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Crocus sativus Pathogens and Defence Responses

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ABSTRACT

Saffron plants in their natural environment are constantly under siege by a multitude of disease-causing organisms including bacteria, fungi, viruses and nematodes. These phytopathogens invade into the plant apoplast and proliferate by assimilating nutrients from plant cells, hence provoking important economic damage to saffron around the world. Most pathogenic species affect the corm, causing pre- and post-development of this organ, which in turn affects saffron viability, propagation and yield. However, only a relatively small proportion of these pathogens is capable of invading the host plant successfully and causing disease. Plants depend on sophisticated defensive strategies to resist this invasion, using both preformed and inducible defence responses. This ability to resist disease also depends on soil conditions such as structure, compaction, drainage, temperature and level of biological activity, along with farming practices that influence plant development, such as planting date and application of fertilisers or herbicides. Our ability to exert sustainable control over saffron diseases relies on a two-fold understanding of saffron development and defence mechanisms.

Keywords: bacteria, *Crocus*, fungi, genes, nematodes, transposable elements, virus

Abbreviations: **BYMV**, *Bean yellow mosaic virus*; **CMV**, *Cucumber mosaic virus*; **CW**, cell wall; **EST**, expressed sequence tag; **HR**, hypersensitive response; **ISMV**, *Iris severe mosaic virus*; **NMV** *Narcissus mosaic virus*; **PAMP**, pathogen-associated molecular pattern; **PCR**, polymerase chain reaction; **PR**, pathogenesis related; **RT-PCR**, reverse transcription-polymerase chain reaction **TF**, transcriptional factor; **TNV**, *Tobacco necrosis virus*; **TRV**, *Tobacco rattle virus*; **TuMV**, *Turnip mosaic virus*

CONTENTS

DISEASE IN PLANTS

The meristematic cells of a healthy plant divide and differentiate as needed, while different types of specialized cells absorb water and nutrients from the soil; translocate these to all plant parts; carry on photosynthesis, move, metabolize or store photosynthetic products; and produce new reproductive structures for survival and multiplication. When a pathogenic organism interferes with the ability of cells or a plant part to carry out one or more of these essential functions, the activities of the cells are disrupted, altered, or inhibited and the plant becomes diseased. This disease, which is the outcome of a successful infection, rarely kills a plant, if the plant is not infected by a necrotroph pathogen. At first, the infection is localized in one or a few cells and is invisible. Soon, however, the reaction becomes more widespread and affects plant parts, developing changes that are visible to the naked eye. These visible changes are the symptoms of the disease. The visible or otherwise measurable adverse changes in a plant produced by the pathogen infection are a measure of the degree of disease in the plant (Gómez-Gómez 2004). Thus, the disease in plants can be defined as the series of invisible and visible responses of plant cells or tissues to the pathogen that result in adverse changes in the form, function, or integrity of the plant and may lead to the death of plant parts or the entire plant. The kinds of cells and tissues that become affected determine the type of physiological function that will be disrupted (Agrios 2005). For example, infection of roots may cause roots to rot and make them unable to absorb water and nutrients from the soil. Infection of xylem vessels, as happens in vascular wilts interferes with the translocation of water and minerals to the crown of the plant. Infection of leaves, as happens in leaf spots, blights, rusts, mildews, mosaics and so on, interferes with photosynthesis, while infection of phloem cells in the veins of leaves and in the bark of stems and shoots, as happens in crankers and in diseases caused by virus, interferes with the downward translocations of photosynthetic products. The infection of flowers interferes with the proper development of reproductive organs. In addition, each kind of pathogen has evolved a particular way to invade plants. Some species directly penetrate surface layers by using mechanical pressure or enzymatic attack. Others pass through natural openings, whereas a third group invades only wounded tissues.

Crocus sativus is cultivated for its red style branches,

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Invited Review

Fig. 1 Representative samples of *C. sativus* corms severely affected by fungi.

which once dry constitute the saffron spice. *C. sativus* is a triploid sterile plant, propagated by corms. As a subterranean organ, the corm is susceptible to diseases caused by fungi, bacteria, nematodes and viruses (**Fig. 1**). Infected plants die off early, resulting in reduction of corm yield, quality and flower and stigma production. In the following sections we will cover the different pathogens that have been isolated and identified in *C. sativus* and also the strategies which the plant has developed to deal with them.

FUNGI AND OOMYCETES AS SAFFRON PATHOGENS

Fungi and oomycetes cover the majority of eukaryotic plant pathogens, but they represent less than 2% of the approximately 100,000 known fungi and oomycetes species. Both microorganisms show filamentous growth in their vegetative stage, produce mycelia and form spores by asexual and sexual reproduction. Almost all pathogenic fungi spent part of their lives on their host plants and part in the soil or in plant debris on the soil. Thus, these pathogens show different lifestyles and are classified as biotrophs, necrotrophs and hemitrophs. Biotrophs grow and reproduce in living plant tissue and obtain nutrients through intimate interactions with living plant cells. They are regarded as having an intricate biological interaction with their host plants, presumably as a result of co-evolution, since they exhibit a high degree of specialization for individual plant species. Necrotrophs, which feed on dead plant cells, kill host cells by means of toxic molecules and lytic enzymes, subsequently decomposing the plant tissue and consuming it for their own growth (van Kan 2006). The hemitrophs that initially establish a biotrophic relationship with their host, whose cells die as the infection proceeds (Latijnhouwers *et al*. 2003). This switch is usually triggered by increasing nutritional demands as the fungal biomass increases. Some of the world's most devastating phytopathogenic species fall into this category. Finally some fungi are facultative parasites because they can live, grow and multiply perfectly in the soil or elsewhere as saprophytes, although when the conditions are favourable, they have the ability to parasitize and cause disease in the plant. Most of the fungi that have been isolated from *C. sativus* plants belong to this group (**Table 1**).

Uromyces croci is a basidomycetes fungus, belonging to Uredinales order, mainly recorded as attacking only the leaves of *Crocus* species in the Mediterranean countries, developing the so-called crocus rust disease. However, leaf infections are not known to occur in northern Europe, where instead the corms are affected by Teleutospores present in soil. The association of the mycelium with the vascular tissue of the host can lead to the production of an infection in new corms (Boerema and Kesteren 1965).

The genus *Aspergillus* is a diverse and familiar group of ascomycetes which is mostly saprophytic, but includes human pathogens, plant pathogens, and species useful in industrial processes and genetic research. *Aspergillus niger* (**Fig. 2A**) is a fungi found worldwide which is responsible for black mould (**Fig. 2B**). It is transmitted by contaminated

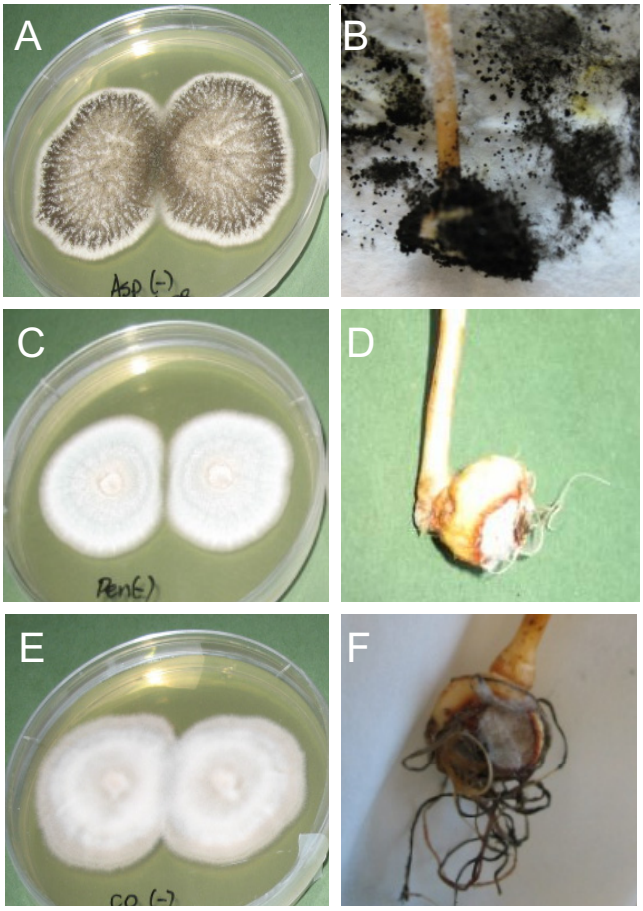

Fig. 2 Fungi as saffron pathogens. (**A**) *Aspergillus niger* isolated from infected saffron corms. (**B**) Saffron corms affected by *A. niger*. (**C**) *Penicillium sp* isolated form saffron corms. (**D**) Saffron corms affected by *Penicillium*. (**E**) *Cochliolobus sp* isolated from infected saffron corms. (**F**) Saffron corms affected by *Cochliolobus.*

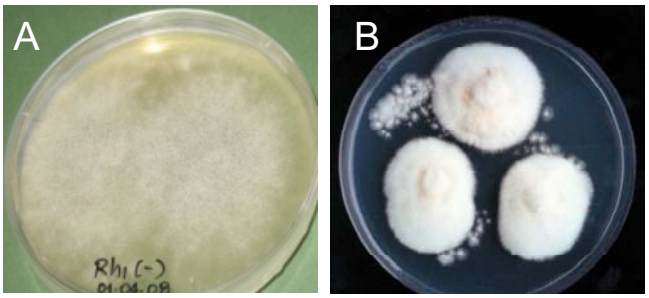

Fig. 3 Other fungi isolated from saffron corms in Spain. (**A**) *Rhizopus sp* isolated from infected saffron corms. (**B**) *Beauveria sp* isolated from infected saffron corms.

corms or soil, with the infection usually starting at the root initiation stage and continuing through corm storage.

Several *Penicillium* species have been isolated from infected saffron corms (**Fig. 2C, 2D**). *Penicillium* rot, more commonly referred to as blue mould rot, is one of the primary agents responsible for crop losses of flower bulbs and vegetables during storage. *Penicillium corymbiferum* Westl., *P. crocicota* Yamamoto and *P. chrysogenum* have been reported as pathogens in crocus (Moore 1939; Yamamoto *et al*. 1956; Saaltink 1971). Saffron plants infected with *P. corymbiferum* manifest damping-off, basal stem rot and drooping or wilting of shoots; the corms often have dark lesions beneath the outer tunic and, sometimes, a blue-green mould on their surface (Capelli *et al.* 1991). It has been demonstrated that a very heavy infection of *P. corymbiferum* on crocus corms at planting can reduce harvested corm yield by more than 20% (Sutton and Walle 1985). The

Table 1 Fungi and oomycetes isolated from *Crocus* in different geographic locations.

Fungi	Life style	Location	Reference
Aspergillus niger	facultative parasite	Spain	This work
Beauveria	facultative parasite	Spain	This work
Botrytis	necrotrophic	The Netherlands	Boerema and Hamers 1989
Burkholderia gladioli	hemibiotrophic	Argentina	Wit *et al*. 2002
Cladosporium	facultaive parasite	Spain	This work
Colchiobolus	hemibiotrophic	Spain	This work
Fusarium oxysporum f.sp gladioli	hemibiotrophic	The Netherlands	McClelland 1945
		Japan	Yamamoto 1954
		Germany	Mes *et al*. 1994
		Italy	Cappelli 1994
Fusarium oxysporum f.sp croci	hemibiotrophic	The Netherlands	Boerema and Hamers 1988, 1989
Fusarium oxysporum f.sp tuberosi	hemibiotrophic	Spain	Castillo and Gómez-Gómez 2009
Fusarium moniliforme	hemibiotrophic	India	Personal communication
		Spain	This work
Penicillium corymbiferum	facultative parasite	UK	Yamamoto *et al*. 1956
		Italy	Capelli *et al*. 1991
Penicillium crocicola	facultative parasite	Japan	Yamamoto *et al*. 1956
Penicillium cyclopium	facultative parasite	Italy	Cappelli and Di Minco 1999
Penicillium spp.	facultative parasite	Spain	This work
Phytium irregulare	necrotrophic	The Netherlands	Van Os *et al*. 1998
Phytium spp.	necrotrophic/ saprophytic/ facultative parasite	The Netherlands	Schenk 1969
Rhizoctonia violacea	necrotrophic and hemibiotrophic	Spain	Pérez-Bueno 1995; De Andrés 1998
Phoma spp.	necrotrophic	India	Madan *et al*. 1966
		The Netherlands	Boerema 1976
		Spain	Pérez-Bueno 1995
Rhizoctonia crocorum	necrotrophic and hemibiotrophic	Greece	Goliaris 1999
Rhizopus nigricans	facultative parasite	Spain	This work
Sclerotium rolfsii	necrotrophic and hemi-biotrophic	India	Kalha *et al*. 2007
Stromatinia gladioli	necrotrophic and hemibiotrophic	The Netherlands	Schenck 1970
Uromyces croci	biotrophic	The Netherlands	Boerema and Van Kesteren 1956

corms grown from infected parent material tend to be smaller than those produced from a clean stock, which would account for the reduced yields. Infection by this fungus has been reported in Italy (Cappelli *et al*. 1991), in Japan (Yamamoto *et al*. 1956; Gould and Miller 1971; Saaltink 1971), Scotland (Sutton and Wale 1985) and Spain (this work) (**Table 1**).

Rhizopus spp are zygomycetes, a primitive fungi, which are either saprophytes or weak parasites of plants and plants products where they cause soft rots or moulds. These fungi enter plants through wounds present on the surface. *Rhizopus nigricans* (**Fig. 3A**) has been isolated from wounds present in *C. sativus* corms (the work in this manuscript).

Beauveria is a well known facultative pathogen and an entomopathogenic fungus. Although the species isolated from *C. sativus* has not been determined (**Fig. 3B**), among the *Beauveria* species identified, *B. bassiana* has been environmentally approved for commercial use against a variety of agricultural pests, including whiteflies, beetles, grasshoppers and psyllids. *B. bassiana* has also been used as a model system to study fungal-mediated tick (Acari: Ixodidae) biological control. The *Beauveria* lifestyle is further unique in that it is a facultative saprophyte and can exist as a plant endophyte and/or form intimate interactions with plants (White *et al*. 2002).

Common root rot is caused by *Cochliobolus* (**Fig. 2E, 2F**) species and produce symptoms identical to the *Fusarium* organism. Infected plants are usually scattered throughout fields rather than in patches and often go unnoticed. Severely infected plants ripen prematurely and "stick out" in green stands. Root development is reduced and plants are easy to pull out of the soil. Lower stems, leaf sheaths, and roots have brown lesions, resulting in a reduced number of new corms.

The genus *Fusarium* comprises several fungal species widely distributed in soils and organic substrates. One of the most relevant species of this genus is the ascomycete *Fusarium oxysporum* (**Fig. 4A**), which causes vascular wilt and root rot in more than 100 species of plants (Berrocal-Lobo and Molina 2008). *Fusarium* corm rot incited by *F. oxysporum* is the most destructive disease in saffron, having caused severe yield losses in Italy (Cappelli 1994). The disease has been referred to by various names, including dry rot, brown rot, basal rot and yellows. The major symptoms of the disease occur during the flowering time when the infected plants show drooping, damping-off, yellowing and wilting of shoots, as well as basal stem rot and corm rot (**Fig. 4B-D**). The pathogen survives in infected corms and in the soil as mycelium, chlamydospores, macroconidia and microconidia (Brayford 1996). Plants may become infected in the field, when germinating spores or mycelia enter the roots directly or through wounds. Most probably, the pathogen may be introduced into new saffron-growing regions via contaminated corms (Cappelli and Di Minco 1999). The disease was detected for the first time in Japan (Yamamoto *et al*. 1954) and was later reported in India (Shah and Srivastava 1984), Spain (García-Jiménez and Alfaro-García 1987) and Italy (Cappelli 1994). From the pathogens isolated from saffron, *Fusarium* has been detected in many different saffron cultivation areas generating the highest losses in corm yield.

The fungi *Rhizoctonia* and *Sclerotium* are soil inhabitant basidiomycetes, which cause serious diseases in saffron. The first report on *C. sativus* plants infected by *Rhizoctonia* was done in 1728, by Duhamel (Orlob 1964; Ecklund 1971), when this author was investigating a disease affecting saffron fields in Gâtinais (France), he discovered that the cause of the disease was a parasitical fungus who named *Tuberoides*, which was later on renamed *Rhizoctonia* by De Candolle (1815). The events occurring during the infection process of *Rhizoctonia* include adhesion, penetration, colonization and host reaction. When *Rhizoctonia* hyphae contact with the external surface of a compatible host, there is a recognition phenomenon that results in profuse hyphal branching and formation of infection structures. The initial steps of the infection process are characterized by both the adhesion of hyphae and an altered growth pattern resulting in directed hyphal growth and the formation of penetrating hyphal structures. Infection structures that are subsequently formed allow the fungus to reach intact plant tissue. In the

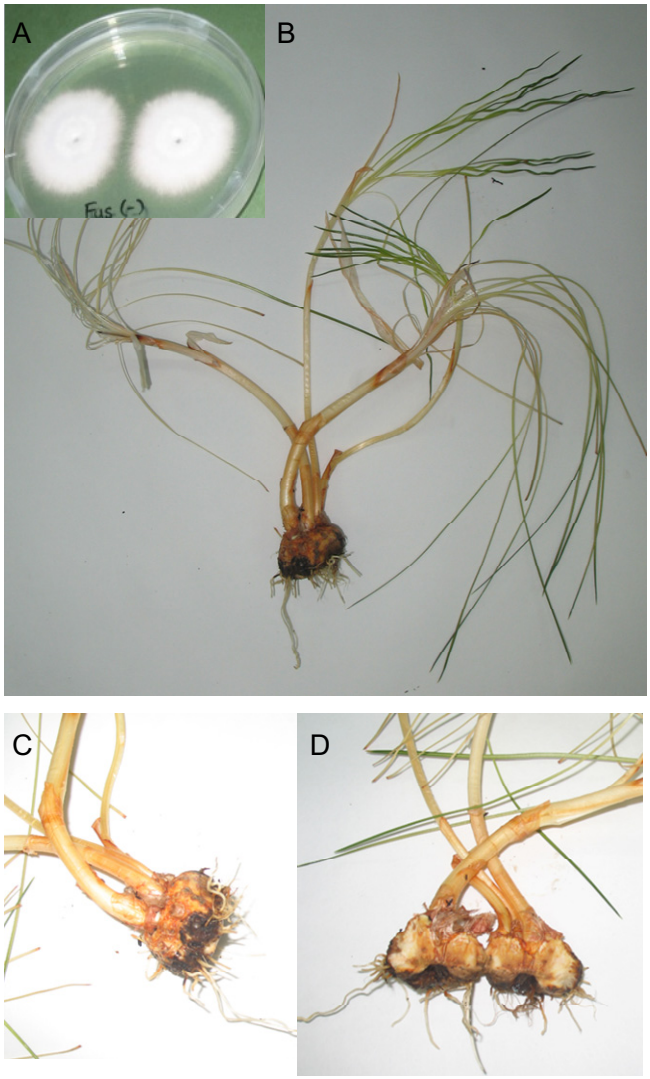

Fig. 4 ***Fusarium oxysporum*** **as an important saffron pathogen.** (**A**) *Fusarium oxysporum* isolated from disease saffron corms. (**B**) Symptoms developed by saffron plants caused by *F. oxysporum*. (**C**) Corm-rot and root-rot caused by *F oxysporum* in infected plants. (**D**) Longitudinal section of affected corms.

course of infection, substances are exchanged between the pathogen and the host plant. These include materials such as extracellular fungal enzymes and host exudates (reviewed by González-García *et al*. 2006). After penetration, colonization of plant tissue is accomplished by the production of hydrolytic enzymes capable of degrading several cell walls beyond the advancing hyphae. Together with cell wall damage, changes in the cytoplasm of cortical cells can be detected before colonization events are produced. From a cytological point of view, pathogenesis in *Rhizoctonia* is characterized by severe damaging or killing of plant cells, before or immediately after penetration and colonization. Thus, as in other fungal pathogen models, penetration and colonization is regarded as a primary process of hyphal growth into highly degraded plant tissue, suggesting a combination of both necrotrophic and hemi-biotrophic behaviour for this fungus on its compatible hosts. In saffron corms, *Rhizoctonia* causes rotten brown areas that may be superficial or may extend inward to the middle of the root system, with severely infected plants having practically no root system. The rotting tissues usually decompose and dry, forming a sunken area filled with the dried plant parts mixed with the fungus mycelium and sclerotia.

The presence of *Sclerotium* as a pathogen in saffron has only been recently reported in India (Kalha *et al*. 2007). In newly infested fields, the disease occurred in small patches that gradually enlarged each year. Symptoms appeared as brown-to-dark brown sunken, irregular patches below corm scales. Lesions were usually 1 mm deep with raised margins. Severely infected corms had foliage that dried from the tip downward, and white fungal mycelia appeared on the bulbs that rotted at later stages of disease development.

The oomycete *Phytium* is not a true fungus but is a fungal-like organism. Several species are facultative parasites of plants and of animals. Some species are parasites of other fungi, and others are primarily saprophytes (Tambong *et al*. 2006). *Pythium* species are ubiquitous non-specific soil-borne vascular pathogens, which under wet, humid conditions cause diseases in many plants including agronomic crops. They possess a high level of diversity with more than 200 species described (Souza *et al*. 2003). Several species of *Pythium* cause severe diseases as sole pathogens or in complexes with *Fusarium* spp. and *Rhizoctonia* spp. Many *Pythium* species can occur simultaneously at the same site and, often, more than one species can often infect a certain host plant. Infections occur on roots, lower stems and soft plant tissues, which are naturally present in seedlings and germinating bulbs and corms, causing root rot (Schenk 1970), stem rot and seedling damping off. Tissue degradation is caused by both cell wall degrading enzymes such as pectinases, hemicellulases, cellulases and proteinases and toxins produced by *Pythium* (Martin 1994). In *Crocus* plants affected by *Pythium irregulare* initially infected roots showed a distinct brown discoloration at the tip. Interestingly, *Crocus* roots are susceptible to the pathogen only during a short period after planting. Therefore, an epidemic may occur when periods of host susceptibility, pathogen activity, and suitable biotic and abiotic environments coincide (Van Os *et al*. 1998).

Stromatinia gladioli (Drayt.) Whetz. is a root-infecting fungus attacking several genera of the *Iridaceae*, notably *Gladiolus*, *Crocus* and *Freesia* (Gould 1958). In the past years the incidence of the disease in commercial crops has declined, following the introduction of routine corm treatments using benzimidazole fungicides (Humphreys-Jones 1971). However, this pathogen is highly persistent in field soils by means of sclerotia, which can remain dormant for long periods of time (5-10 years) and can act as a source of infection for future host plants. Excessive moisture or abundant rainfall and cooler temperatures increase the severity of the disease.

Phoma species are common soil-inhabiting fungi, which appear as wound- and weak parasites. They have been isolated in *Crocus* spp. (Boerema 1976) and *C. sativus* (Madan *et al*. 1966; Perez-Bueno 1955; and the work in this study).

Fungi of the genus *Botrytis* Persoon are important pathogens for many agronomically important crops, such as grapevine, tomato, bulb flowers, and ornamental crops (Jarvis 1977). *Botrytis* diseases appear primarily as blossom blights and fruit rots but also as leaf spots and bulb rots in the field and in stored products. *Botrytis* species are necrotrophs, which induce host-cell death resulting in progressive decay of the infected plant tissue. The pathogen produces abundantly sporulating gray mycelium on infected tissue. Macroconidia (mitotically produced spores) can be transported by wind over long distances. *Botrytis* overwinters in the soil as mycelium in decaying plant debris and as sclerotia, melanized mycelial survival structures. Some species frequently produce a sexual teleomorphic stage in which ascospores are produced in an apothecium. When collected in nature, apothecia are found under cool weather conditions, arising from sclerotia, which have developed on decayed plant parts in moist soil. *Crocus* blight is usually ascribed to *Botrytis croci* (Hennebert 1973, Boerema and Hamers 1989). In addition, it has been recorded that *B. gladiolorum* has been described as grey mould in *Crocus*. The conidial size of the fungus is very similar to that of *B. croci* occurring in crocus, but is distinguished by the production of sclerotia (Boerema and Hamers 1989).

VIRUSES

Plant cells have a robust cell wall which viruses cannot penetrate them unaided. Most plant viruses are therefore transmitted by a vector organism that feeds on the plant or (in some diseases) are introduced through wounds made, for example, during farming operations (e.g. tunic removing and corm progeny separation in the case of saffron). The largest and most significant vector group of plant viruses are insects, especially aphids, which transmit viruses from many different genera, including the *Potyvirus* and *Cucumovirus* viruses that infect *Crocus*. Symptoms of viral infection include tissue yellowing (chlorosis) or browning (necrosis), mosaic patterns, and plant stunting. Plant viruses are biotrophs that all face the same three basic challenges: how to replicate in the cell initially infected; how to move into adjacent cells and the vascular system; and how to suppress host defences and thereby colonize the entire plant.

Turnip mosaic virus (TuMV) has an RNA genome and infects a wide range of plant species, including those of the genus *Crocus* (Hu *et al*. 1996; Chen and Chen 2000). It is probably the most widespread and important virus infecting both crop and ornamental species, and occurs in many parts of the world including the temperate and tropical regions of Africa, Asia, Europe, Oceania and North/South America (Provvidenti 1996). TuMV belongs to the genus *Potyvirus*. This is the largest genus in the largest family of plant viruses, the *Potyviridae* (Ward *et al*. 1995), which in turn belongs to the picorna-like supergroup of viruses in animals and plants. TuMV, like other potyviruses, is transmitted by aphids in a non-persistent manner (Shukla *et al*. 1994). All potyviruses have flexuous filamentous particles 700–750 nm long, each containing a single copy of the genome, which is a single-stranded positive sense RNA molecule about 10000 nt in length. The genomes of potyviruses have a single open reading frame that is translated into a single large polyprotein, which is hydrolysed, after translation, into several proteins by virus-encoded proteinases (Riechmann *et al*. 1992). This virus has been detected in saffron plants from China (Chen and Chen 2000), New Zealand (Ochoa-Corona 2007), France (D'Agostino *et al*. 2007) and Spain (this study) (**Fig. 5**). In these two last studies the virus was detected during the sequencing of ESTs libraries from stigma tissue. The symptoms observed on *C. sativus*, with TuMV varied from chlorosis, mild to severe mosaic, and necrosis.

Another virus infecting *Crocus* ssp. from the same family as TuMV is the *Iris severe mosaic virus* (ISMV) (Langeveld *et al*. 1991; van der Vlugt 1994). This virus causes conspicuous chlorotic stripes and/or mosaic patterns in the leaves, and breaking in the flowers. Symptoms are usually more severe in plants grown at temperatures of 16°C or less, or in those also containing other viruses.

The *Narcissus mosaic virus* (NMV) is also a potyvirus recently found in *Crocus* ssp. by ELISA and RT-PCR (Miglino *et al*. 2005, 2007), although this virus was observed to have a very narrow host range. The symptoms observed are typical of virus infection: stunting, yellowing, necrosis, and flower colour breaking. However, the methodology employed enabled the correct identification of the virus agent. Using the ELISA and RT-PCR methodologies these authors also identified the presence of *Bean yellow mosaic virus* (BYMV) and ISMV. Serological results indicated that BYMV and ISMV were the most commonly encountered viruses, whereas *Tobacco necrosis virus* (TNV) and *Tobacco rattle virus* (TRV) were found occasionally (Miglino *et al*. 2005).

Cucumber mosaic virus (CMV), the type species of the genus Cucumovirus in the Bromoviridae family (Peden and Symons 1973), has been found in most countries of the world and has been identified as the causal agent of several disease epidemics. Its host range exceeds 800 plant species, making CMV one of the most important viruses due to its economic impact. The genome of CMV consists of three capped plus-sense single stranded RNAs (Rybicki 1995). CMV isolates have been classified into two subgroups based on visual symptoms, serology and nucleic acid hybridization tests, with the isolates from *Crocus* belonging to subgroup II (Chen *et al*. 2001). Symptoms consist in retarded growth, various mosaics, streaking, spotting, and distortion of flowers and leaves.

Other virus particles have been isolated and described in *Crocus*, but have not been identified (Ehrig *et al*. 1997). Sometimes the virus is restricted to certain parts of the plant (e.g. the vascular system; discrete spots on the leaf) without spreading throughout the plant to cause a systemic infection. Furthermore, infection does not always result in visible symptoms (such as *Carnation latent virus* and *Lily symptomless virus*, both members of the genus Carlavirus). The presence of additional viruses in *Crocus* could therefore be overlooked.

Fig. 5 *Crocus sativus* plants showing the symptoms caused by TuMV.

TRANSPOSABLE ELEMENTS AS GENOME PATHOGENS IN SAFFRON

Besides viruses, transposons can be considered as another group of special pathogens affecting the plant genome. The difference between transposons and viruses is that transposons simply hop around within the genome of a host cell, whereas viruses colonise new cells. Viruses, in contrast to transposons, can survive outside cells and invade new cells. To achieve this, they need some extra machinery compared to transposons. Active or inactive transposons constitute a large proportion of the repetitive DNA fraction of plant genomes which are dispersed throughout the genome. Transposable elements are separated into two major groups depending on their mode of transposition (Flavell *et al*. 1994). Class I transposable elements include retrotransposons and other retroelements, which are almost certainly viral in origin. These elements, like retroviruses, propagate intracellularly through transcription and translation. This Class I includes long terminal repeat (LTRs) retrotransposons: *gypsy*-like and *copia*-like retrotransposons (Xiong and Eickbush 1990), and non-LTR retrotransposons, which can be divided into LINEs (long interspersed nuclear elements) and SINES (short interspersed nuclear elements) (Schmidt 1999). Class II elements move as DNA copies and include the *Ac/Ds* (Activator/Dissociation), *En/Spm* (Enhancer/Suppressor) and *Mu* (Mutator) transposons, together with MITES, which are most likely non-autonomous transposons (Wessler *et al*. 1995).

The analysis of sequences from expression libraries obtained from corms and stigmas of *C. sativus* has allowed the identification of transposons and retrotransposons in its

Table 2 Transposable elements identified in *Crocus sativus*.

Transposable elements	GeneBank accession number	Library
Polyprotein	Ex148312, Ex148595	stigma
Transposon	Ex148329, Ex148296	stigma
Transposon	BM005533, BM005531, BM005532	corm
Retrotransposon	BM005537, BM956290	corm
Ty-copia	BM005527, BM005538, BM005528, BM005534, BM005530, BM956292	corm
Ty3-gypsy	BM005529, BM005535, BM005526, BM005536, BM956293, BM956291	corm

genome, from Class I and Class II (**Table 2**). Transpositions are influenced by developmental and perhaps environmental signals and may play a role in temporal and spatial patterns of gene expression.

NEMATODES

More than 20 genera of nematodes, i.e. round worms approximately 1 mm in length, cause plant disease. All the sedentary endoparasitic nematodes have evolved the ability to induce morphological changes in plant cells to form feeding cells (Sijmons *et al*. 1994). The juvenile form migrates through the soil towards the root system. Once it reaches the root epidermis, it enters the plant preferentially in the differentiation and elongation zone by perforating cells using a combination of intensive stylet thrusting, enzymatic softening of the cell walls and mechanical force. The nematode then begins migration towards a site in the plant for suitable feeding. At this time, these nematodes become immobile and completely dependent on the successful induction and maintenance of specialized feeding cells. This intimate relationship persists for approximately two months until the end of the nematode's life cycle. These parasites are therefore biotrophic: they do not kill the cells they feed from but instead modify them into efficient food sources. The mechanism of feeding cell formation is different and specific for each infecting nematode.

The nematode *Dytilenchus destructor* severely affects bulbs, corms and tubers (Ortuño and Oros 2002). *D. destructor* is unable to withstand excessive desiccation, and for this reason is usually only important in cool, moist soils. Because nematodes attack only subterranean and not aerial parts of plants, there are generally no obvious symptoms in the aerial parts of the plant. In the tissues affected, feeding initially produces yellow necrosis lesions, which then turn dark-brown. These lesions can be observed in the corms when cut longitudinally or transversally. In advance stages of disease, the corms are also infected by other parasites, especially fungi, which infect the plant and finally mask the responsibility of the nematode for the observed fall in yield (Melakeberhan 2003).

Pratylenchus penetrans, known as lesion nematode or meadow nematode, and *P. pratensis* have been identified in *Crocus* spp. and *C. sativus* (Metkalf 1903; Schenk 1970). These nematodes attack the external cells of radicles and penetrate the tissues little by little whatever the stage of development. They create cavities in which they reproduce and tissues may contain thousands of individuals. The parasites gradually make their way to parts of the parenchyma that are still sound, with the affected areas being rapidly destroyed by a characteristic necrosis. When conditions are unfavourable, e.g. the roots decompose; nematodes leave the root and travel freely within the soil until they come across another host root.

The nematode *Aphelenchoides subtenuis* was identified for the first time by Cobb in 1926 (Steiner and Buhrer 1932) in *Narcissus* bulbs, but was classified as a bud and leaf nematode, although studies on bulbous plants showed that *A. subtenuis* behaves in these plants as a root nematode. *A. subtenuis* has been detected in corms and leaves of *Crocus* spp. in different countries (Koliopanos and Kalyviotis-Gazelas 1979; Decker 1989; Ortuño and Oros 2002; McCuiston *et al*. 2007). It is not strictly an endoparasite because it can survive in dried leaves, but not in the soil.

Recently, a PCR-based approach has detected *Meloidogyne chitwoodi* and *M. fallax* which affect root growth and yield in *Crocus* plants (Zijlstra and Van Hoof 2006), even though these plants are poor hosts. Both nematode species spend the winter as eggs or juveniles and can survive extended periods of sub-freezing temperatures.

PLANT DEFENCE RESPONSES

The surfaces of plant organs, both above and below ground are continuously and permanently exposed to a diverse range of enemies, including microbial pathogens, nematodes and insects. As a result, plants have evolved intricate mechanisms to recognize and defend themselves against the wide array of these disease-causing agents. Plants can directly detect the pathogen presence of elicitors, either by non-self recognition of PAMPs, which are molecules that are highly characteristic of a whole class of microorganisms (Gómez-Gómez and Boller 2002); or by monitoring the integrity of their own cell walls (i.e. 'intact self' or 'degraded self') (Hèmaty *et al*. 2009). This recognition takes place through receptor molecules present in the plant, which are specific to the elicitor molecule (Gómez-Gómez 2004).

After pathogen perception, two types of plant resistance response to potential pathogens can be distinguished: the non-host resistance response (frequent); and the race/cultivar-specific host resistance response (comparatively rare). In the non-host resistance response, plants are equipped with a variety of defence mechanisms, including preformed defences such as waxes, cell wall components, and secondary metabolites. Upon pathogen detection, plants activate a number of responses that can include a hypersensitive response (HR; rapid localized cell death at the site of infection), increased expression of defence related genes [e.g. pathogenesis-related (PR) genes], and the oxidative burst (Gómez-Gómez 2004).

PREFORMED OR PASSIVE DEFENCE MECHANISMS IN SAFFRON

Preformed defence is the first obstacle a pathogen faces when invading a plant. For example, the plant cell wall, and in the case of saffron the presence of the fibrous tunic, protects the corm from pathogens, insects and water loss. But plants constitutively produce a plethora of secondary metabolites, many of which can act as antimicrobial compounds during defence against microorganisms (Dixon 2001). These compounds may be present in their biologically active forms or may be stored as inactive precursors that are converted to their active forms by host enzymes in response to pathogen attack or tissue damage. These compounds include saponins, phenolics, cyclic hydroxamic acids, cyanogenic glycosides, isoflavonoids, sesquiterpenes, sulphur-containing indole derivatives and many others. Altogether, these compounds represent the first chemical barriers to infection and are associated with non-host resistance. Saponins are glycosylated triterpenoid, steroid, or steroidal alkaloid molecules with antifungal activity (Osbourn 1996). Saponins are constitutively produced in many plants and can also be induced as a result of a pathogen infection. Saffron is characterized by the presence of saponins in stigma (Hosseinzadeh and Younesi 2002) and in corm tissue (Rubio-Moraga 2003) where they can play antifungal roles. Saffron corms also show the presence of 12

Table 3 Defence genes identified in *Crocus sativus*.

Protein	GenBank accession numbers	Mechanism of action	Library
PR-1	EX146574	Unknown.	stigma
PR-2	EX148616, EX146887	Hydrolysis of the structural 1,3-β-glucan present in the fungal cell wall.	stigma
Acidic chitinase	BM005616	Cleave cell wall chitin polymers *in situ*.	corm
Chitinase	EU446024	Cleave cell wall chitin polymers *in situ*.	corm
Chitinase	BM005637	Cleave cell wall chitin polymers *in situ*.	corm
Chitinase	EX146541	Cleave cell wall chitin polymers *in situ*.	stigma
PR-5	EX147388	Not completely understood. Some cause fungal cell permeability changes, others bind to 1,3-β-glucan and exhibit 1,3-β-glucanase activity.	stigma
PR-5	BM005643	Not completely understood. Some cause fungal cell permeability changes, others bind to 1,3-β-glucan and exhibit 1,3-β-glucanase activity.	corm
PR-10	Ex147729, Ex148310, EX147960	Ribonuclease activity.	stigma
PR-12	EX146849	Fungal inhibition probably occurs through an ion efflux mechanism.	stigma
PR-13	BM005615	Fungal inhibition probably occurs through an ion efflux mechanism.	corm
LTP (PR-14)	BM956322	Lipid transfer protein. Probably involved in plant-fungi interactions.	corm
LTP (PR-14)	EX148692, EX148514, EX148522, EX148506, EX148483, EX148428, EX148387, EX148382, EX148282, FJ997554, FJ997555	Lipid transfer protein. Probably involved in plant-fungi interactions.	stigma
WRKY2	EX148608	Activates the transcription of PR encoding genes.	stigma
WRKY4	EX144622	Activates the transcription of PR encoding genes.	stigma
Polygalacturonase inhibitor	BM005638, BM005626	Inhibition of fungal polygaraturonases.	corm
Elicitor response	BM005628		corm
PAL	EX147644.1	Phenylalanine ammonia-lyase.	stigma
Peroxidase	EX148603		stigma
β-1,3-galactosyltransferase	EX148441, EX146940, EX147392	Elicitor response protein.	stigma
SOD	EX147952	Superoxide dismutase.	stigma

PR: pathogenesys related; LTP, lipid transfer protein; PAL, phenylalanine ammonia-lyase

different phenolic compounds of which pyrogallic acid, kaempferol, p-coumaric acid and gallic acid, involved in stress response, have been tentatively identified (Crungoo *et al*. 1986; Ebrahimzadeh *et al*. 1997). Furthermore, *C. sativus* phenolic extracts show important antimicrobial activity (Senghum *et al*. 2009) and phenolic compounds together with peroxidases and catalases counteract reactive oxygen species (ROS) in order to survive and prevent molecular harm and damage by microorganisms, insects, and herbivores (Nicholson and Hammerschmidt 1992). Peroxidase, catalase and superoxide dismutase activities have been detected in saffron corms in different developmental stages (Keyhani and Keyhani 2004; Keyhani *et al*. 2006), and genomic approaches have enabled the identification of partial sequence homologues for these enzymes (**Table 3**).

INDUCIBLE DEFENCE MECHANISMS IN SAFFRON

The second obstacle an invading pathogen has to face is the inducible plant defence mechanisms. The induced mechanisms are associated with local changes at the site of pathogen infection, such as the hypersensitive response (HR), one of the most efficient forms of plant defences (Dixon and Paiva 1995). Besides causing accumulation of antimicrobial compounds, such as phenolic compounds, phytoalexins (Ortega *et al*. 2005) and antimicrobial peptides, HR also leads to an increase in the activity of peroxidases (Kortekamp and Zyprian 2003) and polyphenol oxidase enzymes (Agrios 2005) involved in defence responses (Thipyapong *et al*. 2004). The response to a pathogen also involves transcriptional activation of numerous defence-related genes, opening of ion channels, modifications of protein phosphorylation status, and activation of preformed enzymes to undertake specific modifications to primary and secondary metabolism (**Fig. 6**). In addition, a range of secondary signalling molecules are generated to ensure coordination of the defence response both temporally and spatially, resulting in rapid containment of the pathogen.

As previously stated, phenolic compounds, namely pterocarpans, coumarins, flavonols, and isoflavones (Dixon and Paiva 1995; Harbone 1999), are an important group of secondary metabolites involved in resistance to pathogens due to their antimicrobial activity, which biosynthesis increases after pathogen detection. The accumulation of phenolic compounds at the challenge site is a common feature of cell wall reinforcement in plant-microbe interactions. Cell wall reinforcements are generally accompanied by localized production of reactive oxygen species (ROS), which drive cell wall cross-linking, have antimicrobial activity, and are involved in defence-related signalling (Bradley *et al*. 1992; Levine *et al*. 1994). Besides, ROS responses orchestrate the HR response. The HR is one of the earliest visible manifestations of induced defence reactions and resembles programmed cell death in animals (Dangl *et al*. 1996). Concurrent with HR development, defence reactions are triggered in both local and distant parts of the plant and accompanied by a local and systemic increase in endogenous salicylic acid (SA) levels and the upregulation of a large set of defence genes, including those encoding pathogenesis-related (PR) proteins. The PR proteins are not or only present at basal concentrations detectable in healthy tissues, but upon pathological conditions, accumulation at the protein level is detected. These proteins encompass several different families of structurally and functionally unrelated proteins. There are 17 PR families: the PR-1 and PR17 proteins, whose biological function is still enigmatic; β-1,3-glucanases (PR-2), plant chitinases which are represented by several families (PR-4, PR-4, PR-8, PR-11), thaumatin-like proteins (PR-5), proteinase-inhibitors (PR-6), endoproteinases (PR-7), peroxidases (PR-9), ribonuclease-like proteins (PR-10), defensins (PR-12), thionins (PR-13), lipid transfer proteins (PR-14), oxalate oxidases (PR-15) and oxalate oxidase-like (PR-16). Although some of these PR proteins exhibit potential *in vitro* antimicrobial activities and their accumulation in plants is related to plant resistance responses, a direct functional role in defence could not be demonstrated for all (Sels *et al*. 2008). The target structures of the antifungal proteins range from the outermost part of the fungal cell, the cell wall, to the plasma membrane and finally to several intracellular targets. Therefore, these proteins exhibit a very wide diversity of action mechanisms, including, for example, inhibition of the synthesis of the fungal cell wall or disruption of its structure and/or function, membrane channel and pore formation, damage to cellular ribosomes, inhibition of DNA synthesis and inhibition of the cell cycle (Theis and Stahl 2004).

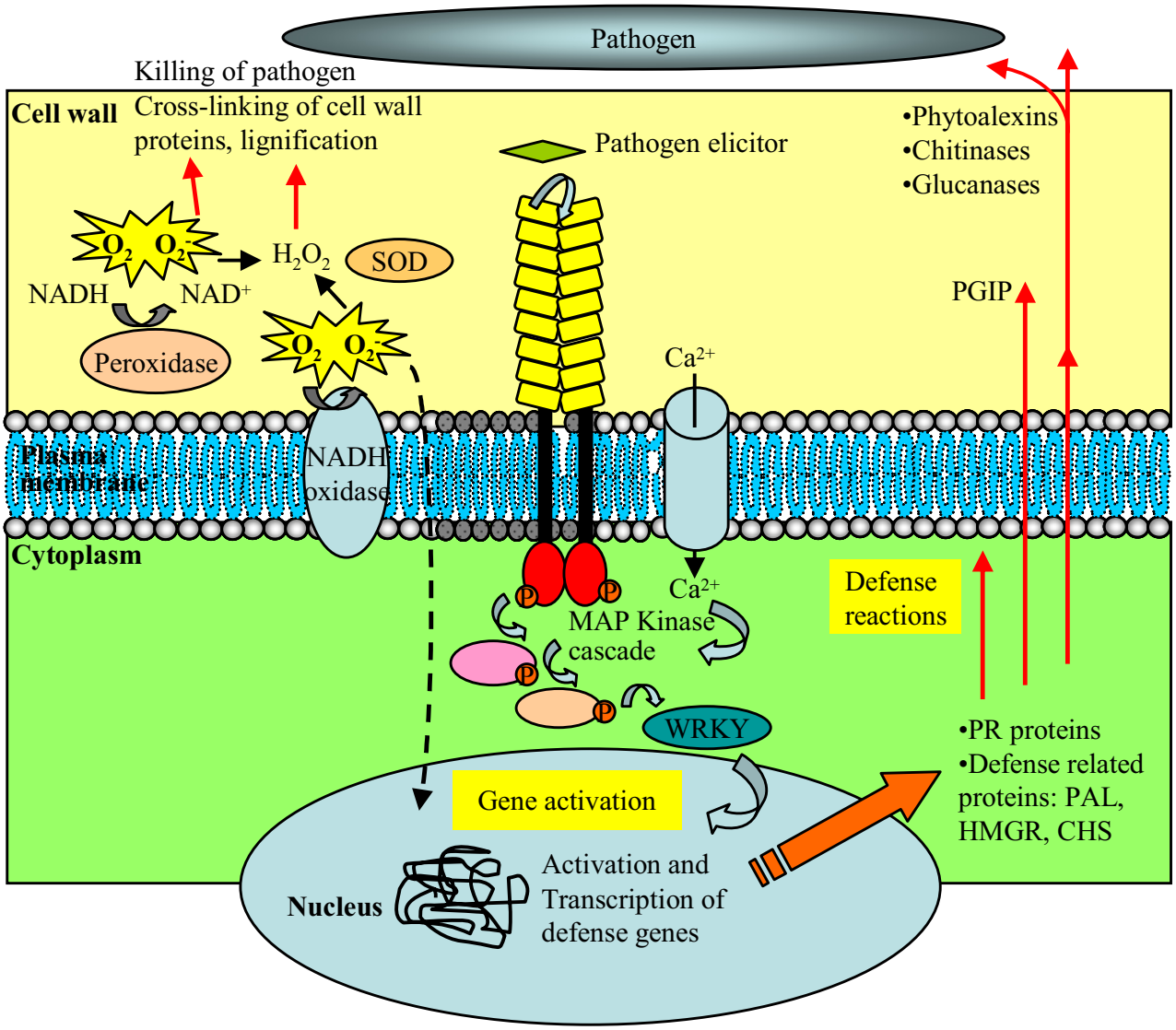

Fig. 6 Overview of the plant cell signalling components mediating responses upon pathogen attack. Plant cells possess a variety of membranes receptors that perceive endogenous (e.g. plant cell wall (CW)-derived fragments) or exogenous (e.g. PAMPs) signals, which can be either peptides or oligosaccharides (or both in the case of proteoglycans). A given ligand activates a specific receptor, which initiates downstream signalling events (a MAP kinase cascade). Most pathogens trigger a common/ interconnected plant signalling network. The graded transcriptional responses associated with the defence response clearly indicate the existence of a complex regulatory circuitry comprising transcriptional activators and repressors fine tuning the expression of defence genes. CHS, chalcone synthase; HMGR, 3-hydroxy-3-methyl-glutaryl-CoA reductase; PAL, phenylalanine ammonia-lyase; PGIP, Polygalacturonase inhibiting protein; SOD, superoxide dismutase.

Nevertheless, the mode of action of most of these proteins remains to be elucidated *in vivo*.

The analysis of corm and stigma libraries from saffron have identified several protein-encoding genes associated with defence responses (Álvarez 2003; D'Agostino *et al.* 2007) (**Table 3**). Antimicrobial proteins, PR, involved in virus resistance and in fungi recognition identified in corm and stigma, could be implicated in the resistance response of *C. sativus* to pathogen infection. Among the PR protein families, the different classes of chitinase conform an heterogeneous group. In fact, a new chitinase from corms, which shows antifungal activity, has recently been isolated (Castillo and Gómez-Gómez 2009). Chitin is the major cell wall component in filamentous fungi (BeMiller 1965). It is therefore not surprising that *C. sativus* synthesizes a large number of defence proteins capable of binding to chitin and chitin oligosaccharides (**Table 3**).

The expression of *PR* and defence related genes are modulated by members of several transcription factor (TF) families. In particular, zinc-finger type WRKY factors play a broad and pivotal role in regulating defences (Eulgem and Somssich 2007). Members of this family contain at least one conserved DNA-binding region, designated the WRKY domain, comprising the highly conserved WRKYGQK peptide sequence and a zinc finger motif (CX4–7CX22–23HXH/C). This domain generally binds to the DNA element termed the W box (C/TTGACT/C).The majority of the analysed WRKY genes respond as well to pathogen attack (Pamdey and Somssich 2009). Two homologues to WRKY TF have been identified in *C. sativus*: WRKY2 and WRKY4 (**Table 3**). Ectopic expression of grapevine VvWRKY2, the homolog to saffron WRKY2, resulted in enhanced resistance to the necrotrophic fungi *Alternaria tenuis*, *B. cinerea*, and *Pythium* (Mzid *et al.* 2007), while the homolog to WRKY4 in *Arabidopsis thaliana* also plays a positive role in plant resistance toward necrotrophic pathogens (Lai *et al.* 2008).

CONCLUSIONS

Many different classes of pathogens including viruses, nematodes and especially fungi severely affect saffron yield and quality. While new pathogenic fungi, e.g. *Fusarium*, are currently being described as important saffron pathogens, there are no recent reports on detection associated to important losses caused by historic fungi, such as *Rhizoctonia*. Surprisingly, pathogenic bacteria have not been detected as responsible for important saffron losses, suggesting the

presence of important defence barriers in saffron, which prevent bacteria colonization. Genomic approaches have permitted the identification of several defence genes in saffron, although a number of important previously known genes are still missing, as is the case of elicitor-receptors for pathogen perception and recognition. Future research on saffron pathogenesis will aim at identifying more of these genes as well as their pathogen-derived elicitors, thus enabling the discovery of their plant receptors.

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