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ABSTRACT

Tomato (*Solanum lycopersicum*) can establish symbiotic interactions with arbuscular mycorrhizal (AM) fungi, and can be infected by several pathogenic viruses. Here we investigated the impact of mycorrhization by the fungus *Glomus mosseae* on the *Tomato spotted wilt virus* (TSWV) infection of tomato plants , by transcriptomic and hormones level analyses. In TSWV-infected mycorrhizal plants, the AM fungus root colonization limited virus-induced changes in gene expression in the aerial parts. The virus-responsive up-regulated genes, no longer induced in infected mycorrhizal plants, were mainly involved in defence responses and hormone signaling, while the virus-responsive down-regulated genes, no longer repressed in mycorrhizal plants, were involved in primary metabolism. The presence of the AM fungus limits, in a salicylic acid-independent manner, the accumulation of abscisic acid observed in response to viral infection. At the time of the molecular analysis, no differences in virus concentration or symptom severity were detected between mycorrhizal and non-mycorrhizal plants. However, in a longer period, increase in virus titer and delay in the appearance of recovery were observed in mycorrhizal plants, thus indicating that the plant's reaction to TSWV infection is attenuated by mycorrhization.

INTRODUCTION

Arbuscular mycorrhiza (AM) is the term used to describe the mutualistic association between most land plants and fungi from the phylum Glomeromycota (Parniske 2008). The ability to form this association is widely distributed throughout the plant kingdom, and involves most agricultural, horticultural and hardwood species (Bonfante and Genre 2010). The symbiosis develops in roots where the fungus colonizes the cortex and obtains carbon from the plant while facilitating the transfer of mineral nutrients to the root cells, through differentiated highly branched intracellular fungal structures called arbuscules (Bonfante and Anca 2009). The benefits of the AM symbiosis on plant fitness are well described, including a better mineral nutrition and increased ability to overcome biotic and abiotic stresses (Aroca et al. 2008; Hildebrandt et al. 2007; Gernns et al. 2001; Pozo and Azcòn-Aguilar 2007; Pozo et al. 2010; van der Heijden and Sanders 2002).

The AM symbiosis can have important impacts on plant interactions with pathogens. Alleviation of damage caused by soil-borne pathogens has been widely reported in mycorrhizal plants. Most studies deal with the reduction of incidence and/or severity of soil-borne disease mainly root rot or wilting caused by fungi such as *Rhizoctonia*, *Fusarium*, or *Verticillium*, and root rot caused by oomycetes, including *Phytoftora*, *Phytium* and *Alphanomyces* (for a review see Whipps 2004). A reduction in the deleterious effects by parasitic nematodes such as *Pratylenchus* and *Meloidogyne* has also been reported (Li et al. 2006; de la Peña et al.,2006).

Different mechanisms may explain the protective role exerted by AM fungi, namely, improved plant nutrition, competition for colonization sites or photosynthesis, changes in the root apparatus and/or in the microbial rhizosphere communities and also activation of plant defence mechanisms (Wehnera et al. 2010; Whipps 2004; Pozo and Azcòn-Aguilar, 2007). The requirement of a well-established AM symbiosis for induced resistance is generally accepted. Dealing with defence mechanisms, accumulation of reactive oxygen species (ROS) (Blee and Anderson 2002), activation of phenylpropanoid and isoprenoid metabolism (Volpin et al. 1994, 1995, Harrison and

Dixon 1993; Strack and Fester 2006), induction of specific isoforms of hydrolytic enzymes such as chitinases and glucanases (Dumas-Gaudot et al. 2000, Pozo et al. 2002) and alterations of hormone levels (Lopez-Raez et al., 2010, Hause et al. 2007) have been reported in mycorrhizal roots.

Experimental evidence also supports the hypothesis that AM symbiosis preconditions plant tissues for a more effective activation of defence responses against stress. This phenomenon, which is also induced in plants by necrotizing pathogens, plant growth-promoting fungi and rhizobacteria, or treatment with various natural and synthetic compounds, is known as priming (Conrath et al. 2006; Beckers and Conrath 2007, Pozo and Azcòn-Aguilar 2007). Several results illustrate that primed responses are not restricted to areas colonized by the AM fungus, but they occur in the whole root system (Cordier et al. 1998; Pozo et al. 2002; Benhamou et al. 1994; Yao et al. 2003; Li et al. 2006).

Recently, more attention has been given to unraveling the functions of the root system in plant resistance and tolerance to above ground attack and evidence for the importance of roots as active modulators of shoot defence and resistance against herbivores and pathogens has been obtained (Erb et al. 2009 and references therein). However, the effects of the AM symbiosis in the protection against shoot pathogens remain to be elucidated and the few data available are contrasting (Pozo and Azcòn-Aguilar 2007; Pozo et al. 2010). Mycorrhization is known to reduce symptoms caused by a phytoplasma (Lingua et al., 2002), the necrotroph *Alternaria solani* (Fritz et al. 2006) and the bacterial pathogen *Xanthomonas campestris* (Liu et al., 2007) but the mechanisms on the basis of these responses are largely unknown. Shoots of mycorrhizal plants showed accumulation of insect anti-feedant compounds (Gange 2006). More recently, a microarray approach showed that the AM symbiosis induces the transcriptional regulation of many defence-related genes in shoots of *Medicago truncatula*. This was associated with an increased resistance to *X. campestris* (Liu et al., 2007). Alterations in phytohormone levels were also hypothesized to mediate shoot responses to biotic stress (Pozo and Azcòn-Aguillar 2007, Fiorilli et al. 2011). On the

other hand, a higher susceptibility to fungal pathogens (Lindermann 1994; Dugassa et al. 1996; Gernns et al. 2001) and aphids (Gange and West, 1994) has been reported. Very little is known concerning the interaction with virus infection. Mycorrhizal colonization was shown to increase the multiplication of some viruses (Daft and Okusanya 1973; Dehne 1982). Only one report described the disease symptoms: Shaul et al. (1999) demonstrated that leaves of *Nicotiana tabacum* mycorrhizal plant infected by tobacco mosaic virus (TMV) showed an enhanced disease severity compared to the controls.

The main goal of this work is to evaluate the impact of colonization by the AM fungus *Glomus mosseae* on infection by *Tomato spotted wilt virus* (TSWV) in tomato, their natural host. TSWV is an RNA virus of ambisense polarity belonging to the genus *Tospovirus*, able to cause huge crop losses in hundreds of horticultural and ornamental crops worldwide. In addition to the phenotypic observations, we used the TOM2 microarray platform, which contains around 12,000 genes, to monitor transcriptional changes in the roots and shoots of mycorrhizal plants two weeks after infection with TSWV. The microarray experiment described here is part of a broader microarray experiment where the single combinations tomato-TSWV and tomato-*G. mosseae* were also investigated (see Catoni et al. 2009 and Fiorilli et al. 2009). The levels of the phytohormones salicylic acid, jasmonic acid and abscisic acid, involved in regulating defence responses to pathogen infection, together with the effect of mycorrhization on virus multiplication and recovery are also analyzed.

RESULTS

Timing of the analysis and phenotypic observations

Transcriptional and hormone analyses were performed 42 days after the AM fungus inoculation, corresponding to 14 days after the TSWV inoculation. The 28-day time lapsed between the symbiont and the virus inoculation has guaranteed the establishment of a good level of root colonization before the infection by the pathogen. Sample collection was performed 14 days after virus inoculation when the first typical TSWV systemic symptoms, such as bronzing on leaves and distortion of the plant apex, appeared (Catoni et al. 2009). Apex and young expanding leaves (hereafter called "shoots") and radical terminal portions of roots (hereafter called "roots") were harvested.

At the time of sampling, viral symptoms on leaves were quite comparable between mycorrhizal and non-mycorrhizal plants. To better evaluate whether colonization by the AM fungus influences viral infection, the biomass of epigean and hypogean parts was measured (Fig. 1A). Mycorrhizal plants show a growth increase if compared to non-mycorrhizal ones (compare M and C). Viral infection dramatically suppresses the AM-induced growth increase (compare M and MV), especially in the roots. No differences in the amount of viral RNA in shoots and roots of MV and V plants were evidenced in a semi-quantitative analysis by dot-blot (Fig.1B).

Comparative analysis of transcriptional responses of mycorrhizal and non mycorrhizal plants to TSWV infection

The condition here considered, i.e. virus-infected mycorrhizal plants, involves the complex interaction of three organisms, plant, virus and fungus. Since it is known that even if microarray experiments are repeated with identical settings, considerable variation occurs between experiments, we set up a microarray experiment with an experimental design where each of the

three biological conditions - TSWV-infected (V), mycorrhizal (M) or TSWV infected mycorrhizal plants (MV) - were pair wise compared to mock inoculated plants (C). The analysis was carried out on both shoots and roots. The results obtained by the analysis of the plant-TSWV and the plant-*G. mosseae* interactions were already validated and described in Catoni et al. 2009 and Fiorilli et al. 2009 respectively. In this work, we focused our attention on the tripartite virus-fungus-plant interaction. The differential expression of a subset of genes regulated in mycorrhizal TSWV-infected plants, not yet validated in the previous works, was confirmed by quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR), using Ubiquitin (X58253) as reference gene (Table 1 and Supplemental Table S1). For the complete list of differentially expressed genes in at least one of the conditions considered, see Supplemental Table S2 and for an overview of the GO categories represented in the differentially expressed (DE) genes see Supplemental Fig. S1.

The overall changes in gene expression detected in virus-infected plants (V), in mycorrhizal plants (M) and in virus-infected mycorrhizal plants (MV) are represented in the Venn diagram of Fig. 2. The mycorrhizal fungus had a lower impact on the plant (655 genes in roots and 422 in shoots) than the virus (1,166 in roots and 2,385 in shoots). In the roots of MV plants, where both microorganisms are presents ,a higher number of genes (1,587) were differentially expressed if compared to the number of DE genes measured in the single interactions (V and M). On the contrary, in MV shoots, where only the virus is physically present, the impact of both virus and fungus (1,532 DE genes) appeared intermediate between that observed for the virus (2,385 genes) and the mycorrhizal interaction (422 genes) separately.

A pool of 215 genes in shoots and 579 in roots were specifically regulated when both TSWV and *G. mosseae* were present. Several of these genes were related to carotenoid metabolism, defence, metabolism of sugars, transport and response to hormones (with predominance of salicylic acid and abscisic acid); however no statistically significant GO category overrepresentation was observed.

The presence of the mycorrhizal fungus limited the gene response in the aerial part of MV plants, thus reducing the number of both up- and down-regulated genes, if compared to the V plants (Fig. 2). To better investigate this effect, we focused our attention on the list of genes induced (580) or repressed (505) in shoots in response to viral infection of non-mycorrhizal plants but not longer regulated when the virus infected mycorrhizal plants (Supplemental Table S3). The genes repressed in the shoots of V plants but no longer down-regulated in MV plants were mainly involved in primary metabolism pathway. The analysis of overrepresented GO categories among genes up-regulated in the shoots of V plants and no longer induced in MV plants highlighted a statistically significant enrichment of genes involved in defence mechanisms and response to hormones. In particular, a reduction in the expression of PR-proteins, heat-shock proteins, glutathione-S-transferases and WRKY transcription factors in the shoots of MV plants was observed if compared to the V plants (Supplemental Table S5). For PR-proteins we observed both a reduction in the number of induced genes and a decrease in the fold change levels in MV shoots if compared to V shoots (Fig. 3 and Supplemental Table S5).

Hormones measurements

Jasmonic acid (JA) and salicylic acid (SA) have been identified as important players in induced defence of the plant against invading organisms (Dong 1998; Penninckx et al. 1998). Recently, these molecules were related to specific stages of root colonization by AM fungi (Hause et al. 2007; Isayenkov et al. 2005; Herrera-Medina et al. 2008; Blilou et al. 2000) and have been proposed to contribute to the balance between compatibility and defence in mutualistic as well as parasitic biotroph-root interactions (Gutjahr and Paszkowski 2009). Our transcriptomic analysis revealed that several genes up-regulated in the shoots of V plants and no longer induced in MV

plants were involved in the response to hormones. Therefore, we decided to measure the levels of SA and JA in our samples, at the same time of the microarray analysis, i.e. 14 days post TSWV-inoculum. We found that TSWV induced a significant increase of SA in both shoots and roots, regardless of the presence of the AM fungus (Fig. 4). The physiological amount (in mock inoculated plants) of SA detected in shoots was twice that measured in roots. After TSWV infection, the SA concentration increased dramatically (about 1,000 times) in the shoots and only moderately (about 3 times) in the roots. No effect of mycorrhizal colonization on SA levels was observed.

The physiological level of JA in shoots was under the detection limit of the method, and no increase was observed in any tested condition. In roots, a significant increase of the JA level in mycorrhizal plants was detected, in both M and MV plants. No significant effect of the virus was observed (Fig. 4).

In a previous work (Catoni et al. 2009), an involvement of abscisic acid (ABA) in the plant response to TSWV infection was suggested. Moreover, an antagonistic interaction between ABA and ethylene during AM formation was recently observed (Martín-Rodríguez et al. 2011). Thus we measured the level of this hormone in mycorrhizal and non-mycorrhizal tomato plants infected by TSWV or mock-inoculated. The presence of TSWV induced a significant ABA accumulation in shoots of both V and MV plants. However, in the MV plants, the level of ABA was significantly lower than in V plants (Fig. 4). In tomato roots the quantity of ABA did not show any significant difference in any of the tested conditions (data not show).

Long term analysis

Microarray analysis gave us a picture of the transcriptomic changes occurring in the plants in response to virus infection and/or mycorrhizal colonization at the selected time point, i.e. at 14 days post virus inoculation, when the first TSWV symptoms became evident. At this time, a

significant reduction in the response of genes involved in defence mechanisms and response to hormones was observed in the shoots of MV plants if compared to the shoots of V plants (see above). This prompted us investigate how the tripartite interaction evolves in time, compared with the bipartite ones, in order to highlight if the reduced gene response can have an effect on the viral infection in a longer period of time. A set of plants representing all the four conditions (C, V, M, MV) was maintained in the same growth conditions for a period of up to 56 days post inoculation with TSWV. Changes in mycorrhization level, viral symptoms development (recovery) and virus concentration were analyzed.

AM fungal colonization was evaluated in M and MV plants at the end of the experiment, measuring several parameters; only the percentage of arbuscules within infected areas (a%) was statistically higher (Fig. 5A).

Virus-infected plants can be occasionally subjected to a phenomenon called "recovery", characterized by disappearance or reduction of symptoms in plants initially showing severe disease and by immunity to re-inoculation with the same virus (Pennazio 2010). In order to estimate if the presence of the fungus can interfere with development of the viral infection, we measured the frequency of spontaneous recovery in MV and V plants. Thirty-four days post TSWV-inoculation, 65% of V plants and 25% of MV plants showed recovery. The difference between these two sets of plants decreased at 44 dpi and disappeared at 56 dpi (Fig. 5B).

When the amount of viral RNA was measured by qRT-PCR, the results indicated no differences at 14 dpi; however, a significant increase of viral concentration was measured in MV plants compared to V plants at 34 and 56 dpi (Fig. 5C).

DISCUSSION

Effect of mycorrhiza on viral infection

In natural ecosystems, plants simultaneously interact with a broad panel of microorganisms, both pathogens and symbionts, giving rise to a complex system where the final outcome is not just the sum of the specific response to each microbe. Here we investigated how tomato plants transcriptionally respond to the concurrent presence of two microorganisms with a different lifestyle and naturally occurring in the environment: the pathogenic virus TSWV and the mycorrhizal fungus *G. mosseae*. Since TSWV invades all parts of the plant, mainly developing symptoms in the aerial part, and the symbiotic fungus colonizes the roots, we considered for the analysis both the aerial (shoots) and the underground (roots) part of the plant.

If compared to the corresponding bipartite interactions (TSWV-infected plants and mycorrhizal plants), shoots and roots of mycorrhizal plants appeared to differently respond to TSWV infection: we observed an "additive" effect (higher number of regulated genes compared to the single interactions) of the transcriptional responses in roots, likely due to the physical co-presence of the two organisms, while, in the aerial part of plants, the presence of the mycorrhizal fungus limited gene response due to viral infection, strongly reducing the number of both up- and down-regulated genes. The number of genes perturbed by the TSWV infection in the shoots of mycorrhizal plants is lower than that perturbed by the virus in non-mycorrhizal plants (Fig. 2). In particular, fewer genes involved in defence response and hormone response are up-regulated and fewer genes involved in primary metabolism are down-regulated (Supplemental Table S3). Activation of defence-related genes is a common process in several plant-virus interactions (Senthil et al. 2005; Dardick 2007; Catoni et al. 2009). On the other hand, it was shown that in shoots of tomato plants colonized by the *G. mosseae*, several genes involved in defence mechanisms are down-regulated (Fiorilli et al. 2009). In the shoots of TSWV-infected mycorrhizal plants we observed an intermediate situation. Several classes of genes involved in defence, i.e. those coding

for pathogen-related (PR) proteins, WRKY transcription factors, heat-shock (HS) related proteins, chitinases and glutathione-s-transferases (GST), activated in response to virus (Catoni et al. 2009), were less (decreasing fold change) or not activated in virus-infected mycorrhizal plants (Fig. 3). This is in agreement with a lower accumulation and a delay in activation of PR1 and PR3 proteins observed in mycorrhizal tobacco (Shaul et al. 1999) and was associated with enhanced virus infectivity.

A reduction in the number of down-regulated genes involved in primary metabolism was also observed in TSWV-infected mycorrhizal plants compared with the TSWV-infected non-mycorrhizal ones (Supplemental Table S3). Repression of primary metabolism was observed previously in several plant-virus interactions and is commonly linked to the development of symptoms (Senthil et al. 2005; Dardick, 2007 Catoni et al 2009). In particular, effects on carbohydrate allocation have been associated with virus infections (Maule at al. 2002; Clark and Adams 1977) and some studies have shown that active infection sites can function as photosynthetic sinks (Herbers et al. 2000; Shalitin and Wolf 2000; Tecsi et al. 1996), in order to expand availability of resources for virus replication and movement.

In spite of such a differential transcriptional response, no changes in virus accumulation or symptom severity was observed between V and MV plants, at the time of the microarray analysis (14 day post virus inoculation). However, by extending the time elapsed since virus inoculation, we did observe a continuous increasing in virus concentration in infected-mycorrhizal plants compared with infected non-mycorrhizal individuals, with a maximum at 56 dpi. Therefore we speculated that mycorrhizal plants could become somehow more sensitive to viral presence over time.

Our data are in agreement with previous studies indicating an increase in symptom development and virus accumulation in leaves of virus-infected mycorrhizal plants (Daft and Okusanya 1973; Shaul et al. 1999). AM fungi are known to increase the uptake of nutrients, especially phosphorous and nitrogen into their host plants (Javot et al. 2007; Guether et al. 2009).

Increasing phosphorous content has been associated with an increase in virus infection (Daft and Okusanya 1973, Borer et al 2010). However, by artificially raising the level of phosphorus, Shaul and colleagues (Shaul et al. 1999) failed to reproduce the increased susceptibility observed in TMV-infected mycorrhizal tobacco plants. Even in cases where the AM symbiosis improved the tolerance to the pathogen the differential response of mycorrhizal plants was not related to enhanced phosphate nutrition (Wehnera et al. 2010).

It is also interesting to note that the "recovery" phenotype is delayed in MV plants. Recovery is an unstable condition of virus-infected plants that appears and disappears over time. Our observations were limited to 56 dpi, when both type of plants showed the same number of individuals with "recovery"; however long-term experiments could again highlight changes between mycorrhizal and non-mycorrhizal plants.

At the end of the experiment (56 dpi), the only difference in AM fungal colonization between M and MV plants was the increased percentage of arbuscules within infected areas (a%) (Fig. 5A). This increase indicates that the fungal arbuscules are more concentrated in MV roots if compared to M roots. This could suggest that the fungus is slightly more active in MV plants. However, it worth notice that the other parameters of fungal colonization didn't show any statistical differences and the mycorrhiza-dependent phosphate transporters were similarly active in both M and MV roots (data not shown). Therefore we couldn't currently highlight a clear effect of the virus on the mycorrhiza.

Hormone signaling in mycorrhizal infected plants

Hormones are active molecules regulating several biological processes, including responses to abiotic and biotic stresses. Salicylic acid (SA) and jasmonate (JA) are well known to play an important role in regulating the defence responses against pathogens (Kovač et al. 2009, Singh et al. 2004, Alvarez 2000) and a fast-growing number of studies are demonstrating that ABA is also

implicated in the stress-response signalling network (Asselbergh et al. 2008b). Moreover, JA and, more recently ABA, have been shown to be prominent in the establishment and maintenance of the mycorrhizal symbiosis (Gutjahr and Paszkowski 2009; Hause et al. 2002; Isayenkov et al. 2005; Hause et al. 2007; Herrera-Medina et al 2007).

An enrichment in genes responsive to SA and ABA but not to JA was observed in the TSWV-infected mycorrhizal plants and the levels of SA, ABA and JA were affected differently by the virus and the fungus.

SA and JA

SA is well known to increase in response to infection by viruses and other biotrophic pathogens (Chaturvedi and Shah 2007; Singh et al 2004). SA levels were enhanced in TSWV-infected plants, with an increase significantly higher in shoots than in roots (Fig. 4). The same hormone was not affected by the presence of the AM fungus, in agreement with observations that the role of SA in mycorrhiza formation is local and limited to the site of interaction (Hause et al 2007, Pozo and Azcòn-Aguilar 2007). In support of these observations, NPR1 (non-expresser of PR genes1; SGN-U229275), an important regulatory component of SA signaling involved in the activation of PR genes (Dong 2004), was induced in the shoots of both V (FC=1.9) and MV (FC=1.5) plants, but not in the M plants. A consequent induction of PR proteins was observed mainly in the V shoots, and, with lower intensity, in the MV shoots (Fig. 3).

JA is usually associated with defence against necrotrophic pathogens and does not have a systemic role in defence against viruses although recently it was observed to have a limited effect in early local defenses (Kovač et al., 2009). It is widely accepted that JA is involved in the process of mycorrhiza formation in roots, as demonstrated in several host-fungus interactions (reviewed by Hause et al., 2007), and as well specifically in tomato (Herrera-Medina et al. 2008). In our

conditions, increased levels of JA in mycorrhizal roots were detected irrespective of the presence of TSWV infection.

To date, several reports have provided evidence of improved resistance in mycorrhizal plants against a broad panel of pathogens, leading to a proposed mycorrhiza-induced resistance (MIR). The partial suppression of the SA-dependent response in the plant, compensated by an enhancement of the JA-regulated response is one hypothesis for the "priming" of the JA dependent defence mechanism in MIR (Pozo and Azcón-Aguilar 2007). During TSWV infection, no significant change was observed in SA accumulation in mycorrhizal and non-mycorrhizal plants. Similar levels of SA were coupled with similar levels of virus accumulation and symptom development (at 14 dpi) indicating that mycorrhization was unable to counteract TSWV infection. Moreover, the significant lower up-regulation of PR proteins occurring in MV plants compared to V plants, together with the temporary decrease in recovery and with the increase in virus accumulation in the late steps of infection, suggests a more complex cross-talk among phytohormones, not limited to SA and JA only.

ABA

Recently it became evident that beside SA and JA, ABA can play important roles in defence responses (Bari and Jones 2008). In both V and MV plants, we measured an increase of ABA in response to TSWV infection. However, in MV plants, the level of ABA was lower than that measured in V plants. We already observed (Catoni et al. 2009) that in the roots of V plants, the gene coding for the 9-cis-epoxycarotenoid dioxygenase (SGN-U214605), a key enzyme in the ABA biosynthesis, was up-regulated (FC=3.1) and the gene coding for the ABA 8'-hydroxylase CYP707A1 (SGN-U222532), the key enzyme for the ABA degradation, was down-regulated (FC=0.3). No differential expression of these genes was observed in the shoots, where we measured an increase in ABA level. A similar situation can be observed in MV plants, where the same genes

were still respectively up- (FC=4.8) and down-regulated (FC=0.3) only in the roots while the ABA increase was measured in the shoots. On the other hand in the aerial part of both V and MV plants, we observed the induction of ABA-responsive elements-binding factors (AREB), that mediate ABA-regulated gene expression: two (SGN-U224515 and SGN-U224383) were both up-regulated in MV shoots (FC=2.2 and FC=1.6 respectively) and one of them was up-regulated in V shoots (SGN-U224383, FC=1.6). It is known that ABA can move from roots to shoots (Wilkinson and Davies 2010), and we suppose that modifications in ABA synthesis in roots can affect the level of this hormone in shoots.

The precise role of ABA in plant-pathogen interactions in still unclear. In tomato, high ABA levels are predominantly associated with high susceptibility but in *Arabidopsis*, both positive and negative effects have been reported (Asselbergh et al. 2008a). It is known that Tobacco mosaic virus (TMV) infection is able to increase ABA concentration in tobacco (Whenham et al., 1986; Fraser 1982) and that infecting bacteria enhance ABA levels in early stages of interaction in order to promote plant infection (Maksimov 2009). Mutants unable to synthesize ABA show enhanced resistance to several biotrophic and necrotrophic fungi (reviewed in Maksimov 2009; Mauch-Mani and Mauch 2005). The positive effect of ABA against virus infection is mainly related to its ability to enhance callose deposition by activating callose synthases and suppressing beta-1,3-glucanases (Ton and Mauch-Mani 2004; Flors et al. 2008). Callose accumulation at plasmodesmata is considered a defence mechanism against viruses, producing a physical barrier restricting virus movement in both compatible and incompatible interactions, while its degradation promotes pathogen spread (Zavaliev et al. 2010). Beta-1,3-glucanases are pathogenesis-related proteins able to degrade callose (van Loon and van Strien 1999). Plants deficient in basic beta-1,3-glucanases are more resistant to viral infection (Beffa et al. 1996). Increased expression of beta-1,3-glucanases in virus-infected cells can promote the spread of the virus by enhancing degradation of callose, leading to an increase of the plasmodesmatal size exclusion limit and consequently increasing local lesion

size (Bucher et al. 2001). We found two beta-1,3-glucanases (SGN-U215958 and SGN-U214527) up-regulated both in V (FC=8.8 and FC=4.2 respectively) and MV shoots (FC=4.3 and FC=3.1 respectively) and two other beta-1,3-glucanase isoforms (SGN-U218121 and SGN-U220005) down-regulated in V shoots (Catoni et al 2009), but no longer regulated in MV shoots. This expression pattern correlates well with the ABA level, lower in MV than in V plants (Fig.4).

Conclusion

The aim of this work was to investigate how the colonization by an AM fungus can influence plants' response to virus infection, providing new knowledge on the complexity of this naturally occurring tripartite virus-fungus-plant interaction. Our data showed that mycorrhization causes a dramatic modification in the levels of plant transcripts, particularly in shoots, where, out of the total number of DE genes, more than 1,000 were regulated in V plants but not in MV plants. This indicates that the root colonization has a strong systemic effect, also highlighted by the fact that ABA level in MV shoots was intermediate between that measured in M and V shoots. Curiously, these changes were not paralleled by differences in virus symptoms or concentration, at least at the time of the transcriptomic analysis. Differences in plant phenotype could be detected later on, both in the development and evolution of recovery, and in virus concentration. In mycorrhizal plants, the recovery phenotype was delayed and the concentration of TSWV became higher. These results highlighted the complexity of the dialogue between the host plants and the microbes, indicating that the protective effect induced by mycorrhizal fungi is not a general phenomenon but it is the result of a delicate equilibrium depending on these organisms' different life styles.

MATERIAL AND METHODS

Biological materials

Solanum lycopersicum L. (cv. Moneymaker) seeds were surface-sterilized by washing in 70% ethanol with a few drops of Tween 20 for 3 min and in sodium hypochlorite 5% for 13 min, and rinsed three times in distilled water for 10min. The seeds were placed in agar:H2O (0.6%) in Petri dishes, incubated for 5 days in the dark (25°C) and then exposed to light for 4 days. The seedlings were transferred to pots with sterile quartz sand. Inoculation of *Glomus mosseae* Gerd. & Trappe BEG12 (Biorize) was performed by mixing the inoculum with sterile quartz sand (30% v / v). The plants were grown in a growth chamber under a 14 h light (24°C) / 10 h dark (20°C) regime, and watered at a rate of 125 ml per plant twice a week with water, and once a week with a modified Long Ashton solution containing a low phosphorus concentration (3.2 µM Na2 HPO4·12H2O; Hewitt 1966). For recovery analysis, form 14 dpi to 56 dpi, plants were watered with a modified Long Ashton solution containing an higher phosphorus concentration (32 µM Na2 HPO4·12H2O; Hewitt, 1966) compared with that used before. Plants were inoculated with a Tomato spotted wilt virus (TSWV, genus *Tospovirus*) isolate (T1012, IVV collection) from Italy 28 days after planting (at the four leaf stage). The inoculum was prepared from systemically infected leaves of TSWV-infected tomatoes. Approximately 1g of infected leaf tissue was homogenized in 10 ml inoculation buffer (10 mM sodium diethyldithiocarbamate, 5 mM ethylene diamine tetracetic acid, 20 mM sodium sulfite). The inoculum was applied to the upper side of leaves by gentle rubbing with carborundum. Mock-inoculated plants, used as control, were subjected to the same protocol, using non-infected leaf tissue.

Plants were harvested 14 days post-inoculation (dpi); in order to check virus infection, freshly cut petiole of the youngest available leaf of every plant was printed on a positively charged nylon membrane (Roche, Mannheim, Germany). Membranes were then hybridized with a digoxigenin-labeled TSWV-specific probe (Vaira et al. 1995). Only infected plants were retained for further

analysis. Root and shoot fresh biomass of control, virus-infected plants, mycorrhizal and virus-infected mycorrhizal plants were measured and evaluated with ANOVA test. Samples were then immediately frozen in liquid nitrogen and stored at -80°C. Mycorrhization level was assessed according to the method of Trouvelot et al. (1986). Only roots showing high percentages for the four parameters considered (frequency of mycorrhiza formation (f %) > 50%, intensity of mycorrhiza formation (M %) >10%, percentage of arbuscules within infected areas (a %) > 60% and percentage of arbuscules in the root system (A %) > 10%) were used for RNA extraction.

DOT BLOT analysis

Semi-quantitative analysis of virus concentration was performed using dot blot hybridization. A mock inoculated sample was used as control. A starting quantity of 150 ng of total RNA, and 5-fold serial dilution, were blotted onto a positively charged nylon membrane (Roche, Mannheim, Germany) using the Minifold system (Schleicher & Schuell). Membranes were then hybridized with a TSWV-specific probe as described above.

RNA extraction and microarray experiment

Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA, U.S.A) following the manufacturer's instructions. RNA quantification was performed using Nanodrop 1000 Spectrophotometer, and RNA integrity was examined with Bioanalyzer 2100 (Agilent Technology). RNAs were pooled in four biological replicates for each condition, each pool containing three/four plants; for mycorrhizal roots the samples were grouped considering comparable F (frequency of mycorrhization) and M (intensity of mycorrhization) values in each. The same pools were prepared for shoot and root samples.

The TOM2 microarrays were obtained from the Center for Gene Expression Profiles (CGEP; Cornell University, Ithaca, NY, USA). Each microarray contains 11769 oligonucleotide probes

designed based on gene transcript sequences from the *Lycopersicum* Combined Built # 3 unigene database (http://www.sgn.cornell.edu). Three biological replicates were analysed and a 'dye swap' approach was adopted. Total RNA (500 ng) was used to generate direct fluorescently labelled cRNA using the Low RNA Input Linear Amp Kit (Agilent) according to the manufacturer's instructions.

Array slides were pre-hybridized, hybridized, washed and scanned as described by Fiorilli et al., 2009. Fluorescence data were processed using ImaGene software (version 5.6; BioDiscovery Inc., El Segundo, CA) using default quality controls and segmentation values with appropriate adjustment according to the signal intensity of each slide. Normalization and analysis of microarray data were performed using Limma package (Smyth et al., 2005). Within and between arrays normalization was performed (Lowess normalization). A gene was considered differentially expressed when i) its false discovery rate (FDR) was < 0.05 and ii) its fold change (FC) was ≤ 0.67 or ≥ 1.5 (log2 ratio ≤ -0.17 or ≥ 0.17).

Functional and metabolic analysis.

GO annotation was obtained using Blast2go software (Conesa et al. 2005), with default parameters. The lists of up- or down-regulated genes were searched for overrepresented GO terms. P-values were computed with Fisher's exact test and a P value $< 10^{-3}$ was considered statistically significant (Bluthgen et al. 2005). The analysis was performed using a set of Perl and C programs available from the authors upon request.

Real-time qRT-PCR analysis.

The RNA samples used for the hybridization experiments were treated with Turbo DNase free (Ambion, Foster City, CA, USA) according to the manufacturer's instructions. DNA contaminations were evaluated by RT-PCR using 18S rRNA specific primers of tomato and One

Step RT-PCR kit (Qiagen). Single-strand cDNA was obtained from ~1500 ng of total RNA using Oligo-dT (Invitrogen) primers and StrataScript Reverse Transcriptase (Stratagene, La Jolla, CA, USA). The volume of RNA samples was brought to 40 µl and then 10 µl of Mix (composed of 0.6 µl of 500 ng/µl Oligo-dT and 9.4 µl of distilled water) was added. The samples were incubated for 5 min at 65°C and for 10 min at room temperature. A master mix (8.5 µl) containing 5 µl of StrataScript RT buffer, 1 µl of RNase inhibitor (40 U µl-1), 2 µl of dNTPs (10 mM) and 0.5 µl of RT StrataScript enzyme was then added. The samples were incubated at 42°C for 1 h. Real-time PCR assays were carried out using Platinum Sybr Green qPCR SuperMix-UDG (Invitrogen) in an iCycler iQ apparatus (Bio-Rad). The reactions were conducted in a total volume of 25 µl, containing 12.5 µl of 2xPlatinum PCR Supermix-UDG, 300 nM of each primer (Supplemental Table S6) and 20 ng of cDNA template. The PCR cycling program consisted of: 50°C for 3 min, 95°C for 3 min and 40 cycles each consisting of 95°C for 30 s and 60°C for 30 s.

A melting curve (55–95°C with a heating rate of 0.5°C per 10s and a continuous fluorescence measurement) was recorded at the end of each run to assess amplification product specificity. All reactions were performed with three technical replicates and three biological replicates. PCR efficiency was determined from standard curves constructed of serial dilutions of tomato genomic DNA. The comparative threshold cycle method (Rasmussen 2001) was used to calculate the relative expression level using ubiquitin (accession no. X58253) as housekeeping gene.

Virus concentration

Virus concentration was measured by qRT-PCR (see above for details), with primers q-TSWV_492(+) (5'-TGTCTTGGCTATATATCAGGATGCA-3') and q-TSWV_570(-) (5'-TAAGGCTTCCCTGGTGTCATACTT-3'), amplifying a portion of the N gene of the TSWV genome. Relative quantity of virus was calculated using ubiquitin (accession no. X58253) as reference gene.

Hormone detection

Plant shoots and roots harvested at 14 dpi were immediately frozen in liquid nitrogen. Samples were transferred to 2 ml tubes and ground in a bead beater (Qiagen) with 3 mm tungsten beads at 25 Hz/s for 3 min. For each shoot sample, two aliquots of about 20 mg of powdered tissue (~220 mg fresh weight) were weighed and extracted with 500 µl of extraction buffer (10% v/v methanol containing 1% v/v acetic acid). One aliquot was added with internal standards (10 ng ABA, 15 ng JA and 200 ng SA) and used for recovery calculation. For each treatment an extraction control without plant material was performed. Samples were extracted in an orbital shaker at 4°C in the dark for 1h and then centrifuged at 13,000 xg for 10 min at 4°C. Supernatant was carefully removed and the pellet re-extracted twice with 300 µl of extraction buffer as before..The supernatants were pooled and centrifuged again to pellet any particulate material. Root extraction was performed on an initial amount of about 30-40 mg; extraction buffer volumes were 1000 µl, 500 µl and 500 µl for first, second and third extraction respectively. 200 µl of supernatant were directly analyzed for SA concentration while the remnant was purified through a 1ml Weak Anion Exchange & Reverse Phase column (Strata™-X-AW; Phenomenex) previously activated with 1 ml of methanol and equilibrated with 1 ml of extraction buffer. Columns were washed with 1 ml of 25mM sodium acetate and samples were eluted in 1ml in methanol with 2% v/v formic acid. Samples were concentrated in speed-vac at one third of their initial volume, and were analyzed by LC-MS/MS (ACQUITY UPLC System, Waters town UK). The LC was equipped with a 2.1mm_50mm_1.7µm ACQUITY UPLC BEH C18 column and was used with a binary solvent system comprising water (A) and acetonitrile containing 0.05% acetic acid (B). Separations were performed using a gradient of increasing solvent B content with a flow rate of 0.6 ml min-1. Gradient was increased linearly from 3% B to 30% B over 1.5 min and then 95% B at 1.6 min. After 0.5 min of exponential increase until 98% B, the initial condition was restored (3% B) and allowed to equilibrate for 2 min.

Retention times of the compounds were 1.07 min (SA), 1.88 min (ABA), and 1.97 min (JA). MS/MS conditions were follows: capillary (kV)=2.6, source temperature (°C)=120, desolvation temperature (°C)=400, cone gas flow (litre h-1)=0, desolvation gas flow (litre h-1)=800, multiplier (V)=650, cone voltage: 35 (ABA), 32 (JA), 31 (SA), collision energy: 12.0 (ABA), 14.0 (JA), 16.0 (SA), MS/MS transition (m/z): 263.2/153 (ABA), 209.1/58.9 (JA), 136.9/92.8 (SA).

Microarray data are available in the ArrayExpress database under the Accession Number E-MTAB-620

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TABLES

Sgn ID	Description	qRT-1	qRT-2	qRT-3	Array	Validated
Shoots						
SGN-U230270	receptor-like protein kinase ark1	21.11	68.59	25.99	8.86	**yes**
SGN-U231884	myb transcription factor myb117	10.80	27.22	8.98	7.94	**yes**
SGN-U219598	putative similar to receptor protein kinase	24.82	48.50	24.82	3.60	**yes**
SGN-U215018	acid phosphatase	0.14	0.02	0.01	0.16	**yes**
Roots						
SGN-U231884	myb transcription factor myb117	101.59	122.22	101.59	22.11	**yes**
SGN-U222064	ap2 domain transcription factor-like	132.51	337.79	922.88	28.24	**yes**
SGN-U219598	putative similar to receptor protein kinase	4.29	3.40	4.00	2.03	**yes**
SGN-U217373	basic helix-loop-helixfamily protein	0.29	0.16	0.29	0.15	**yes**
SGN-U216203	c-repeat binding factor	43.21	125.08	86.42	9.06	**yes**
SGN-U216297	ap2 domain transcription factor	32.00	71.01	15.82	14.31	**yes**
SGN-U216204	c-repeat binding factor	10.67	56.04	20.89	8.81	**yes**

Table 1. Validation of differentially expressed genes in response to TSWV infection in arbuscular mycorrhizal (MV) plants according to microarray data. Quantitative RT-PCR was performed on the three biological replicates (qRT-1, -2, -3) in shoots and roots. Expression values are indicated as fold changes of the differential expression in MV plants in respect to C plants. Ubiquitin was used as reference gene.

FIGURES

Fig.1. A, Biomass of epigean (left)and hypogean (right)parts of mock-inoculated (C), mycorrhizal mock-inoculated (M), TSWV-inoculated (V), and mycorrhizal TSWV-inoculated (MV) tomato plants, 14 days post TSWV inoculation (= 42 days post mycorrhizal inoculum). Bars represent standard errors. Different letters indicate significant differences (ANOVA). **B,** Dot-blot of total RNA extracted from shoots (left) and roots (right) of TSWV-inoculated, mycorrhizal TSWV-inoculated and mock-inoculated plants, 14 days post-inoculum. Hybridization was performed with a TSWV-specific probe, on the three pools of RNA (V and MV) used in the microarray experiment. A mock-inoculated plant was used as a negative control. Numbers above the panel indicate the amount (ng) of total RNA loaded in each square.

Fig.2. Venn diagrams showing differentially expressed genes in response to TSWV infection in shoots (left) and roots (right) of arbuscular mycorrhizal (MV) plants, compared with those differentially expressed in TSWV-infected tomato shoots and roots (V, from Catoni et al. 2009), and in mock-inoculated mycorrhizal plants (M, from Fiorilli et al. 2009). Bold numbers indicate differentially expressed genes; numbers between blankets indicates up-regulated (first number) and down-rugulated (second number) genes. The number of genes responsive in more than one condition is shown in the overlapping portion. The circle area is a proportional representation of the gene number.

Fig.3. Defense-related gene families showing significant decrease in the expression values between V (black diamonds) and MV (gray diamonds) shoots; diamonds represent genes belonging the each gene family; gray lines join the expression value of the same gene in each condition; bold line

represents the expression trend line. Significance was tested by t-test. PR, pathogenesis-related proteins; HSP, heat shock proteins; GST, glutathione-S-transferases; WRKY, WRKY transcription factors.

Fig.4. Hormone levels in tomato shoots and roots of mock inoculated (C), mycorrhizal (M), TSWV infected (V), and TSWV infected mycorrhizal plants (MV). Values are the average of 14 (shoots) and 7 (roots) samples. Bars represent standard errors. Different letters indicate significant differences (ANOVA). SA, salicylic acid; JA, jasmonic acid; ABA, abscisic acid; DW, dry weight.

Fig.5. A, Mycorrhization levels of mycorrhizal (M) and TSWV-infected mycorrhizal (MV) plants were assessed according to the method described in Trouvelot et al. (1986). F%, frequency of mycorrhization; M%, intensity of mycorrhization; a%, percentage of arbuscules within infected areas; A%, percentage of arbuscules in the root system. (*) indicates statistically significant differences (Kruskal-Wallis). **B,** percentage of plants showing recovery; plants with not clear or not stable recovery were not considered. **C,** virus concentration in shoots (young leaf), measured by qRT-PCR. (*) indicates statistically significant differences.

SUPPLEMENTAL DATA

Supplemental figure S1 – GO annotation of differentially expressed genes

Supplemental table S1 - Differentially regulated genes in infected mycorrhizal plants chosen for validation.

Supplemental table S2 – Expression matrix

Supplemental table S3 - GO enrichment analysis of gene regulated in shoots of infected non mycorrhizal plants and no longer regulated during infection in presence of mycorrhizal interaction

Supplemental table S4 - List of genes regulated in virus-infected roots (V) but no longer regulated in virus-infected mycorrhizal roots (MV)

Supplemental table S5 – List of defense-related gene families showing significant decrease in the fold change values between V and MV shoots.

Supplemental table S6 - List of the primers used for validation by qRT-PCR