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Specific Isolation of RNA from the Grape Powdery Mildew Pathogen *Erysiphe necator*, an Epiphytic, Obligate Parasite

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Abstract
RNA expression profiling of obligately parasitic plant microbes is hampered by the requisite interaction of host and parasite. This can be especially problematic in the case of powdery mildews, such as *Erysiphe necator* (syn. *Uncinula necator*), which grow superficially but tightly adhere to the plant epidermis. We developed and refined a simple and efficient technique in which nail polish was used to remove conidia, appressoria, hyphae, conidiophores, and developing ascocarps of *E. necator* from grapevine (*Vitis vinifera*) leaves and showed that RNA isolated after removal was not contaminated with *V. vinifera* RNA. This approach can be applied to expression analyses throughout fungal development and could be extended to other epiphytic pathogens and saprophytes.

Introduction
Powdery mildews are caused by obligately parasitic fungi in the Erysiphales that superficially colonize plant hosts and are among the most economically important and widespread plant pathogens (Bélanger et al., 2002). In spite of their importance, molecular characterization of powdery mildews is poor, with the notable exception of *Blumeria graminis*, the sole species of a phylogenetically distinct tribe of powdery mildews whose members infect grasses (Glawe, 2008). Aside from *B. graminis*, only 23 expressed sequence tags (ESTs) have been deposited at GenBank for the Erysiphales (National Center for Biotechnology Information, 2009). A major contributing factor to this paucity of expression data for powdery mildews is the inability to isolate and maintain the fungus in axenic culture. By contrast, for another economically important ascomycete, *Botryotinia fuckeliana*, which can be cultured axenically, 10982 ESTs have been deposited at GenBank (National Center for Biotechnology Information, 2009).

In general, powdery mildew pathogenesis begins by deposition of ascospores or conidia onto susceptible host tissue, initiating an infection process (Pearson and Goheen, 1988). In Fig. 1a, this is depicted by fluorescence microscopy of *Erysiphe necator* (syn. *Uncinula necator*), causal agent of grape powdery mildew. Germination and appressorium formation are host-independent processes that can occur on inert surfaces (Ficke et al., 2003). However, infection requires successful penetration of the host cuticle and cell wall under the appressorium, and establishment of a haustorium (the 'feeding' structure) in a host epidermal cell (Pearson and Goheen, 1988). Following formation of the haustorium, hyphae will elongate, branch, and form additional appressoria, each potentially giving rise to haustoria (Fig. 1a). Thus, with the exception of the haustoria, the pathogen is wholly external to the host. Several days after infection, conidiophores and conidia are produced (Fig. 1b). When hyphae of opposite mating types adjoin, sexual reproduction occurs and chasmothecia (syn. cleistothecia), the overwintering structures that contain ascospores, are initiated (Fig. 1c). Sexual reproduction of *E. necator* typically occurs late in the growing season (Gadoury and Pearson, 1988).

To obtain powdery mildew DNA free of contaminating plant DNA, fungal tissue can be harvested from infected plant tissue by vacuum (conidia) or by scraping (conidia and some mycelia) prior to DNA isolation (McDermott et al., 1994; Jankovics et al., 2008). Such approaches have been used to develop rando, amplified polymorphic DNA and amplified fragment length
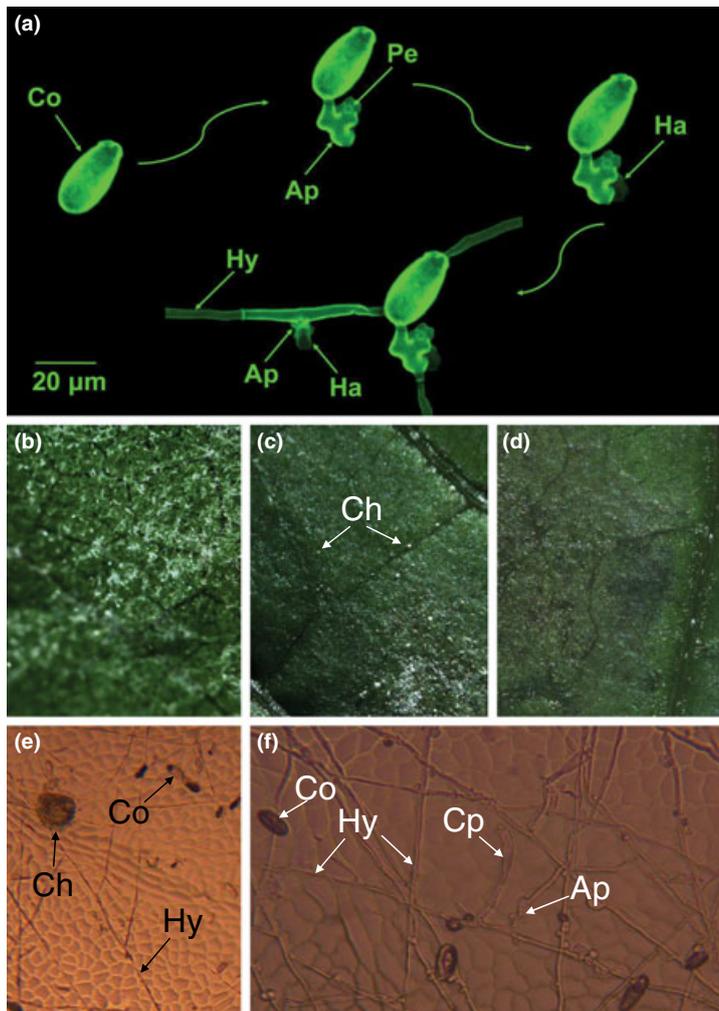

Fig. 1 *Erysiphe necator* developmental stages and removal of developmental stages from grape leaves using nail polish. After staining with wheat germ agglutinin-conjugated AlexaFluor 488 (Invitrogen, Carlsbad, CA, USA), confocal microscopy was used in (a) to visualize structural components of *E. necator* infecting a leaf of *Vitis vinifera* cv. 'Chardonnay.' Here, a z-series has been stacked to show structures on top of the leaf surface in addition to haustoria within non-fluorescing plant epidermal cells. Nail polish was applied to (b) a 10-day-old conidiating colony of *E. necator* and (c) a colony several weeks old. After removal of the nail polish (d) a grape leaf remains intact with undamaged trichomes along the leaf vein. Upon close inspection of the removed nail polish, all powdery mildew structures except for haustoria can be clearly observed at (e) 100× and (f) 200×, along with impressions of the plant epidermal cells. Ap, appressorium; Ch, chasmothecium (immature); Co, conidium; Cp, conidiophore; Ha, haustorium; Hy, hypha, Pe, penetration pore.

polymorphism molecular markers and to characterize two divergent genetic groups (Delye et al., 1995; Péros et al., 2005; Núñez et al., 2006). Similarly, RNA expression analyses have also been conducted on powdery mildew structures capable of being separated from the plant tissue, such as germinated conidia and appressoria on glass plates (Justesen et al., 1996). However, the inability to conduct open platform expression analyses of other structures and infection stages severely limits our understanding of powdery mildew biology at the molecular level.

To facilitate such RNA expression analyses, we developed a system for separating all powdery mildew tissues except haustoria from host tissue prior to RNA isolation. Clear nail polish had previously been used for plant surface impressions of stomata and for observing saprophytic fungi growing on leaf surfaces (Baudoin, 1986; Lee and Hyde, 2002; Oerke et al., 2006). Here, we used nail polish to remove fungal tissue from grape leaves prior to RNA isolation.

Materials and Methods

Separation of fungal tissue

Clear nail polish (Naturalistics™, Del Laboratories, Farmingdale, NY, USA) was painted onto powdery mildew-infected grape leaves and allowed to air dry for 5 min. The leaf was then torn to expose a clear tab of dried nail polish, and the tab was pulled using forceps and care was taken to avoid obtaining the grape tissue.

RNA isolation

Up to 10 cm^2 of the peeled, dried nail polish and associated fungal tissue were directly transferred to a 2 ml RNase-free eppendorf tube containing one 5-mm stainless steel bead and at least fifteen 0.4-mm silica beads (OPS Diagnostics, Bridgewater, NJ, USA). The tube was filled at least three times with liquid nitrogen to initiate disruption of fungal cell walls (DuTeau and Leslie, 1991). After the liquid nitrogen completely boiled off, the tube was sealed and shaken in a Geno/Grinder 2000 for 90 s at 500 strokes/min to obtain a powder. Total RNA was isolated using the Plant RNA kits NucleoSpin® (Clontech, Mountain View, CA, USA) or RNeasy™ (Qiagen, Valencia, CA, USA), as per the manufacturers' directions.

RNA quantification and quality checks

Total RNA was quantified by measuring absorbance at 260 nm and observed on a 2% agarose gel. To
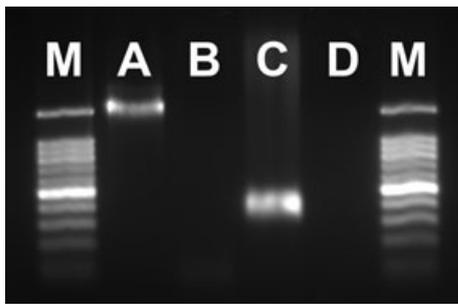
Fig. 2 Confirmed isolation of RNA specifically from *Erysiphe necator*. We conducted (a) reverse transcription PCR using grape RNA and primers specific to grape chalcone synthase (*VvCHS*, GenBank: X75969), (b) RT-PCR using *E. necator* RNA and grape chalcone synthase primers, (c) RT-PCR using *E. necator* RNA and primers specific to *E. necator* β-tubulin (*EnTub2*, GenBank:AY074934) and (d) PCR (without reverse transcription) using *E. necator* RNA and β-tubulin primers. (**M**) 100 bp markers (Promega, Madison, WI, USA). The lack of a product in lane B indicates that the isolated RNA is free from contamination by grape nucleic acids.

assess the purity of powdery mildew RNA, reverse transcription-PCR (RT-PCR) experiments were conducted using primers specific to grape chalcone synthase (*VvCHS*, GenBank: X75969) or to *E. necator* β-tubulin (*EnTub2*, GenBank:AY074934).

Results and Discussion
By light microscopy, all fungal tissues except for haustoria were observed to be imbedded in the clear nail polish and impressions but not tissue of leaf epidermal cells and trichomes were visible (Fig. 1d–f). While this process worked well for one brand of nail polish, most brands (e.g. L'Oréal™, Clichy, France) permeated the cuticle and remained tacky, not fully solidifying and preventing the clean removal of fungal tissue from host tissue. Similarly, nail polish failed to solidify on young, translucent leaves, also preventing removal of fungal tissue.

RNA isolations repeated with more than 100 heavily infected leaves suggested that this protocol, with either Plant RNA kit, NucleoSpin® or RNeasy™, typically yields 4–6 μg total RNA from an estimated 50–200 mg fungal biomass of conidiating *E. necator* infected leaves. Similar results were obtained with each kit.

Successful amplification of *E. necator* β-tubulin (Fig. 2, lane C) and lack of amplification of grape chalcone synthase (Fig. 2, lanes B) by RT-PCR suggested that the RNA was specifically from *E. necator* and lacked contaminating grape RNA. While the *E. necator* target successfully amplified, no grape RNA was detected in any of eight independent experiments using this method of RNA isolation. The RNA samples were treated with DNase prior to RT-PCR; thus, no contaminating fungal DNA was detected (Fig. 2, lane D).

The ability to isolate powdery mildew RNA in the absence of contaminating plant RNA or of any DNA enables open platform expression analyses. This approach is being applied for studying the regulation of conidiation and sexual reproduction in *E. necator* (Wakefield et al., 2007) and could have widespread application in expression analysis not only for powdery mildews but for all superficially growing pathogens and saprophytes.

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