A scourge of mankind: From ancient times into the genomics era*

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Rust diseases cause major losses in yields of crops of global importance. The first step in successful parasitism by a rust fungus is the 'blind' spore germ tube sensing the surface topography of the plant to feel its way to the stomatal opening for entry into the host. The parasite forms specialized infection structures for delivery of effector proteins into the host cells. Each of the several pathogen effector protein interacts with a specific host protein. The protein complexes function as signalling molecules. In the resistant host plant, the protein complexes activate a defence pathway resulting in a hypersensitive reaction causing death of infected cells and thereby limiting spread of the parasite. In the susceptible host plant, the effector proteins presumably act as transcription factors to subvert metabolic pathways and host physiology. The parasite becomes a powerful sink of host-made carbon compounds. The host plant is starved and injured as the parasite produces countless spores capable of aerial dissemination to new host plants. Due to accumulation of mutations in the genes determining pathogenicity, the parasite frequently evolves into newer races that overcome the effects of rust-resistant genes in crop plants introduced from their wild relatives. A 'race' continues between the plant breeders and the coevolving rust races, requiring newer strategies of controlling the rust disease. These could be anti-adhesion fungicides, preventing the adhesion of pathogen on host surface; or by the modification of the host topography by metabolic engineering, such that germ tubes are misdirected from reaching the stomata.

Keywords: Coevolution, effector proteins, host parasite, rust fungi.

PRIOR to the discovery of the role of fungi in causing plant diseases, the rust disease of wheat and other grain crops caused by *Puccinia graminis* (Figure 1) was believed to be the result of a curse for the wanton act of a boy who had caught a fox stealing his father's poultry and punished the animal by tying straw around its tail and igniting it[1]. The ancient Romans held a ceremony annually on 25 April in which a dog resembling the colour of the lesion was sacrificed to appease Robigus, the God who protected crops. Around 1870, the coffee crop in Ceylon (now Sri Lanka) was destroyed by a rust fungus *Hemiliea vastatrix*. The disease spread to Java, Sumatra, Arabia, Liberia and Africa, forcing the farmers to grow tea in lieu of coffee. As a result, coffee exports to England ceased, and the British were compelled to change their drinking habit to tea. During the British Raj, tea cultivation in India received a big boost, particularly in the northeast Himalayan mountain ranges.

Current threats

The rust fungi, with more than 7000 species described, are one of the largest groups of plant pathogenic fungi. A species, such as *P. graminis tritici*, may comprise several races that differ in pathogenicity on varieties of wheat (cultivars). Though the parasite does not have its alternate (sexual) host plant where the wheat crop is grown and therefore its rapid evolution by recombination does not occur, with cycles of rapid asexual multiplication by prolific production of urediospores, a few mutants that arise could multiply under favourable conditions. The East African highlands are a 'hotspot' for the evolution of new rust races since they have year-round favourable environmental conditions and existence of host allowing perpetuation of obligately parasitic rust fungi[2]. Currently, a new race of wheat stem rust *P. graminis tritici* discovered in Uganda, designated Ug99, is a threat since the wheat varieties in its likely migration path (Figure 2) are susceptible to this race. Aided by hurricane Ivan, the rust on soybean caused by *Phakopsora pachyrhizi* has reached North America from its centre of origin in China[3,4]. The rust fungi are fearsome pathogens of plants[5].

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Infection strategy

Rust fungi are biotrophic (obligate parasites). They rob and starve the host plant of nutrients rather than kill it. (In contrast, the necrotrophic fungi kill the cells and derive nutrition from the dead cells.) The rust parasite diverts the normal pattern of translocation of carbon compounds from leaves to the developing grains or seeds towards itself. As a consequence, the grains or seeds are either shrunken or not formed at all. The breeding of wheat varieties containing the stem rust-resistance *Sr2* gene complex from wild relatives by the American scientist, Norman Borlaugh, was one of the factors in the Green Revolution. In 1970, Borlaugh was awarded the Nobel Prize for Peace, 'for peace will not – and cannot – be built on empty stomach'.

Researches in the 21st century

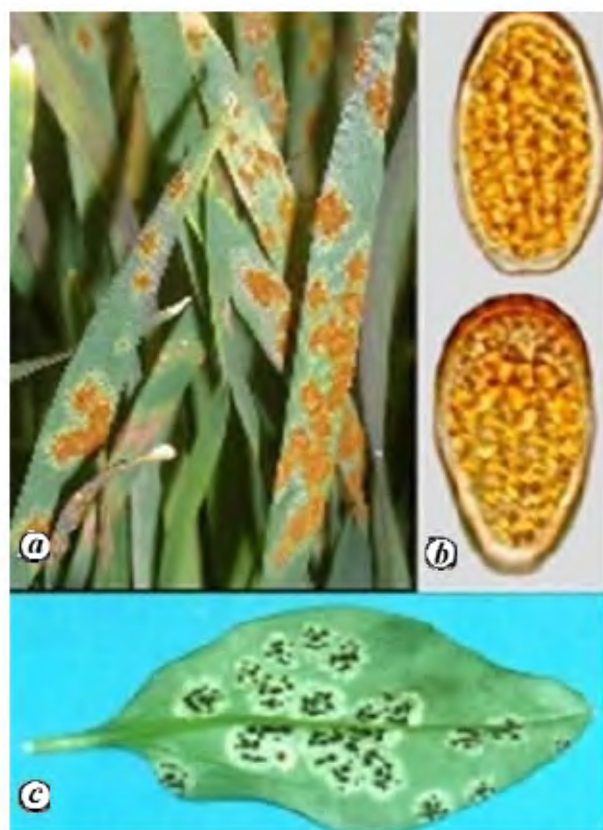The basic questions in rust pathogenesis are: How, in the first place, does the fungus locate stomata for entry into the host? How does it recognize a specific plant species or a cultivar as a compatible host? How does it counteract the host defensive molecules? How does the rust-infected 'island' become a sink of nutrients? What is the biochemical basis of compatibility of a virulent race of the fungus with its host? Are the resistant genes in different plants similar, and if so can these be transferred among plants?

Although a pathogen must possess several attributes to become successful, this discussion will be limited to those properties of rust fungi that strike me of special importance in their biotrophic lifestyle, and can form the basis for new strategies of controlling rust disease. These are events occurring at the external surface itself, and those occurring inside the host cell cytoplasm.

Role of host surface

Figure 3 shows the route of germinating urediospores on the host surface. Germ tubes bypassed nearby stomata and did not take the shortest path to gain entry through the stomata, suggesting that the route is prescribed by the topography of the host surface rather than by the generally entertained view of a chemoattractant being associated with the stomata. Recent scanning electron and atomic force microscopic examinations of plant surfaces have revealed that the cuticle has an intricate topography due to: (a) concavities and convexities of underlying epidermal cells (Figure 4), (b) foldings of cuticle layer as striations (Figure 5), and (c) crystalline deposits of secreted wax molecules[6–9].

Though an effective physical barrier to entry of microbes, paradoxically, certain observations over more than

Figure 1. *a*, Urediospore pustules of *Puccinia graminis tritici* on wheat leaf. Infection is mainly on stems which shrivel as they do not receive carbohydrates from photosynthesis and their translocation to the grains is severely affected. *b*, One of the spore types that causes epidemics. *c*, Urediospore pustules of *P. antirrhini* on a snapdragon leaf. (*a*) and (*b*) courtesy respectively of Jacolyn A. Morrison and Tamar Eilam, USDA, Agricultural Research Service, Cereal Disease Laboratory, St. Paul, MN, USA.

Figure 2. Projected migratory route of airborne urediospores of race Ug99 of wheat stem rust pathogen based on monthly wind-vector data. High- and low-wheat production areas are shown in red and green respectively. Route A via the Arabian peninsular, Middle-East and South Asia is considered to have a higher probability. Reproduced from Singh[2], with permission of CABI.

half a century have hinted to the cuticle (a thin heterogeneous lipid polyester layer comprising cutin and wax covering the aerial surface of all plants to prevent non-stomatal water loss by evaporation, and serving as a filter against harmful ultraviolet radiation) being, in fact, required for infection by the rust fungi. These observations were: (1) Germ tubes oriented and grew at right angles to the repetitive lines formed on (stretched) artificial membranes resembling the cuticle that were prepared by pouring a solution of nitrocellulose on a sloped glass[10], or on

Figure 3. Forced penetration of germinating urediospores of *P. antirrhini* by appressorium (light microscopy). (Inset) Magnified appressorium. Bypass of nearby stomata by germ tubes suggests that stimulus for entrance into host is thigmotropic.

naturally occurring repetitive lines on the cuticle[11]. (2) Sterile, excised stem pieces or leaves could be infected if the epidermis was intact, but not if it was stripped prior to inoculation[12]. (3) Host tissue (callus) cultures, which lack epidermis/cuticle, could not be infected by germinating urediospores or rust mycelium[13]. (4) Germ tubes formed appressorium (a penetration structure) on enzymatically isolated plant cuticle[14], or on artificial replicas of the plant (leaf) surface[15], or on artificial grooved substratum[16]. These observations are manifestations of thigmoresponse phenomenon in the rust fungus. Successful establishment of infection is determined at a very early stage when the fungus has just begun to grow while tightly adhering to the cuticle.

Figure 5 illustrates the external surface of a plant tissue that was recently featured on the cover of a plant journal, *The Plant Cell*. The most striking structures in this image are the longitudinally oriented ridges (appearing as striations) on pavement cells surrounding the stomata. Since the journal did not contain a comment on this structure, enquiries were made from several plant scientists, but without response. Why the fuss on the contour lines/striations? As referred to above[11,17], the rust fungi sense the closely spaced ridges and grooves, and orientate the germ tubes at right angles to the long axis of ridges and grooves, i.e. germ tubes grow transversely, and not along the ridges and furrows. Let us predict the behaviour of germ tube(s) in a gedanken experiment: Urediospores are germinated on a leaf surface, as in Figure 5. Considering the orientation of the ridges in relation to arrangement of the stomata, the germ tubes that grow transversely on
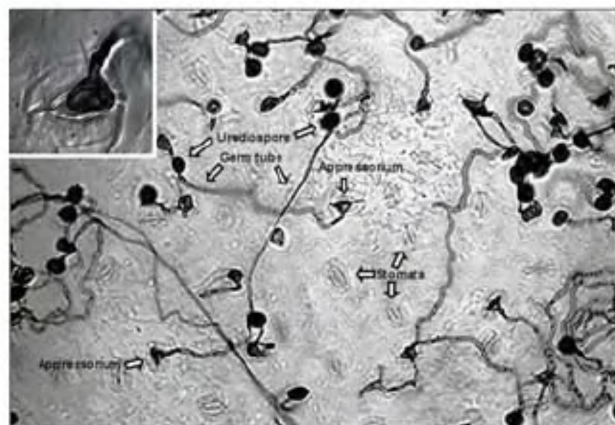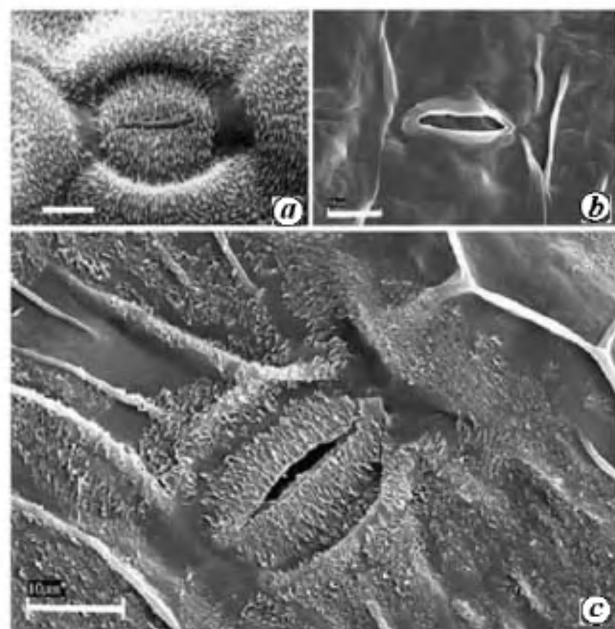

Figure 4. Scanning electron microscopic image of cuticle showing crystalline wax deposits around stomatal pore. With permission from Koch *et al.*[8], © Springer.

Figure 5. Scanning electron microscopic image of surface topography of *Arabidopsis thaliana* sepal (http://www.plantcell.org/content/vol17/issue10). © American Society of Plant Biologist.

striations will have the best chance of encountering the stomata for securing penetration. Indeed, urediospore germ tubes grow precisely in this manner (Figure 6), both on host and non-host plant surfaces. A 'touch and feel' mechanism guides the growth of urediospore germ tubes towards the stomata!
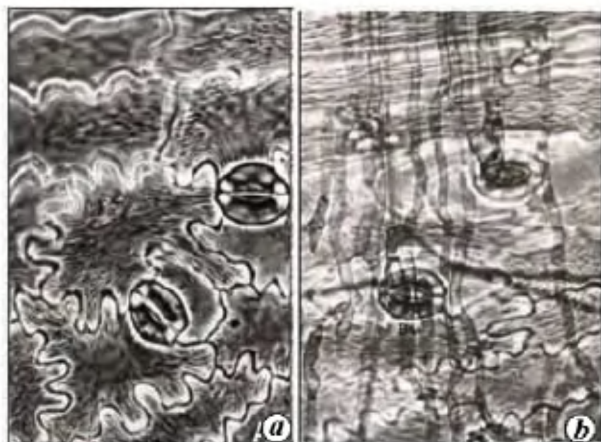

Figure 6. Directional growth of urediospore germ tubes on the surface of snapdragon leaf. *a*, Light microscopic image of cuticular ridges. *b*, Germ tubes oriented perpendicular to the ridges.
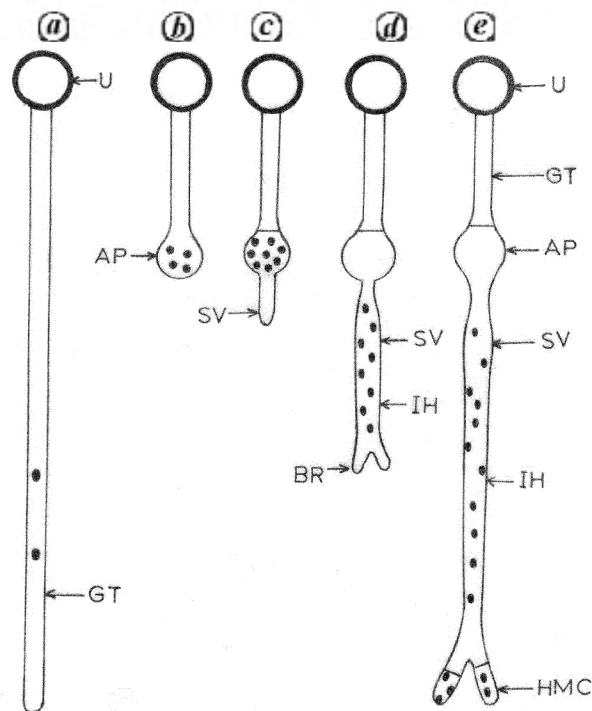

Figure 7. Thigmoinduction of nuclear and cell divisions in urediospores on nitrocellulose membrane[18] (diagrammatic). *a*, On non-hydrophobic membrane (control). The two urediospore nuclei migrate into the germ tube but do not divide. *b–e*, Nuclear and cell division during infection structure formation on hydrophobic membrane. Note germinating urediospore replicates nuclear division only in contact with hydrophobic membrane. AP, Appressorium; SV, Substomatal vesicle; IH, Infection hypha; BR, Branch and HMC, Haustorial mother cell.

Another thigmoresponse to the cuticle or to an artificially-prepared simulated surface is the development of multicelled structures, called infection structures – similar to those formed in nature (described below). The urediospore germ tube, otherwise capable of only a limited (<1 mm) polarized growth and without nuclear replication is transformed through infection structures into a mycelium that forms haustoria (the feeding structures) and proliferates in three dimensions. The formation of infection structures is a prelude to the development of parasitic mycelium, and this is accompanied by nuclear and cell divisions (Figure 7)[18].

The importance of topographical signals in thigmoresponsive phenomena has been confirmed using artificial surfaces. Hoch *et al.*[16] demonstrated that urediospore germ tubes of bean rust develop infection structures on microfabricated silicon wafers having multiple ridges. Closely spaced (1.5 μm) ridges which were 2.0 μm high, provide the most inductive topography for the wheat stem rust fungus[19]. The other fungus in which thigmotropism (Greek, *thigmo* = touch) operates during penetration into the host includes the powdery mildews[20]. However, the biophysical mechanism which orientates the germ tubes perpendicular to the parallel ridges remains unknown. Hitherto, mechanisms in pathogenicity were thought to reside only in the cytoplasm; however the touch-induced development of infection structures throws light on unknown mechanisms that operate at the external surface of a host plant itself.

Priming pathogen development

The entry of rust fungus through the stomata involves the formation at the germ tube tip of a bulbous cell called appressorium – a structure with adhesive properties to attach it to the surface. In other pathogenic fungi where this has been measured, it has a pressure equivalent to that in a car tyre. Since appressorium is the first in a sequence of cells formed by the parasite leading to the formation of feeding structure (haustorium), inhibiting its development is a target for chemical control. From its undersurface emerges a tubular extension, called the infection peg, that pushes through the stomatal opening into the host plant, subsequently swelling into a vesicle inside the substomatal cavity. From the vesicle develops an infection hypha that adheres to the host cell wall and develops a haustorium comprised of a stalk and a body inside a discrete environment created by navigation of host cell membrane[14]. Following the recognition of a susceptible host, the parasite redirects the host metabolism and proliferates intercellularly. Results from application of various polysaccharide constituents on urediospores germinated on collodion membranes suggested that contact of hypha with the plant cell wall could be important in development of haustoria[21].

A specific and crucial signal

To perceive the minute topographical features, the germ tube must closely adhere to the surface. The thigmotropic signals must then be relayed to an yet unidentified structure at the apex in order that the growing germ tube is accurately navigated towards the stomata. What determines the germ tube to form the appressorium over the stomata? To determine whether the contour lines around the stomata have a role in initiation and positioning of the appressorium over the stoma, Hoch and co-workers microfabricated ridges and grooves of defined height, width and spacing on inert templates by electron beam lithography[16] (Figure 8). In the bean rust fungus *Uromyces appendiculatus*, the 0.2 μm ridges guided the germ tube to grow perpendicularly, but did not induce appressorium formation, whereas the 0.5 μm ridges induced nuclear and cell division, resulting in differentiation of appressorium over the ridge. Scanning electron microscopy showed that this elevation of the ridge corresponds almost exactly to the elevation of guard cells in the bean leaf, demonstrating that sensing of topographical signal by the rust pathogen is highly precise. It is the right combination of height, width and spacing of the surface ridges and furrows on the surface of the host that determines the direction of germ tube growth and formation of appressorium over the stomata. The region of germ tubes responsive to topography was determined by perturbing the germlings using a glass

Figure 8. *a*, Signalling for growth orientation and cell differentiation by surface topography. Urediospore of bean rust germinating on artificial surface on silicon wafer microfabricated with 0.2 μm ridges. This elevation does not provide an inductive stimulus for nuclear division in germ tube (Gt), fluorescing objects in the right panel. *b*, Silicon wafer microfabricated with 0.5 μm ridges. The tip of the germ tube at 0 time contains two-nuclei. A germ tube that has crossed 15 ridges (60 min) has differentiated an appressorium (Ap) containing four-nuclei (right panel). Images were viewed under scanning electron microscope or fluorescence microscope. Courtesy Harvey C. Hoch and Richard C. Staples.

micropipette[23]. Appressorium developed when the micropipette was left inserted parallel to the germ tube tip at the uppermost 10 μm for >20 min. The intimate relationships between the host and the pathogen described above suggest coevolution of plant host–rust parasite system.

Events inside the host-plant cell

Having gained entry inside the host, what determines that the intruder becomes a pathogen? The complex interrelationship between the pathogen and the host is being researched on the basis of a model that was formulated more than six decades ago by Flor, to determine the genetic basis of race specificity[24]. Flor selected a group of flax varieties (genotypes within a single species of *Linum*) by means of which he was able to identify physiological races of flax rust fungus *Melampsora lini* based on visible differences in the degree of resistance or susceptibility. By studying inheritance of pathogenicity using differential varieties of flax that differed in genes governing rust reaction and parallel crosses between rust races that differed in genes governing rust reaction (genes for virulence and avirulence), Flor found that the resistance genes possessed by the flax varieties and the genes for virulence possessed by their rust races were numerically equal. This is interpreted as for each of the several genes conditioning resistance reaction in the host, there are specific related genes conditioning virulence or pathogenicity in the rust.

Flor's theory known as 'gene for gene' has greatly impacted research on pathogenesis. Using present-day knowledge of genetics and biochemistry, the theory is that the pathogen secretes/translocates Avr protein molecules (effectors) at the haustorium–host interface[25]. These effector protein molecules physically associate with the *R*-gene products (proteins) in the host. Disease is triggered if a resistance (*R*) gene product in the host-plant cell recognizes a specific virulence (*Avr*) gene product secreted by the parasite. The Avr–R protein complex is hypothesized to activate an innate defence pathway in the host that results in rapid death of the infected cells (termed the hypersensitive response), thereby blocking proliferation of the pathogen[26,27]. Susceptibility to a particular race occurs when either one of the genes is inactivated by mutation as a result of which a physical interaction between specific R–Avr proteins does not occur. The pathogen subverts host metabolism, redirecting the nutrient flow to the infection site in order that the pathogen can obtain a 'dowry' of nutrients that can be provided to spores for their initial development until the parasite can establish a haustorial connection with a specific host. The R–Avr protein interaction provides an explanation for specificity of host–rust parasite relationship at the intracellular level. The protein complex may interact with proteins in the signal transduction pathway leading to change
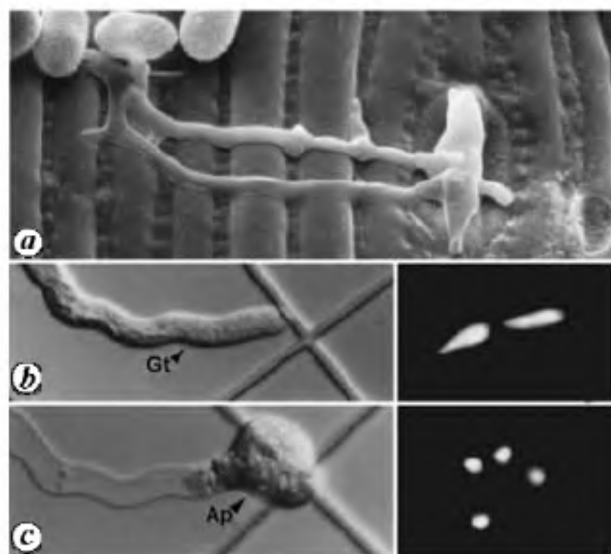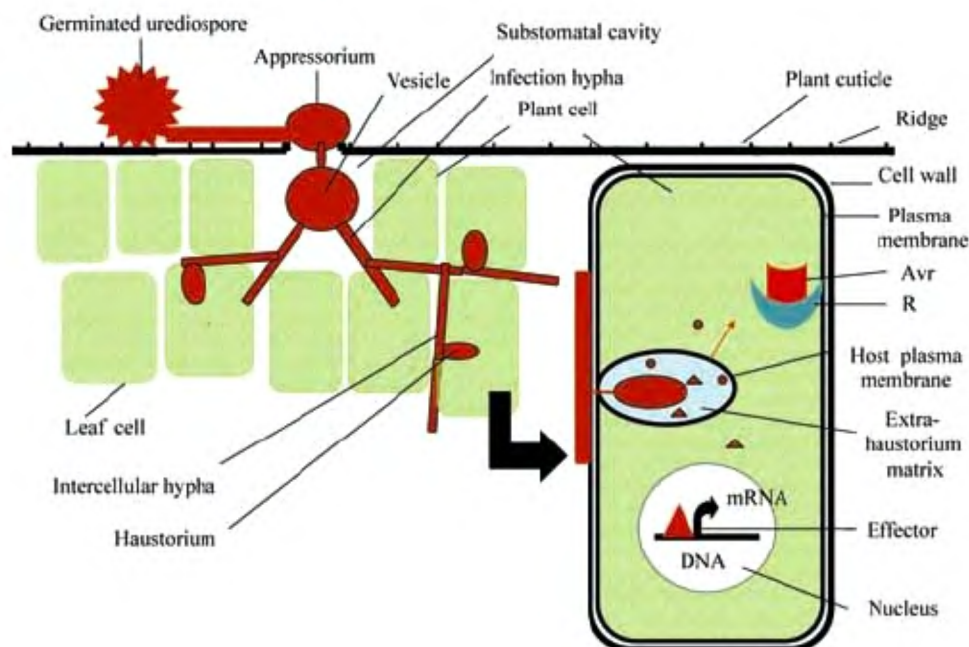
Figure 9. Host–rust parasite interaction. Schematic diagram showing interaction between rust fungus and susceptible host is initiated on external surface. Pathogen-secreted molecules inside the host cell (●, ▲) suppress host defence and enhance susceptibility. Enlarged infected cell is on the right.

in gene expression, enzymatic activity, and metabolic modifications in the infected host plant cell, rendering the cell hospitable for conversion of the photosynthetic host-made nutrients into fungus-specific compounds (e.g. chitin, trehalose) for it to produce disseminative spores. Alternatively, the R–Avr protein complex signals activation of programmed cell death in infected cells in resistant host to arrest pathogen development. There may be other defence responses too[28]. The protein complex may act as a transcription factor (Figure 9). Hitherto haustoria were thought to be structures involved only in nutrient uptake. An additional function of haustoria has emerged, i.e. it delivers several effector proteins in the infected cell that in susceptible cultivars suppress signalling pathways leading to defence reaction and to remodel the host physiology[27,29]. In summarizing this section, recognition of a pathogen inside the host cytoplasm is mediated by proteins. Race-specific effectors (Avr) induce resistance responses only in a host plant variety carrying the corresponding resistance (R) gene. Disease results when either or both of these gene products are absent.

Research in the genomics era

A flurry of research activity has begun on the identification of functionally important genes in rust fungus–host plant relationship[30]. A method was developed to isolate haustoria from homogenates of rust-infected tissue by lectin-affinity chromatography and a heterologous probe

was used to demonstrate that nutrient (hexose and amino acids) transporters are localized in haustorial plasma membrane, providing a basis for considering that haustoria have a specialized function in proton-coupled uptake of nutrients from the infected host cells[31–33]. Using a haustorium-specific cDNA library, the generation of EST (expressed sequence tag) sequences and analysis for their coding region with homologies to known genes in other fungi, about 30 *in planta* induced genes (*PIG*s) were identified[32]. Several genes involved in ribosome biogenesis and translation, glycolysis, amino acid metabolism, stress response, and detoxification showed an increased expression in the parasitic mycelium. These data indicate a strong shift in gene expression in rust fungi between germination and the biotrophic stage of development. A consequence of the gene-for-gene theory is the molecular dialogue between the parasite and host through the haustorium. Transfer of protein from the rust into the host has been detected using heterologous antibody probes and immunofluorescence microscopy[34,35]. Identification of the elicitors will enable to combine the genes in rational ways in new varieties for providing maximum resistance to rust races.

New strategies of disease control

Research on mode of entry of pathogen has prompted thoughts on newer methods of controlling disease. For example, the biotrophic powdery mildew fungus, *Phyl-*

lactinia corylea, which parasitizes mulberry plants, forms special lateral hyphal branches (stomatopodia), functionally analogous to appressorium, that firmly attach to the host surface to perceive fine topographical signals prior to entry into susceptible host[36]. In resistant varieties the stomatopodia developed away from the stomata[37], precluding the entry of the pathogen. Thus, a potentially promising method of curtailing rust infection is spraying of anti-adhesion chemicals. Alternatively, the topography of the cuticle may be distorted by alterations in its wax constituents using molecular genetic approaches[38–40]. However, because of mutation, and evolving newer and virulent races of rust, no plant variety will remain permanently resistant. A genetic change in one species of host plant causes a genetic change in the coevolving parasite, i.e. there is an ongoing 'arms race' between the plant breeder and the pathogen. Currently, the focus of research is to understand recognition events that activate defence response in the resistant host. The gained knowledge may be applied to induce resistance response in crop plants to reduce crop losses due to rust infection, for example, by engineering resistance to disease in crop plants by transfer of disease resistance genes from one plant species to another. It has been proposed to develop more durable cultivars by multiple transformations with *R* genes. However, cells have evolved mechanisms to silence repeat copies of genes introduced by transformation.

Summary

Specific interaction between the host and the rust pathogen is initiated soon after the urediospores have landed on the plant surface. Control measures may be based on anti-adhesion fungicide, and/or repatterning host topography through genetic engineering of wax molecules deposited on the cuticle such that the germ tube is misdirected from reaching the stomata. With the sequencing of wheat stem rust genome being completed (www.broad.mit.edu/annotation/genome/pucciniagraminis), the databases are being searched to identify the *Avr* genes and effector molecules secreted into the host cytoplasm. The understanding of the nature and modus operandi of effector proteins could provide an understanding of the molecular mechanisms by which the pathogen subverts the host cell physiology for deriving materials to make reproductive spores for its own survival.

Concluding remarks

The rust fungi remain challenging organisms to study. The genome of the wheat stem rust fungus has been sequenced. It is hoped that research programmes combining plant pathology, cell biology (transmission electron microscopy of haustoria–host interface) and genomics will help understand the nature of the host–pathogen interaction and how a rust fungus has continued to live as a successful parasite since ancient times, without decimating its host.

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