deposition of a thin sheath of callose around infection hyphae, which weakly fluoresced (Figs. 4C to E) at 30 to 72 hai. The latter, weaker reaction was the predominant reaction observed during the interaction between *U. hordei* and the wheat host. Occasionally, two penetration events, IH^1 (infection hyphae 1) and IH^2, occurred within a single plant cell. For the IH^1 event (Fig. 4D), a thin callose sheath was evident, whereas, in the IH^2 event, callose directly accumulated around the penetrating hypha. This phenomenon was also evident in the micrograph shown in Figure 4E, in which IH^1 was the initial invading hypha that continued to penetrate the plant cell, despite the apparent accumulation of some callose around it. Meanwhile IH^2, a coinfecting hypha in the same cell, rapidly became encased within a callose deposit at 30 to 48 hai, which coincided with the cessation of growth of secondary penetration hyphae. These results suggested that individual cells are potentiated by the initial penetrating hypha and reacted more rapidly to subsequent penetration attempts by rapidly depositing a callose plug around the invading hypha in this nonhost interaction. This phenomenon was not observed following multiple infections of a single cell in *Ruh1* interactions in Hannchen (Fig. 3C).

In most instances, fungal growth in Neepawa stopped at the initial penetrated cell, although occasionally, growth in a second adjacent cell was observed (Fig. 4E). A characteristic of nearly all attempted penetrations in this nonhost interaction was the limited development of inter- and intracellular infection hyphae, within the epidermal cell layer and beyond, that prevented displacement of all the cytoplasm from the surface infection hyphae (Fig. 4C), and consequently, the occurrence of remnant cytoplasm in the remnant ghost cells on the surface was observed. This contrasts with observations involving Odessa (Fig. 2C and D) and Hannchen (not shown), in which all cytoplasm of the surface-borne infection hyphae moved into the rapidly developing inter- and intracellular hyphae in the coleoptile. In defense responses involving primary infections, fibrous strands extending from the cell wall towards the invading hyphae were evident at 48 to 96 hai (Fig. 4F). Eventually, after 72 to 144 hai, all infection hyphae became encased in callose, which originated as small droplets associated with the cell wall and deposited around infection hyphae (Fig. 4B and G). Successive layers of callose deposition were evident prior to encasement of the entire hypha (Fig. 4G). At 144 hai, a remnant fluorescent callose plug around the infection site was the only evidence remaining of the penetration attempt (Fig. 4H). Similar to Hannchen, no evidence of HR was observed.

Autofluorescence associated with penetration from 24 to 168 hai was interpreted as a measure of host defense responses. Our results clearly showed such fluorescence expression to be more rapidly induced in nonhost Neepawa compared with incompatible Hannchen (Fig. 5). Fluorescence associated with penetration sites was evident as early as 48 hai in Neepawa (Figs. 4A and 5) and rose to approximately 80% at 72 hai compared with a later occurrence in incompatible Hannchen (Figs. 3, 4, and 5). Susceptible Odessa exhibited a much weaker, diffuse fluorescence (Fig. 2E) that developed slightly later than in Hannchen, and infection site numbers rose to a maximum at 120 hai (Fig. 5).

DAB staining for hydrogen peroxide detection showed that the oxidative burst generally remained localized to a region directly adjacent to the penetration site (Figs. 2A and 3B), rarely extending beyond the borders of the affected cell. The occurrence of the DAB stain reached maximum levels at 48 hai and remained elevated until 96 hai, after which the percentage of stained infection sites declined (Fig. 6). In noninoculated control plants, DAB-stained cells were not observed (data not shown). The time of occurrence of the oxidative burst did not vary among the three interactions and the percentage of infection sites staining with DAB was similar. This suggested that the oxidative burst occurred during early penetration and infection events, characteristic of both nonhost and host interactions, and that the hydrogen peroxide product of the oxidative burst was transiently maintained until 96 hai, after which levels dropped.

Microarray profiling.

For gene expression analysis during the incompatible interaction involving *U. hordei* and Hannchen (*Ruh1*), Odessa, and nonhost Neepawa, we performed transcript profiling using the Affymetrix GeneChip Wheat Genome Array representing

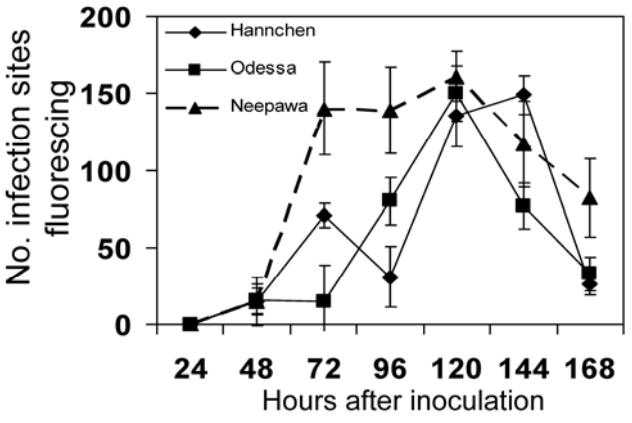

Fig. 5. Number of fluorescing infection sites observed on the surface of coleoptiles following *Ustilago hordei* germination on susceptible Odessa barley, resistant Hannchen barley that possesses *Ruh1*, and nonhost Neepawa wheat. Coleoptiles were inoculated with *U. hordei* isolate Uh4857 in petri dishes on sterile filter paper and were incubated at 20°C. Coleoptiles were sampled from 24 to 144 h after inoculation. Bars indicate standard errors.

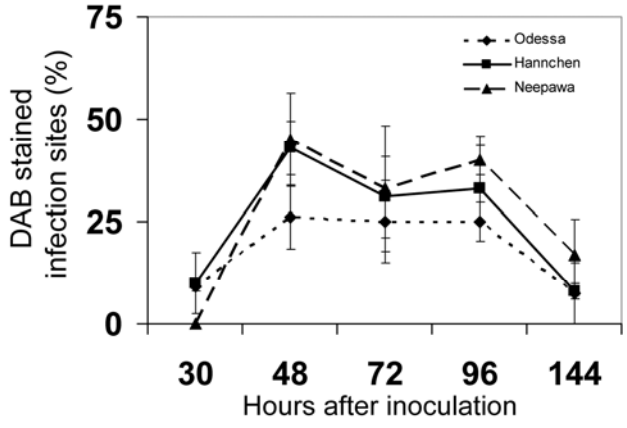

Fig. 6. Percentage of infection sites staining positively with 3,3′-diaminobenzidine on the surface of coleoptiles following germination of susceptible Odessa barley, resistant Hannchen barley possessing *Ruh1*, and nonhost Neepawa wheat in petri dishes on sterile filter paper, inoculated with *Ustilago hordei* isolate Uh4857 and were incubated at 20°C. Coleoptiles sampled from 30 to 144 h after inoculation. Bars indicate standard errors.

61,200 gene transcripts. Seeds were inoculated with *U. hordei* and coleoptile samples were taken at 48 and 96 hai in three independent experiments. Changes in gene expression were calculated relative to noninoculated control samples. Differentially regulated genes were assigned different functional categories based on supplied annotation and additional manual annotation (Table 1). The greatest number of differentially up- and downregulated genes in all categories were observed in Neepawa, followed by host-susceptible Odessa and Hannchen. The more rapid, stronger response at the transcriptional level in nonhost Neepawa coincided with observed morphological defense responses. More than half of the 893 differently regulated genes in Neepawa, representing 1.4% of detectable transcripts, were expressed early, at 48 hai, and approximately 25 percent fell into the category of defense- and stress-related genes. Examples included many PR proteins, including chitinases and nonspecific lipid transfer proteins (ns-LTP), peroxidases, PAL, eight different WRKY genes, heat-shock proteins, a NAC transcription factor, ethylene (ET) and abscissic acid precursors, and several receptor-like protein kinases. Interestingly, more than 15 genes were associated with the jasmonic acid(JA)/ET signaling pathway (Table 1), suggesting that the JA/ET pathway predominates in nonhost interactions involving *U. hordei*; no genes related to the salicylic acid (SA) pathway were expressed in this interaction.

Comparing the inoculated Hannchen and Odessa coleoptiles over the two timepoints, 98.6% of the detected transcribed genes showed similar expression profiles during infection. *U. hordei* inoculation of Odessa and *Ruh1* Hannchen resulted in differential expression of only 486 genes (0.80% of detectable transcripts) and 194 genes (0.31% of detectable transcripts), respectively, during the course of infection. Common to both Hannchen and Odessa were 124 genes that showed similar expression patterns in both. This suggested that the *Ruh1* reaction in Hannchen involved the differential expression of relatively few genes. The majority of differentially expressed genes in Odessa and Hannchen were expressed later, at 96 hai (Table 1). These results demonstrate that the gene complement in Hannchen and Odessa responds very similarly to the early stages of infection by this *U. hordei* isolate. While both the host interactions evoked involved differential upregulation over time of defense- and stress-related genes related to the JA/ET pathway, Hannchen exhibited an earlier upregulation, at 48 hai, of three different transcripts. One encoded for a beta vacuolar processing enzyme, beta-VPE, known to be involved in seed-storage protein processing (Shimada et al. 2003) and implicated in SA-induced HR in tobacco infected with *Tobacco mosaic virus* (Hatsugai et al. 2004). This gene was upregulated 1.0- to 1.5-fold ($\log_2$ scale) in Hannchen and Neepawa, respectively, compared with the noninoculated controls but not in Odessa. Expression of this same gene was not different from the controls at 96 hai in all three cultivars (data not shown). A second gene encoding a putative hypersensitivity-related protein previously identified in *Arabidopsis* (transcript ID = Ta.29307.1, accession number CK163859) was upregulated onefold ($\log_2$ scale) at 96 hai only in Hannchen. Additionally, a gene encoding for lipoxygenase (LOX) involved in JA biosynthesis was up-regulated 1.2 fold ($\log_2$ scale) in all three cultivars at 96 hai.

Expression of defense-related genes during penetration.

Additional defense-related genes evaluated in the present study (Table 2) were selected because of the differential regulation revealed during a compatible and incompatible interaction of wheat involving the *R* gene *Bt10* and the common bunt pathogen *Tilletia tritici* (Gaudet et al. 2007; Lu et al. 2005a) and analysis of transcriptome profiling using the Affymetrix GeneChip as described above. The pronounced differences in time of appearance of visible host responses among the three interactions warranted an investigation into the expression of various known defense-related genes; a possible correlation of such genes with the described reactions might allow the development of diagnostic molecular assays.

Using qPCR, the change in transcript levels of defense-related genes encoding for callose synthase (CS), ns-LTP, a basic pathogen response protein 1.1 (PR-1.1b), PAL, EDS1, PAD4, beta-VPE, isochorismate synthase (ICS), and LOX was analyzed in a timecourse experiment. Clear differences in expression levels were evident when comparing the three interactions with the corresponding noninoculated control treatments. The transcript levels for *CS* in nonhost Neepawa were generally highest early in infection at 29 hai, decreased at 48 hai, then steadily increased again from 96 to 144 hai (Fig. 7). Conversely, the transcript levels for Hannchen increased steadily and maintained high expression after 48 hai. Expression levels for *CS* in compatible Odessa were only significantly different from noninoculated controls late in the timecourse (96 hai). For *ns-LTP* expression, no differences were observed in the inoculated controls in either of the incompatible interactions involving Hannchen or Neepawa during any of the timepoints. However, in the susceptible interaction on Odessa, continuing downregulation of the *ns-LTP* transcript was observed until

Table 1. Differentially up- and downregulated genes in Odessa, Hannchen, and Neepawa at 48 and 96 h after inoculation (hai)[a]

	Odessa				Hannchen				Neepawa			
	Up		Down		Up		Down		Up		Down	
Category	**48 h**	**96 h**	**48 h**	**96 h**	**48 h**	**96 h**	**48 h**	**96 h**	**48 h**	**96 h**	**48 h**	**96 h**
Uncategorized	3	40	0	13	8	8	1	1	56	25	5	7
Unknown	0	25	0	6	3	5	0	0	45	26	2	1
Defense and stress	14	91	2	15	12	42	7	3	194	175	23	6
Jasmonic acid/ethylene pathway	2[b]	5[bc]	0	0	0	7[bdef]	0	0	6[bcef]	8[bcef]	1[d]	0
Salicylic acid pathway	0	0	0	0	2[gh]	0	0	0	0	0	0	0
Other hormone-related	0	6	0	3	0	1	0	0	10	5	0	0
Other categories	11	184	1	65	4	65	23	1	148	120	17	13
Total	30	351	3	102	30	128	31	5	459	359	48	27

[a] Values represent numbers of genes with greater than twofold difference between inoculated and noninoculated treatment ($P < 0.05$).
[b] 12-Oxophytodienoate reductase ($n = 5$).
[c] 1-Aminocyclopropane-1-carboxylate oxidase ($n = 2$).
[d] Lipoxygenase ($n = 2$).
[e] Ethylene-responsive methionine synthase ($n = 1$).
[f] Ethylene-forming-enzyme-like dioxygenase-like protein ($n = 2$).
[g] Beta-VPE ($n = 1$).
[h] Hypersensitivity-related protein ($n = 1$).

144 hai, at which time a sixfold downregulation of transcription was observed (Fig. 7). This suggested that the host-compatible interaction may involve selective downregulation of specific constitutively expressed defense-related genes. *PR-1.1b* transcripts were up-regulated six- to eightfold early during Hannchen and Neepawa infection by *U. hordei* compared with the corresponding noninoculated controls at 29 and 48 hai, respectively, before a secondary increase at 96 hai. Expression of *PR-1.1b* in Odessa remained unchanged until 96 hai, when it was elevated to levels similar to those in the incompatible interaction. For *PAL*, a fourfold upregulation was observed at 48, 72, and 96 hai in Neepawa, Hannchen, and Odessa, respectively, indicating earlier expression of this gene in the incompatible interactions (Fig. 7).

In the nonhost Neepawa interaction, a twofold difference in transcript levels was evident for both *EDS1* and *PAD4* genes at 29 hai (Fig. 7). Expression of *EDS1* and *PAD4* differed from noninoculated controls in incompatible Hannchen, in which, after 48 to 72 hai, four- to fivefold and twofold ($\log_2$ scale) increases were observed, respectively, followed by a gradual decline. The expression levels of both genes in Odessa were not significantly different from those of the noninoculated controls at any of the timepoints. For *beta-VPE*, transcript levels were up-regulated very early in Hannchen (at 29 hai) and late in Odessa and Neepawa (at 144 hai). The hypersensitivity-related protein was not tested due to the difficulty in designing specific primers that amplified a single fragment. ICS followed a similar expression pattern to beta-VPE, being early upregulated in Hannchen and Neepawa at 29 hai (Fig. 7) and downregulated from 48 hai to 72 hai, compared with Odessa, in which expression levels increased later on at 96 and 144 hai. There were two expression peaks of *LOX*, one at 29 hai in Neepawa and Hannchen and one at 96 hai for all three cultivars (Fig. 7). Among the remaining candidate genes *chit3*, *gluc3*, and lipase (Table 2), no significant changes in expression among the three interactions were observed (data not shown). Globally, these results indicate that both SA- and JA-responsive genes are early activated in the incompatible interactions compared with that observed in Odessa, whereas the JA-responsive genes appeared to be nonspecifically induced late in all three interactions. Additionally, at the time that clear evidence of morphological defense-related responses are being observed in the incompatible interactions Neepawa and Hannchen, *EDS1* and *PAD4*, *PAL*, and *PR-1.1b*, and *CS* were all coincidently up-regulated, whereas *ns-LTP* was down-regulated in the compatible interaction in Odessa.

Expression of defense-related genes in response to SA, JA, and ET.

Genes representing both SA and JA pathways were represented on our microarray, and differential responses were observed during profiling. We determined the transcriptional

Table 2. DNA primers used to assay the gene expression by quantitative real-time polymerase chain reaction (PCR)

Gene	Clone	Forward primer (5′ > 3′) Reverse primer (5' > 3')	Tm (EC)[a]	Size (bp)[b]	Gene description
Ef1a	M90077[c]	GGTGATGCTGGCATAGTGAA GATGACACCAACAGCCACAG	55 56	126	Translation elongation factor 1 alpha-subunit (TEF1)
CS	CA501322	TGAAGACCTCGAATCTGCATT TCTGGTTTCCCTTCTCCTTTC	60 60	150	Callose synthase
LTP-1	DN551584	ACGTAGGTACTCCTCTCGCTGT GTTGATCGACCACTTCTTCTCA	59 55	148	Wheat lipid transfer protein
LTP-1 Hv	AK250392	GCTGATCGACCACTTGCTGT CCATGCAGAGCTGGCTCAAC	58 59	150	Barley lipid transfer protein
Lipase	DN551653	GATCATGGCGACAAGGCG TTTCCACTGGCCATCGGT	57 57	150	Wheat lipase
Lipase Hv	AK249383	CACAAGATGCTCACCCACCAC ACCTGGTGTTCGTCATTCAGC	59 58	150	Barley lipase
Chit3	L34211	TGCTCAACAACCCGGACCT ACCGTTGATGATGTTGGTGAT	59 55	150	Class 1b neutral chitinase
PR-1.1b	AJ007348	CGAGAAGAAGGACTACGACTA TCGTAGTTGCAGGTGATGAAG	53 55	149	Basic *PR-1.1b*
Glu3	AK250565	CTTCAGTACGTCGTCAATGCA TCATCTTTTGTGGGTTCTTGC	55 54	144	Basic β-1,4-glucanase
PAD4	CJ934977	GGAGGTTGTTGCGGTGATA GCCAGTTTTGGGATGGTG	56 56	149	PHYTOALEXIN DEFICIENT 4
EDS1	*CA650876*	CAGTCCGTTCTGGATTGGTT GCTAGTGCATCCCATGAAAGA	60 60	150	ENHANCED DISEASE SUSCEPTIBILITY 1
PAL	X99705	CAGTGTTGCCAAAGGAGGTC GTCTTCTCCCCGGTCAAGTA	60 60	151	Phenylalanine ammonia-lyase
Beta-VPE	EF682132	CTTGTCTTTGGGTTCGACAAG CATGCCGTACTGAGTGAGTGA	60 60	144	Beta vacuolar processing enzyme
Beta-VPE Hv	AM941111	GCATCCAATACCTACGCAAAA ATTCCTGGGCAGTATGATCCC	60 60	152	Beta vacuolar processing enzyme
ICS	CV769104	CGAGCAAGAGGGAAAACAAG GCTGGGATTGACAACAACCT	60	150	Isochorismate synthase
LOX	CD935100	CACTATATTAGCATGCCCCAAT AGGGTACGTCTCAGGGAAAGT	60 60	154	Wheat lipoxygenase
LOX Hv	AJ966349	ATATACTTTGGGCCGGGAAC CATGTCCCAACTTTTGAACAGA	60 60	158	Barley lipoxygenase

[a] Tm values represent melting temperatures for primers.
[b] Size of PCR product generated from the primer pair.
[c] Codes represent GenBank accession numbers.

response of common defense-related genes following exogenous application of these signaling compounds in intact young Neepawa wheat and Odessa barley. ET, SA, or methyl-jasmonate (meJA) were applied to seedlings at the three-leaf stage, and transcription profiling using qPCR was analyzed for eight defense-related genes. In general, the gene transcript levels in response to the hormone applications were similar in both Odessa and Neepawa, although small differences were noted (Fig. 8). *PR1.1b* was strongly up-regulated by all three hormones in both barley and wheat. Conversely, the remainder of defense-related genes were only modestly up-regulated compared with the untreated controls (Fig. 8). Thus, most defense-related genes in wheat and barley responded similarly to all three defense-signaling hormones.

DISCUSSION

A detailed characterization of morphological and molecular responses of host-parasite interactions on coleoptiles between the barley smut pathogen *U. hordei* and a susceptible host, a *Ruh1*-mediated resistant host, and a wheat nonhost is presented. We employed microarray and qPCR to profile, in parallel, transcript expression of common host resistance and defense factors during these nonhost, host *R* gene–resistant, and susceptible interactions. We demonstrated that there were no differences in prepenetration processes between these three interactions but obvious time-dependant morphological- and resistance-dependent gene expression differences between host and nonhost interactions occur in the coleoptiles during penetration. The expression of autofluorescence and the deposition of callose in close association with the invading fungal hyphae remain common morphological markers for all three interactions, although the timing and extent of expression of these markers varied greatly, depending on the nature of the interaction. Microarrays revealed a discrete upregulation of 1.4% of the detectable transcripts early at 48 hai in the nonhost interaction in Neepawa following the interaction with *U. hordei*. The response in Neepawa was more rapid compared with the later expression of 0.8 and 0.3% of detectable transcripts at 96 hai observed in Odessa and Hannchen, respectively, following inoculation with the same pathogen. Among the differentially regulated genes, the most interesting were the early upregulation of *EDS1*, *PAD4*, and some genes encoding PR proteins, including *PR-1.1b* and *PAL*, and their expression coincided with the cytological expression of incompatibility in both Hannchen and Neepawa. The downregulation of a *ns-LTP* and a large number of other gene transcripts in the microarray was observed in Odessa in comparison with the incompatible interactions. While no evidence of a HR was observed, we noted overexpression of the hypersensitivity-related beta-VPE gene and ICS, its associated SA synthesis regulator, early during the host-resistance reaction, suggesting that a SA-related micro-HR might be involved. Upregulation of LOX and other genes involved in JA synthesis early in the nonhost Neepawa interaction implicates the JA/ET defense pathway in PTI to nonadapted smut fungi in wheat. In both compatible Odessa and *Ruh1* Hannchen interactions, some JA-related genes were also up-regulated but later during infection.

Germination, sporidial development, dikaryotization, subsequent fungal development on the coleoptile surface, and appressorium formation were similar among host, *Ruh1*, and nonhost interactions, demonstrating that *U. hordei* does not have to overcome preformed host barriers prior to penetration on the wheat and barley coleoptile. In this respect, *U. hordei* appears unique, since preformed host barriers such as specific surface topography (Hoch et al. 1987), wax composition (Tsuba et al. 2002), and antimicrobial substances (Papadopoulou et al. 1999) are frequently cited as important components of nonhost

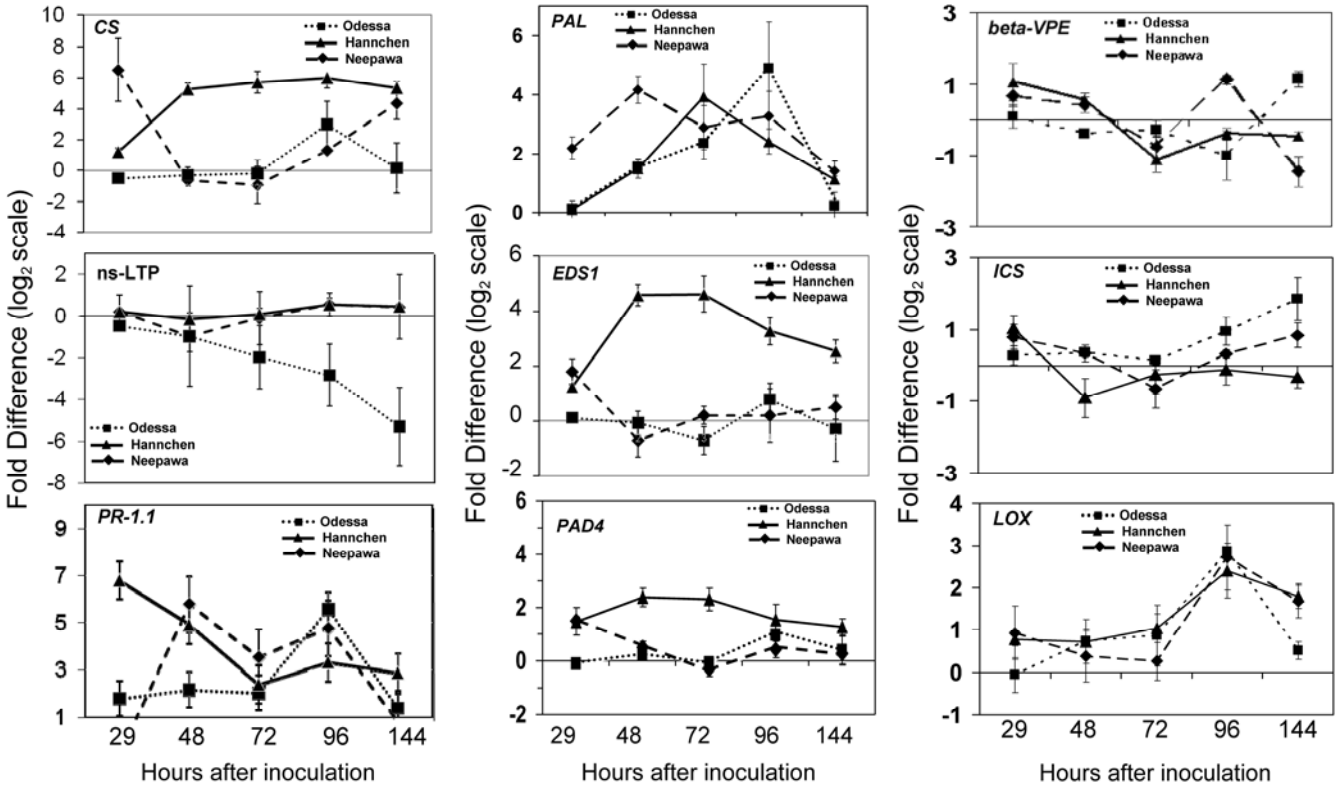

Fig. 7. Changes in transcript levels of wheat genes *CS*, *ns-LTP*, *PR-1.1b*, *PAL*, *EDS1*, *PAD4, beta-VPE, ICS*, and *LOX* in susceptible Odessa barley, resistant Hannchen barley possessing *Ruh1*, and nonhost Neepawa wheat after inoculation with *Ustilago hordei* isolate Uh4857 at 29, 48, 72, 96, and 144 h after inoculation. Values represent $\log_2$ fold differences between the inoculated and corresponding noninoculated treatments ($P \leq 0.05$). Bars indicate standard errors.

resistance (Lipka et al. 2008; Numberger and Lipka 2005; Thordal-Christensen 2003).

Penetration of the epidermis, inter- and intracellular growth, and spread to the internal tissues of the coleoptile by *U. hordei* in the susceptible barley host Odessa was rapid and was associated with a diffuse, spreading autofluorescence later in infection at 72 to 144 hai. Peak accumulation of *PAL*, *CS*, and *PR1.1b* transcripts at 96 hai preceded the maximum expression of fluorescence at 120 hai in Odessa. The PAL pathway is involved in the synthesis of lignin and other simple wall-bound phenolic compounds (Maher et al. 1994; Nicholson and Hammerschmidt 2003). The late accumulation of callose and phenolic compounds and PR-protein transcripts was evidence of a weak and delayed defense response in Odessa compared with those in the incompatible interactions in Hannchen and Neepawa. Details of infection in the compatible interaction involving Odessa were similar to those observed for compatible interactions involving *U. hordei* in barley (Hu et al. 2002, 2003; Kozar 1969) and *T. tritici* in wheat (Gaudet et al. 2007). This was corroborated by the microarray results that demonstrated the downregulation of a large number of genes, including genes involved in defense responses. Specifically, a *ns-LTP* was down-regulated in the compatible interaction in Odessa, whereas no change in expression was observed in the incompatible interactions in Hannchen and Neepawa. This particular ns-LTP was isolated from a differential subtraction library involving the *Bt10* gene for resistance to the bunt fungus *T. tritici* (Lu et al. 2005b) and exhibited in vitro toxicity to *T. tritici* following ectopic expression in the methylotrophic yeast *Pichia pastoris* (Sun et al. 2008). Therefore, repression of this *ns-LTP*

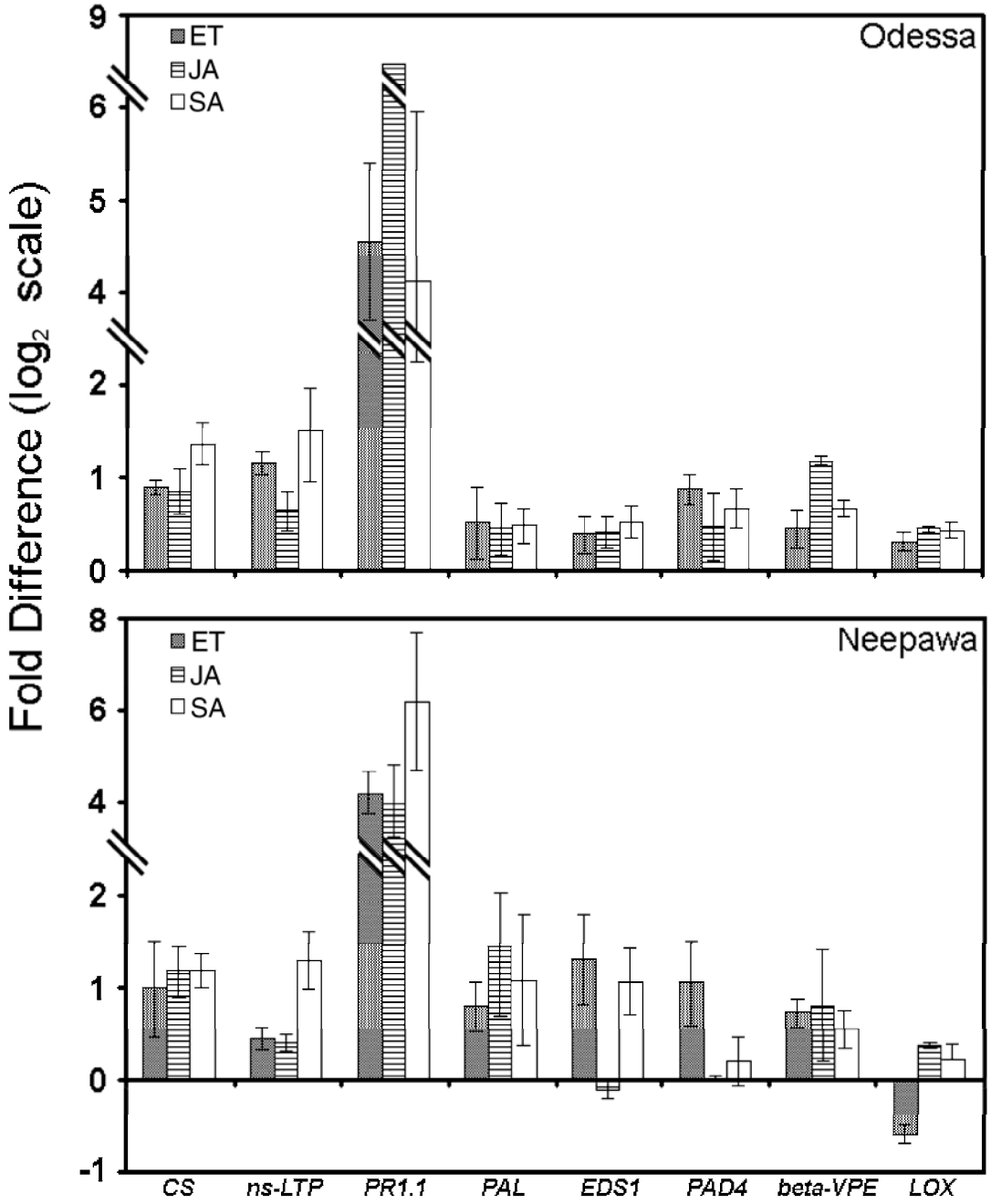

Fig. 8. Changes in transcript levels of genes *CS*, *ns-LTP, PR-1.1b*, *PAL*, *EDS1*, *PAD4, beta-VPE*, and *LOX* in susceptible Odessa barley and nonhost Neepawa wheat 48 h following application of ethylene (ET), jasmonic acid (JA), and salicylic acid (SA). Values represent $\log_2$ fold differences between the hormone treatments and controls ($P \leq 0.05$). Bars indicate standard errors.

and other defense-related genes observed during transcript profiling could be important in determining compatibility in the *U. hordei*–Odessa interaction. Downregulation of defense-related genes involved in basal resistance has been reported for bacteria and nematodes (Caillaud et al. 2008; Truman et al. 2006). In this respect, the active suppression or delay of such host-defense responses is hypothesized to be caused by secreted *U. hordei* effectors (Jones and Dangl 2006). Upregulation of a Bax-inhibitor and cystatin, genes involved in cell-death suppression, has been reported in the compatible *U. maydis*–maize interaction (Doehlemann et al. 2008). No differential regulation of genes with homology to a Bax inhibitor was observed in this study.

During the incompatible interaction involving *Ruh1* in Hannchen, dikaryotic infection hyphae penetrated the epidermis and appeared to develop internally with equal rapidity as in the compatible interaction in Odessa from 48 to 72 hai. Similar to Odessa, callose in Hannchen accumulated initially around the penetration sites of the ghost cells at the cell junctions. However, in Hannchen, callose accumulation continued to extend inwards along the ghost cells into the interior of the coleoptile, in which large deposits of callose originating from adjacent cell walls eventually completely engulfed hyphae, and this coincided with higher callose synthase gene expression during infection between 48 and 144 hai. These observations confirm those of previous studies of the same incompatible interaction by Hu and associates (2003) and in the incompatible interaction involving *T. tritici* and the *Bt10 R* gene in wheat (Gaudet et al. 2007).

No clearly visible HR was observed in this study involving *Ruh1* Hannchen interaction with *U. hordei* nor in any other interaction involving seed-borne smuts (Gaudet et al. 2007; Hu et al. 2003; Kozar 1969). However, a very localized HR-like reaction was observed by electron microscopy by Hu and associates (2003) in invaded cells during the same incompatible *Ruh1*-involved interaction. Indeed, microarray results revealed the differential upregulation of *beta-VPE* and a second hypersensitivity-related protein after 48 and 96 hai, respectively, in Hannchen. Beta-VPE is an indicator of implication of the SA pathway during plant defense (Catinot et al. 2008; Creelman and Mullet 1995; Hatsugai et al. 2004). SA, a known activator of systemic acquired resistance, is also associated with the HR (Durrant and Dong 2004; Ward et al. 1991). Beta-VPE was upregulated at an early stage of infection in Hannchen, and its expression pattern was very similar to ICS. This, coupled with the occurrence of callose and the early upregulation of callose synthase (Dong et al. 2008), *EDS1/PAD4* (Jirage et al. 1999), and *ICS* (Catinot et al. 2008; Creelman and Mullet 1995; Hatsugai et al. 2004), all implicated in SA-mediated HR reactions, suggest that some form of SA-mediated micro-HR is occurring in this *U. hordei*–Hannchen interaction. Coleoptiles of monocots possess a limited vascular system (Avery 1930), and as such, spread of systemic signaling compounds such as SA in host-parasite interactions in this organ may be limited, and this may reduce overall HR reaction in coleoptiles. Moreover, the coleoptile is a dispensable organ that is eventually sloughed off early during the seedling stage. It is possible that cereals have not evolved an extensive HR response in coleoptiles as a resistance mechanism to seed-borne smuts because of the transient nature of the coleoptile and because the resistance strategy to seed-borne smuts and bunts is to delay fungal growth, thereby preventing access to meristematic tissues of the crown before floral differentiation and stem elongation occur (Fischer 1953). This strategy would also be effective against most soil-borne fungi that would attack the developing coleoptile.

The early expression of morphological resistance in the nonhost interaction was accompanied by a pronounced transcriptional response including upregulation of defense-related genes, many involved in the JA/ET defense pathway. A similar early and pronounced gene response was observed with nonhost powdery mildew interactions in *Arabidopsis* compared with the host interaction (Laurent et al. 2004). Coleoptiles protect the emerging primary leaves from mechanical damage (O'Brien and Thimann 1965). The wide-scale upregulation of defense- and stress-related genes in response to a nonadapted pathogen suggests that the coleoptile is likely involved in preventing or delaying attack of the emerging stem and primary leaves by numerous soil-borne plant pathogens. The coordinated expression or repression of specific defense-related genes related to the JA defense signaling pathway, appears to be mediating the final outcome of nonhost interactions of wheat challenged with *U. hordei*.

EDS1 and *PAD4* were up-regulated in the nonhost Neepawa at 29 hai and in *Ruh1* Hannchen from 48 to 144 hai but not in Odessa, suggesting a role for these genes in incompatible interactions involving *U. hordei*. This is consistent with cytological observations that demonstrated that resistance was induced early in Neepawa, expressed only later in Hannchen, and was not observed in Odessa. These results are also consistent with the reported roles for *EDS1* and *PAD4* in plant defense in both nonhost and host-incompatible reactions in *Arabidopsis*. A complex that involves *EDS1*, *PAD4*, and a *senescence-associated gene (SAG) 101* has been shown to be involved in both postpenetration basal immunity (Lipka et al. 2005) as well as in TIR-NB-LRR–conditioned *R* gene–mediated resistance (Aarts et al. 1998). The present study suggests that *EDS1* and *PAD4* are also involved in basal immunity in the wheat–*U. hordei* nonhost interaction and the *Ruh1* gene resistance in Hannchen. This complex acts upstream of the oxidative burst and the HR and is required for SA accumulation and for defense potentiation involving the processing of reactive oxygen intermediate–derived signals around infection foci (Aarts et al. 1998; Feys et al. 2001; Rusterucci et al. 2001; Wiermer et al. 2005). To date, no *R* genes for resistance in cereals to the smuts and bunts have been isolated and characterized, and no TIR-resistance genes have been observed in cereals (Bai et al. 2002). Thus it is possible that other types of *R* genes may exist in cereals that interact with this gene complex.

In the present study, the accumulation of peroxides followed similar patterns in the compatible, *Ruh1*, and nonhost interactions. The oxidative burst is known to occur in two phases (Lamb and Dixon 1997); phase 1 is nonspecific and activated in both compatible and incompatible interactions, whereas phase 2 is specific, activated only in incompatible interactions by avirulent races and nonhost interactions (Keppler et al. 1989; Lamb and Dixon 1997; Nurnberger et al. 2004). The timing of the appearance of DAB-stained cells for the three interactions observed in the present study likely represented phase 1, as it is similarly activated in all interactions. It is possible that phase 2 may not exist or is diminished in the coleoptiles during host-parasite interactions involving smut fungi. Phase 2 is most frequently associated with a HR (Lamb and Dixon 1997; Levine et al. 1994; Tenhaken et al. 1995) and, as discussed, this may affect only a few cells in the barley–*U. hordei* interaction.

The majority of defense-related genes studied here were upregulated to approximately the same extent following the application of ET, JA, and SA in both 2-week-old wheat and barley plants. Except for *PR1.1b*, which exhibited high transcript levels, the transcript levels induced for the majority of defense genes were only modestly up-regulated. This would be expected for the upstream nature of genes such as *PAL*, *EDS1*, and *PAD4* with respect to SA and JA signaling. The apparent nonspecific nature of exogenously applied defense signaling compounds to induce *PR-1.1b* gene expression observed in wheat and barley in the present study has not been reported. Schweizer and

associates (1993) demonstrated the application of JA to barley induced the expression of a series of unidentified PR proteins. Molina and associates (1999) observed that *PR1.1* and *PR1.2* were not induced to any extent by treatments in which SA was exogenously applied to 2-week-old wheat plants (Görlach et al. 1996). However, these authors stated that PR1.1 and PR1.2 were clearly divergent from the basic and acidic monocot PR-1 proteins, which may explain the different results. Anaud and associates (2007) observed increases in *PR1.1b* transcripts, especially, only after 5 weeks of seedling growth. Clearly, the various isoforms of PR1.1 proteins within wheat vary in their response to exogenous applications of SA. Native and induced JA and SA levels in monocots such as wheat and barley are very low, near the level of detection for current methods (Vallélian-Bindschedler et al. 1998), compared with those in rice (Silverman et al. 1995), particularly in young wheat plants up to 4 weeks old (Anand et al. 2007). Therefore, the transcriptional response of young wheat plants to inducers of resistance appears to be different from responses of older plants and may be related to plant development. The low level of transcripts from JA- and SA-induced genes may reflect the low endogenous concentrations of these compounds in wheat and barley. However, high levels of *PR1.1b* during the timecourse of infection in the incompatible interaction of *U. hordei* in wheat and barley demonstrates that high levels of these transcripts can accumulate in young seedlings. In any case, the response in wheat and barley to exogenously applied JA, SA, and ET are clearly different from those observed in *Arabidiopsis* and other model dicot plants (Glazebrook 2005).

U. hordei was selected for this study because of its rapid and relatively uniform spore germination on both barley and wheat coleoptiles. To elucidate transcript responses of plants to biotic or abiotic stress, the use of isogenic lines is preferred. However, there are no barley lines available isogenic for *R* genes and no *U. hordei* strain isogenic for a single *Avr* gene has yet been constructed. Although the lack of isogenic lines limited our ability to make certain comparisons, the close similarity between Hannchen and Odessa over the entire transcriptome as detected in the Affymetrix microarray (>99.94%) suggested that the two cultivars were very closely related within the confines of environmental conditions and inoculations employed in this study.

To place the *U. hordei* interactions with hosts Odessa, Hannchen, and nonhost Neepawa within the context of current models of host-parasite interactions (Abramovitch et al. 2006; Bent and Mackey 2007; Chisholm et al. 2006; Jones and Dangl 2006), we propose that the first detection of *U. hordei*-derived PAMPs in all three interactions occurs rapidly, prior to 30 hai, upon the earliest infection events. This is followed by the first expressions of nonhost resistance, observed histologically at 30 hai in Neepawa, that include upregulation of defense- and stress-related genes, including *EDS1* and *PAD4*, *CS*, *PR1.1b*, and the PAL pathway. Infection is terminated shortly after PAMP detection in epidermal layers of the coleoptile with a PTI response consisting of a deposition of callose as a mechanical barrier to pathogen growth. *U. hordei*–produced effectors in Hannchen and Odessa likely suppress PTI, permitting development of the pathogen into the coleoptile (Doehlemann et al. 2009). At this point, the *UhAvr1-Ruh1* interaction and perception prior to 48 hai occurs in Hannchen with the upregulation of *beta-VPE* and initiation of a micro-HR; assuming the product of *UhAvr1* is a secreted effector, it would set off ETI at 72 hai and beyond. ETI also includes upregulation of *EDS1*, *PAD4*, *CS*, *PR1.1b*, and the PAL pathway, firstly via the SA pathway and, subsequently, by the JA pathway. Downregulation of defense genes, including *ns-LTP* in Odessa, was part of the susceptible interaction to reduce host-based defense responses that impeded pathogen growth that led to rapid pathogen development and its establishment in the embryonic meristematic tissues and subsequent colonization of the seed tissues.

In summary, we compared and contrasted the details of *U. hordei* teliospore germination and fungal penetration, oxidative burst, global gene expression, PR protein, and other defense-associated genes and signaling pathway genes in nonhost Neepawa wheat, barley host Hannchen expressing the *Ruh1* gene for resistance, and barley host Odessa, which is universally susceptible. We demonstrate that there is a close association between defense responses at the morphological level in wheat and barley coleoptiles, and the transcriptional responses in defense- and other resistance-related genes. Additionally, we demonstrate that similar resistance pathways are activated during the host *R* gene and nonhost reactions in barley and wheat but that the timecourse expression of individual genes is consistent with very early expression in nonhost Neepawa, followed by later expression in *Ruh1* resistant Hannchen, and latest expression in susceptible Odessa. Furthermore, callose played an important role in plant defense reactions in nonhost and *Ruh1*-mediated resistance. Both the timing of accumulation and total amount of callose deposited appeared critical for resistance. This study will serve as the foundation for future in-depth investigations aimed at characterizing pathways and key constituents imparting resistance to these pathogens. We are continuing to compare the current concepts of compatibility and incompatibly observed in *Arabidopsis* with those occurring in monocots infected by smuts and bunts, including basal or innate resistance and *R* gene resistance triggered by pathogen elicitors and effectors.

MATERIALS AND METHODS

Biological materials, growth conditions and treatments.

The barley (*Hordeum vulgare* L.) cultivars Hannchen and Odessa and Neepawa spring wheat (*Triticum aestivum* L. em. Thell) were employed. Hannchen (CI 531) possesses *Ruh1* (Grewal et al. 2008) and Odessa (CI 934) is susceptible to all known races of *U. hordei* (Tapke 1945). Teliospores from the *U. hordei* isolate Uh4857 possessing *Avr1/Avr1 Avr2/Avr2 Avr6/Avr6 MAT-1/MAT-2* (Linning et al. 2004) were employed as inoculum. This strain is virulent on Odessa, avirulent on Hannchen, and is a nonadapted pathogen to Neepawa wheat.

All seeds were surface-sterilized in 10% bleach plus Tween 20 (0.1% vol/vol; Sigma, St. Louis) for 3 min, were rinsed three times in sterile distilled water, were dried, and were stored at 4°C until used. Seeds were dehulled and placed embryo side up on two layers of Whatman #1 filter paper in 90-mm sterile petri dishes and were hydrated by saturating the filter paper with sterile, distilled water. Immediately following hydration, *U. hordei* teliospores, in a solution of 0.1% Tween 20 (10^8 teliospores/ml) were applied to the embryo end of the seeds, using an artist's paintbrush. Petri dishes were wrapped in aluminum foil and were placed in an incubator at 20°C (±1.5°C) until sampled; 20°C is the optimum temperature for *U. hordei* infection (Kozar 1969). Germination of surface-sterilized seeds in petri dishes (in vitro treatments) essentially represented a sterile environment. In separate studies, in order to determine if similar results were observed in a nonsterile environment, inoculated seedlings were germinated in sterile petri dishes for 48 h but were then planted into rootrainers (Spencer-Lemaire Industries, Edmonton, AB, Canada) containing a soilless potting mixture. Rootrainers were placed in a growth cabinet (Conviron, Winnipeg, Canada) set at 20°C (±1.5°C). Fluorescent and incandescent lights (approximately 240 μmole s^{-1} m^{-2}) in growth cabinets provided an 18-h day length. Noninoculated treatments were also included in the in vitro and rootrainer studies. For

sampling studies described below, the sampling times are expressed in hours after inoculation of seeds with *U. hordei*.

The seeds of barley cultivar Odessa and spring wheat cultivar Neepawa were planted into rootrainers and were placed in a greenhouse for a 16-h light and 8-h dark cycle at 20°C (±1.5°C). Seedlings at the three-leaf stage were subjected to hormone treatments. The seedling leaves were sprayed with 100 μM SA, 100 μM MeJA, and ET released from 100 μM ethephon, at 0 and 24 h. Seedlings treated with sterile water served as the controls. Leaves were sampled at 48 h, were flash-frozen in liquid nitrogen, and were stored at –80°C until used.

DAB staining.

Hydrogen peroxide was detected in coleoptiles using the 3,3′- diaminobenzidine (DAB, 1 mg ml^{-1}; Vector Laboratories, Inc., Burlingame, CA, U.S.A.) uptake method (Thordal-Christensen et al. 1997). Seedlings were sampled at 30, 48, 72, 96, and 144 hai in petri dishes. Coleoptiles sampled from seeds were washed in sterile distilled water, were vacuum-infiltrated in DAB for 3 min, and were allowed to stain for 6 h, according to the manufacture's instructions. Sections were washed three times in sterile distilled water and were mounted on microscope slides in 50% glycerol or lacto-phenol cotton blue. The numbers of DAB-stained sites on both halves of 1-cm coleoptile sections were counted from a minimum of five seedlings per replication. Three replications were employed, and the study was repeated two times.

Cytological studies.

For microscopic studies, plants were sampled at 8, 16, 24, 30, 48, 72, 96, 120, 144, and 168 hai in petri dishes or in rootrainers containing the soilless mix. Plants were gently washed, and crown tissues consisting of a 1- to 2-cm section of the coleoptile surrounding the crown region were excised and split longitudinally in half, and both halves were mounted in lactophenol-cotton (aniline) blue to stain for callose (Hauck et al. 2003; Hood and Shew 1996). Sections were viewed with a Zeiss Ultraphot research microscope using the 330- to 385-nm and 460- to 490-nm excitation and emission filters, respectively, and a HBO103W/2 light source. The numbers of fluorescent infection sites on both halves of the 1-cm coleoptile sections were counted from a minimum of five seedlings per replication. Three replications were employed, and the study was repeated two times. Infection sites were verified by bright-field microscopy to ensure that fungal hyphae associated with the fluorescence were those of *U. hordei* and not potentially containing fungi.

DNA microarray and data analysis.

The Affymetrix GeneChip Wheat Genome Array was used to profile gene-expression patterns in RNA collected from three biological replicates of each tissue type according to the manufacturer's instructions. FlexArray was used for analysis of raw probe-level hybridization data (CEL files). Raw data was normalized using the just RMA algorithm (Blazejczyk et al. 2007), and then, comparisons were made between controls and pathogen-inoculated plants on a per-genotype basis (barley cultivars Hannchen and Odessa and spring wheat cultivar Neepawa) at each timepoint (49 and 96 hai). This allowed the identification of genes specifically induced by *U. hordei.* Expression values were subsequently subjected to a random variance model *t*-test (Wright and Simon 2003) (P-value < 0.05), and changes greater than twofold to be considered differentially expressed.

Expression of candidate defense-related genes.

Transcript profiling of candidate genes (Table 2) in noninoculated and in *U. hordei*–inoculated wheat and barley coleoptiles at 29, 48, 72, 96, and 144 hai was determined by PCR analyses as well as in wheat and barley seedlings under hormone treatment at 48 h. Sampling earlier than 29 hai was not possible due to lack of growth of the coleoptiles, especially in Neepawa wheat. DNA sequences from candidate wheat genes were obtained from the GenBank database (National Center for Biotechnology Information, Bethesda, MD, U.S.A.). Primer pairs for PCR analyses were designed using the software Primer3 (Whitehead Institute and Howard Hughes Medical Institute, Chevy Chase, MD, U.S.A.). Guidelines for primer design were according to recommendations provided by Qiagen (Mississauga, Canada). Methods for RNA isolation and qPCR were according to Lu and associates (2005a). Fold difference levels are presented as $\log_2$ values and represent the difference between the inoculated and corresponding noninoculated treatments.

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