Early *Lotus japonicus* root transcriptomic responses to symbiotic and pathogenic fungal exudates

Marco Giovannetti¹, Alfredo Mari¹,², Mara Novero¹ and Paola Bonfante*¹*

¹ Department of Life Science and Systems Biology, Università degli Studi di Torino, Torino, Italy, ² Scuola Superiore Sant’Anna di Studi Universitari e Perfezionamento, Pisa, Italy

The objective of this study is to evaluate *Lotus japonicus* transcriptomic responses to arbuscular mycorrhizal (AM) germinated spore exudates (GSEs), responsible for activating nuclear Ca²⁺ spiking in plant root epidermis. A microarray experiment was performed comparing gene expression in Lotus rootlets treated with GSE or water after 24 and 48 h. The transcriptional pattern of selected genes that resulted to be regulated in the array was further evaluated upon different treatments and timings. In particular, Lotus rootlets were treated with: GSE from the pathogenic fungus *Colletotrichum trifolii*; short chitin oligomers (COs; acknowledged AM fungal signals) and long COs (as activators of pathogenic responses). This experimental set up has revealed that AM GSE generates a strong transcriptomic response in Lotus roots with an extensive defense-related response after 24 h and a subsequent down-regulation after 48 h. A similar subset of defense-related genes resulted to be up-regulated also upon treatment with *C. trifolii* GSE, although with an opposite trend. Surprisingly, long COs activated both defense-like and symbiosis-related genes. Among the genes regulated in the microarray, promoter-GUS assay showed that *LjMATE1* activates in epidermal cells and root hairs.

Keywords: presymbiotic phase, defense response, microarray, *Lotus japonicus*, germinating spore exudates, chitin oligomers, arbuscular mycorrhizal symbiosis

Abbreviations: CERK1, chitin elicitor receptor kinase 1; COs, chitin oligomers; CSSP, common signaling symbiosis pathway; GSA, glucosidase SA conjugate; GSE, germinating spore exudates; GUS, beta-glucuronidase; LCO, lipo-chitooligosaccharide; LjCCD7, *Lotus japonicus* carotenoid cleavage dioxygenase 7; LjERF19, *Lotus japonicus* ethylene responsive factor 19; LjGST, *Lotus japonicus* glutathione-S-transferase; LjHydr, *Lotus japonicus* hydrolase; LjLeuc, *Lotus japonicus* Bark leucoagglutinin precursor; LjMATE1, *Lotus japonicus* multidrug and toxic compound extrusion protein; LjMTN19, *Lotus japonicus* medicago truncatula nodulin-like; LjPR10, *Lotus japonicus* pathogenesis related 10; LjVap-a, *Lotus japonicus* vapyrin-a; LjVap-b, *Lotus japonicus* vapyrin-b; LysM, lysin motif; LysM RLK, lysin motif receptor like kinase; MtERF19, *Medicago truncatula* ethylene responsive factor 19; MLYK3, *Medicago truncatula* LysM-type receptor-like kinase 3; MtNFP, *Medicago truncatula* Nod factor receptor protein; Myc-LCO, mycorrhizal lipo-chitooligosaccharide; nsMyc-LCOs, non-sulphated mycorrhizal lipo-chitooligosaccharide; NSP1, nodulation signaling pathway 1; NSP2, nodulation signaling pathway 2; PAMP, pathogen-associated molecular pattern; PTI, PAMP-triggered immunity; RAM, required for arbuscular mycorrhization; ROS, reactive oxygen species; SA, salicylic acid; sMyc-LCO, sulphated mycorrhizal lipo-chitooligosaccharide.
Introduction

Since the land conquest about 450 million years ago, plants had to deal and relate with both pathogenic and beneficial organisms. On the one hand, plants had to protect themselves from pathogens, evolving strong defense mechanisms to effectively ward them off (Jones and Dangl, 2006). On the other hand, plants have developed symbiotic relationships based on a fair nutrient exchange (Kiers et al., 2011). Understanding how plants can discriminate between friends and foes is a crucial question in plant biology (Hayashi and Parniske, 2014; Bonfante and Genre, 2015) with a direct effect on agricultural practices. On the pathogen side, a big effort has been invested in the study of fungal and bacterial effectors and plant receptors, focusing on the modulation of plant immunity, plant disease resistance and its application in modern agriculture (Dodds and Rathjen, 2010; Macho and Zipfel, 2014).

Among the beneficial microorganisms capable to form symbiosis with plants, research has mainly been guided by nutritional aspects (Gutjahr and Parniske, 2013) with less focus on immunity and compatibility aspects (Rey and Schornack, 2013). The two most studied symbioses between plants and soil microorganisms are symbiotic nitrogen fixation (Gage, 2004) and arbuscular mycorrhizal (AM; Parniske, 2008). Plant genetics and mutant analyses allowed to characterize several genes required for both root endosymbioses (in primis LjSYMRK/DMI2, LjPOLUX/DMI1, LjCCaMK/DMI3 in the case of the model legumes Lotus japonicus and Medicago truncatula, respectively), thus defining a CSSP (Oldroyd, 2013).

Knowledge on AM signals perception is not yet fully understood: LCOs have been identified in AM roots and in germinating AM spore exudates using the same bioassay that was set up for Nod factor characterization and named Myc-LCO (Maillet et al., 2011). Myc-LCO can induce lateral root formation, calcium spiking and a huge set of gene regulation but all of these actions are dependent on the MtNFP, the Nod Factor receptor (Maillet et al., 2011; Czaja et al., 2012; Genre et al., 2013). By contrast, it has been demonstrated that tetra- and pentamers of N-acetylglucosamine (chitin oligosaccharides: CO4, CO5), contained in spore GSE, activate the CSSP independently of MtNFP and its overexpression increased resistance (Gough and Jacquet, 2013; Rey et al., 2013). Another hint about the existence of parallels between Nod factor-induced and chitin-induced signaling, mediated by the respective LysM RLK, is given by the similarity of Nicotiana benthamiana responses to MtNFP and MtLYK3 (both part of the Nod factor receptor complex) co-production and AtCERK1 production (Pietraszewska-Bogiel et al., 2013). It was recently shown that OsCERK1, known to be responsible of the detection of pathogenic chitin molecules in rice, is also involved in the interaction with AM fungi (Miyata et al., 2014; Zhang et al., 2015).

Both the Nod Factor receptor and AtCerkl and, probably, the still unknown Myc-LCO receptor(s), bind to chitin residues, but the presence of different receptor complexes seems to allow a correct recognition of different chitin molecules and a discrimination between symbiotic and pathogen chitin signals (Antolin-Llovera et al., 2014; Cao et al., 2014; Ried et al., 2014).

Fully colonized and functional AM roots were extensively studied by expression profiling, initially with a whole organ approach (Fiorilli et al., 2009; Guether et al., 2009), then through cell-type-specific microarray (Hogekamp et al., 2011; Gaude et al., 2012), and more recently with RNA-seq approaches (Ruzicka et al., 2013; Handa et al., 2015). But up to date, genome-wide studies analyzing the presymbiotic stages only considered the transcriptomic impact of Myc-LCOs (Czaja et al., 2012) or the stage of hyphopodium formation (Hogekamp and Küster, 2013). The goal of this investigation is to characterize the transcriptome of L. japonicus upon perception of Gigaspora margarita GSE. GSE may contain not only a mix of simple sulfated and non-sulfated LCOs (referred to as sMyc- and nMyc-LCOs; Maillet et al., 2011), COs (Genre et al., 2013), and effectors (Klopholz et al., 2011; Tisserant et al., 2013) – each one probably playing a role during AM presymbiotic phase (Sun et al., 2015) – but also – still unknown – fungal molecules perceived by plants. This exudate could represent an ideal mix to investigate plant responses to AM fungi, since it better mimics the bioactive molecules released by AM fungi in natural conditions during plant–fungal presymbiotic interaction and defense-like responses. As a second goal, we wanted to go deeper in the characterization of plant-defense genes – which are known to be activated by AM fungi not only in roots but also in other organs like shoots and fruits (Fiorilli et al., 2009; Zouari et al., 2014) – in order to understand whether AM exudates may simultaneously activate both symbiotic and pathogenic-like responses. To validate this hypothesis, we treated Lotus seedlings with short (CO5) and long (CO8) COs, since the first elicit the symbiotic calcium spiking (Genre et al., 2013), while CO8 are the chitooligosaccharides which act as elicitors of defense (Hayafune et al., 2014). In parallel, we tested the specificity of a subset of genes by treating Lotus rootlets with GSE from a pathogen fungus such as C. trifolii.

Our genome-wide expression analysis revealed: (i) more than 100 genes induced by the perception of fungal GSE; (ii) a wide and extensive defense-like response in Lotus root 24 h after the perception, and a subsequent down-regulation of defense-like genes after 48 h; (iii) the activation by CO8 of both defense-like and symbiosis-like genes; (iv) similarities between the symbiotic and pathogenic signature elicited by the AM and C. trifolii GSE,
and (v) the localization of one of the activated genes in epidermal cells by means of promoter-GUS assays.

Results

G. margarita GSE Triggers a Specific Gene Expression

To record transcriptional responses toward symbiotic signals, we treated L. japonicus wild-type roots with G. margarita GSE. A microarray experiment with RNA coming from Lotus rootlets treated with GSE allowed us identifying 134 genes differently regulated after 24 h of treatment with GSE and 21 genes after 48 h (Figure 1). One third of the genes resulted to be linked to defense or redox mechanisms: they showed an up-regulation after 24 h and most of them had a dramatic down-regulation after 48 h (Table 1), probably pointing to a defense-like response of the plant to the AM fungal exudates and a subsequent down-regulation of that response (data sheet 1).

Among the most up-regulated genes at 24 h, Table 2 shows a pathogenesis-related protein, a lipase-hydrolase protein and various glutathione- S-transferases (GSTs), revealing a response of the root to microbe signals in line with responses already registered in plant root hairs upon early infection by symbiotic soil bacteria (Libault et al., 2010; Gourion et al., 2015). Among the most down-regulated genes, we identified genes linked to the phospholipid metabolism, such as a putative phosphatidylinositol phosphatidylcholine transfer protein, and to the ion traffic.

AM-GSE Activate Genes in an Opposite Way than Pathogen GSE

Looking at the relevant Lotus defense responses elicited by the GSE (one third of the differentially expressed genes were related to defense) we wondered whether this gene subset represented a part of the genes required for the establishment of AM symbiosis or whether it mirrored mostly a defense response, similar to the one elicited by pathogenic fungi.

To better understand this point, from the microarray data we selected four genes that were strongly down-regulated between 24 and 48 h, one gene that resulted to be up-regulated at both time points and one putative marker gene (LjERF19). We compared the relative expression of these six genes over two time points (24 and 48 h) with the gene induction generated by spore germination exudates from a fungal pathogen, C. trifolii. This biotrophic pathogen is known to trigger early cell responses in the root epidermis (Genre et al., 2009), but its GSE (which contains COsofs variable length) cannot activate the CSSP (Genre et al., 2013). Figure 2 shows that four out of five selected genes (a protein inhibitor-LjPI, a hydrolase-LjHydr, a pathogenesis related protein-LjPR10 and a lectin-like proteins-LjLeuc) when treated with G. margarita GSE are highly overexpressed at 24 h and then dramatically down-regulated, confirming the data coming from the microarray. By contrast, the treatment with C. trifolii GSE resulted in an opposite behavior, showing a slower increase of expression between 24 and 48 h. The only exception was represented by LjPR10 that was down-regulated with a similar expression pattern than AM fungal exudates.

Finally we checked for the trend of a LjGST, one of the few genes that in the microarray was overexpressed at both time points. QPCR confirmed this trend with AM GSE treatment but the transcript was not detectable after treatment with pathogenic GSE. LjGST function within AM development is still unknown but the gene is overexpressed also in later stage of symbiosis (Wulf et al., 2003; Hao et al., 2012; Hogekamp and Küster, 2013).

In addition, to understand if GSE could contain not only bio-active molecules involved in signaling, but also effectors down-regulating host defense pathways, we checked for the expression level of LjERF19. LjERF19 is the Lotus ortholog of MtERF19, a transcription factor targeted and down-regulated by AM fungal effector SP7 (Kloppholz et al., 2011). As reported for Medicago, we show a down-regulation of the transcription factor from 24 to 48 h after AM but not C. trifolii GSE treatment. This result can indirectly show the presence of effectors in G. margarita GSE, specifically targeting LjERF19.

Taken in the whole, the qRT-PCR experiment suggests that the plant genes regulated by pathogenic or symbiotic signals are largely overlapping with a specific trend of expression. The results therefore suggest a possible role represented by the timing of the gene activation that could be responsible of triggering different physiological plant responses.

![FIGURE 1 | Genes specifically activated by Gigaspora margarita spore germination water. The Venn diagram visualize the coactivation of Lotus japonicus wild-type root genes by GSE 24 and 48 h after treatment. Numbers indicate the number of genes with FDR values <0.05 and a fold change cut-off < -1 or > +1. The number into brackets indicate the genes that resulted to be down-regulated.](image)

![TABLE 1 | List of gene categories regulated in the microarray experiment.](image)
TABLE 2 | Most up- and down-regulated genes in Lotus japonicus wild-type roots treated with Gigaspora margarita GSE.

| Lotus GeneChip ID   | Annotation                              | Log2 FC 24 h | Log2 FC 48 h | p-values 24 h | p-values 48 h |
|---------------------|-----------------------------------------|--------------|--------------|---------------|---------------|
| Ljwgs_075692.1_at   | Lipase hydrolase-like protein           | 4.92         | -0.52        | 0.000         | 0.369         |
| Ljwgs_079996.1_at   | PR10-1 protein                          | 4.82         | 0.73         | 0.000         | 0.205         |
| Ljwgs_044810.1_at   | Bark leucoagglutinin | precursor    | 4.57         | 0.77         | 0.000         | 0.334         |
| TM1666.16_at        | Pectinesterase                          | 4.22         | -0.17        | 0.000         | 0.808         |
| Ljwgs_051780.1_at   | UDP-glucose:SA glucosyltransferase      | 3.96         | 4.60         | 0.000         | 0.000         |
| chr1.CM0064.61_at   | Germin-like protein                     | 3.95         | 2.23         | 0.000         | 0.006         |
| Ljwgs_028218.2_at   | Glutathione S-transferase GST 9        | 3.90         | 3.50         | 0.000         | 0.000         |
| Ljwgs_075865.1_at   | Bark agglutinin, polypeptide B precursor| 3.77         | 0.83         | 0.000         | 0.200         |
| chr6.CM0437.7_at    | MTN19 gene precursor                    | 3.21         | 1.56         | 0.000         | 0.016         |
| chr5.CM0909.45_at   | Glutathione-S-transferase GST 15       | 3.15         | 3.36         | 0.000         | 0.000         |
| chr3.CM0208.34_at   | Putative Fe(II) Ascorbate oxidase       | 3.10         | 0.12         | 0.000         | 0.740         |
| chr2.CM0201.55_at   | Auxin-induced protein                   | 3.08         | 3.96         | 0.000         | 0.000         |
| chr4.CM0046.42_at   | Glutathione-S-transferase GST 14       | 3.02         | 2.36         | 0.000         | 0.000         |
| chr1.CM0010.42_at   | Purple acid phosphatase (PAP22)         | -1.52        | -0.25        | 0.000         | 0.300         |
| Ljwgs_066244.1_at   | Putative glucanase                      | -1.74        | -0.10        | 0.000         | 0.689         |
| chr4.CM0126.67_at   | Hypothetical protein                    | -1.80        | -0.25        | 0.000         | 0.422         |
| Ljwgs_122957.1_at   | Cyclic nucleotide and calmodulin-regulated ion channel-like protein | -1.83         | -0.24        | 0.000         | 0.406         |
| Ljwgs_025743.1_at   | Cyclic nucleotide and calmodulin-regulated ion channel-like protein | -1.83         | -0.22        | 0.000         | 0.393         |
| Ljwgs_071032.1_at   | Putative phosphatidylinositol transfer protein | -1.83         | -0.22        | 0.000         | 0.393         |
| chr4.CM0099.52.1_at| AP2 domain containing protein PAP2.11   | -1.91        | 0.01         | 0.000         | 0.979         |
| Ljwgs_007118.2_at   | Unknown protein                         | -1.93        | -0.25        | 0.000         | 0.412         |
| chr5.TM1125.10_at   | Glutathione peroxidase -like protein    | -2.11        | -0.03        | 0.000         | 0.925         |
| Ljwgs_071601.1_at   | Germin-like protein                     | -2.19        | -0.15        | 0.000         | 0.587         |
| chr5.CM0909.45_at   | Glutathione S-transferase GST 15       | 3.15         | 3.36         | 0.000         | 0.000         |
| chr3.CM0208.34_at   | Putative Fe(II) Ascorbate oxidase       | 3.10         | 0.12         | 0.000         | 0.740         |
| chr2.CM0201.55_at   | Auxin-induced protein                   | 3.08         | 3.96         | 0.000         | 0.000         |
| chr4.CM0046.42_at   | Glutathione-S-transferase GST 14       | 3.02         | 2.36         | 0.000         | 0.000         |
| chr1.CM0010.42_at   | Purple acid phosphatase (PAP22)         | -1.52        | -0.25        | 0.000         | 0.300         |
| Ljwgs_066244.1_at   | Putative glucanase                      | -1.74        | -0.10        | 0.000         | 0.689         |
| chr4.CM0126.67_at   | Hypothetical protein                    | -1.80        | -0.25        | 0.000         | 0.422         |
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| Ljwgs_007118.2_at   | Unknown protein                         | -1.93        | -0.25        | 0.000         | 0.412         |
| chr5.TM1125.10_at   | Glutathione peroxidase -like protein    | -2.11        | -0.03        | 0.000         | 0.925         |
| Ljwgs_071601.1_at   | Putative phosphatidylinositol transfer protein | -2.19        | -0.15        | 0.000         | 0.587         |

Gene expression is represented by Log2-fold change values for the two treatments (24 and 48 h after GSE application), indicating also the p-values.

Short and Long Chitin Oligomers Elicit Different Gene Expression

On the basis of insights on the composition of active and non-active chitin-related oligomers contained in AM and C. trifolii exudates (Genre et al., 2013), and our RT-PCR experiments (Figure 2), we wondered whether short (CO5) and long (CO8) COs could partly mimic symbiotic and pathogenic signals, respectively. It is known that CO5 are able to induce Ca2+ spiking (Genre et al., 2013), while CO8 which miss to elicit the symbiotic-calcium spiking, can be associated to the defense-responses previously identified (Liang et al., 2014). Moreover we hypothesized that G. margarita GSE could contain a variety of chitin molecules and therefore able to trigger both symbiosis and pathogenic related genes. To test this, we performed a set of qPCR on Lotus rootlets treated with water, short (CO5), long (CO8) COs, and G. margarita GSE focusing on LjMATE1, a putative promoter region of LjMATE1, a multidrug and toxic compound extrusion protein that resulted to be up-regulated in the microarray experiment (data sheet 1) and is potentially involved in different kind of cellular detoxification. The same gene is involved in citrate transfer within nodules (Takanashi et al., 2013) and upregulated in mycorrhizal roots (Handa et al., 2015) therefore making it a good candidate for a putative function in legume endosymbioses. The putative promoter region of LjMATE1, 2243 bp upstream of the coding region, were fused to the reporter gene GUS. This construct was introduced into Lotus roots by Agrobacterium rhizogenes-mediated transformation. Composite plants were grown in plates, transgenic hairy roots were generated and treated after 4 weeks with long COs (CO8) for 1 h. Roots incubated with GUS buffer overnight at 37°C showed a typical epidermal coloration (Figure 4A) with homogenous blue color coming from root hairs (detail showed in Figure 4B) and outer

Promoter GUS Localization

To localize Lotus transcriptomic responses to GSE, we focused on LjMATE1, a multidrug and toxic compound extrusion protein that resulted to be up-regulated in the microarray experiment (data sheet 1) and is potentially involved in different kind of cellular detoxification. The same gene is involved in citrate transfer within nodules (Takanashi et al., 2013) and upregulated in mycorrhizal roots (Handa et al., 2015) therefore making it a good candidate for a putative function in legume endosymbioses. The putative promoter region of LjMATE1, 2243 bp upstream of the coding region, were fused to the reporter gene GUS. This construct was introduced into Lotus roots by Agrobacterium rhizogenes-mediated transformation. Composite plants were grown in plates, transgenic hairy roots were generated and treated after 4 weeks with long COs (CO8) for 1 h. Roots incubated with GUS buffer overnight at 37°C showed a typical epidermal coloration (Figure 4A) with homogenous blue color coming from root hairs (detail showed in Figure 4B) and outer
root cell layers. In conclusion it seems that the localization pattern of \textit{LjMATE1} is consistent with the functionality of a gene hypothetically involved in the presymbiotic fungal-plant interaction.

Discussion

The combination of untargeted and targeted transcriptomic analyses has allowed us to add some novel information to the still opened question of plant host responses to AM signals during the presymbiotic phases. In addition, the comparison of the effects elicited by exudates released by symbiotic and pathogenic fungi has revealed how the host plant responds activating the same gene subset but with a specific timing.

\textbf{G. margarita GSE Trigger \textit{L. japonicus}}

\textbf{Transcriptomic Responses}

The results of our experiment (the use of the whole GSE which contains a cocktail of known and unknown molecules) led to a first conclusion: \textit{G. margarita} GSE-induced gene regulation in Lotus is highly different than gene expression induced in \textit{Medicago} by purified Myc-LCO (Czaja et al., 2012). Among the 134 genes regulated by GSE in Lotus, just two genes show a significant similarity with \textit{Medicago} genes induced by Myc-LCO treatment, a putative GST (Mtr.18369.1.S1_at) and a putative endoglucanase (Mtr.50565.1.S1_at). These few overlaps could be due to the different biological system or to the different activity exerted by the complexity of GSE.

The induction of gene expression resulted to be mostly transient, consistently with the results obtained with Myc-LCOs and differently from gene regulation by purified Nod-LCOs (Czaja et al., 2012). However, we cannot exclude that the transiency of the response is due to instable nature of AM fungal molecules or, alternatively, to the presence in the GSE of enzymes or other molecules able to inactivate or outcompete for fungal molecules, as it happens with \textit{Cladosporium fulvum} LysM effectors (Sanchez-Vallet et al., 2013). This transiency is well indicated by the low number of regulated genes and the overall highest \textit{p}-value at 48 h. Nor the genes encoding components of the CSSP did show a marked expression change toward GSE, or the genes encoding GRAS transcription factors acting downstream of Ca$^{2+}$ signaling, such as NSP1, NSP2, RAM1 (Gobbato et al., 2012), RAM2 (Wang et al., 2012), or DELLA (Floss et al., 2013): however, it is worth to highlight the fact that all of these genes were identified and characterized through forward genetics and mutant phenotyping and their gene regulation during presymbiotic phase was not described elsewhere.

Among the regulated genes, GSTs represents a major group of detoxification enzymes, regulated \textit{in vivo} by ROS. ROS production at the infection site is the earliest response of PAMP-triggered immunity (PTI). Apart from primary effects, such as cell wall strengthening and induction of antimicrobial activity, ROS function as diffusible second messenger, inducing several resistance responses including synthesis of pathogenesis-related proteins (Zurbriggen et al., 2010), which have been recently demonstrated to be relevant also for symbiotic interaction (Gourion et al., 2015). Other species of oxidative burst are demonstrated to be involved in AM symbiosis as nitric oxide accumulation (Calcagno et al., 2012) and in nodules (Marino et al., 2011).
FIGURE 3 | Relative expression of symbiotic and defense-like genes after treatment with water, short (CO5), long (CO8) chitin oligosaccharides and *G. margarita* GSE, 1 h after the treatment. The expression of genes previously characterized as related to defense or symbiosis was characterized after treatment of Lotus rootlets with short or long chitin oligosaccharides. As controls we used *G. margarita* GSE and water. Bars represent the mean of four biological replicates ±SE. Bars subtended by the same low-case letter do not differ significantly at *p*-value < 0.05, according to the Kruskal–Wallis test and Mann–Whitney Post hoc test. NRQ stands for Normalized Relative Quantities. LjPR10, pathogenesis related protein; LjLeuc, Bark leucoagglutinin precursor; LjMATE1, multidrug and toxic compound extrusion gene; LjERF19, ethylene responsive element 19; LjVap, vapyrin-like gene; LjCCD7, carotenoid cleavage dioxygenase 7.

Lotus japonicus medicago truncatula nodulin-like 19 (LjMTN19) seems to be a possible key gene in both nodule and AM formation since its expression is induced both during mature AM colonization in Lotus (Guether et al., 2009) and during nodule formation (Moreau et al., 2011; Naya et al., 2014). Its biological role and function is still unknown but it was shown that Mtn19-like gene from pea increases also in pods treated with the insect elicitor Bruchin B (Doss, 2005) and thus it has been proposed to be involved in plant biotic responses. Our findings support this hypothesis also for Lotus.

Overall many of the genes that showed a highest regulation were previously characterized as involved in plant–microbe interaction: in virus-tobacco interaction, salicylic acid (SA) can activate plant resistance and its levels increase systematically following the hypersensitive response. The SA increase in the inoculated leaf coincided with the appearance of a GSA thanks...
to the higher activity of UDP-glucose: SA glucosyltransferase (Enyedi and Raskin, 1993). The same class of genes resulted to be regulated in our set up but also involved in Arabidopsis resistance against Pseudomonas syringae (Boachon et al., 2014).

Altogether novel genes activated by AM fungal exudates have been identified and could constitute a target for future analyses.

**A Cocktail of Specific and Non-Specific Responses**

Due to the fact that the majority of genes regulated in the microarray seems not to be specific of AM interactions but belonging to generic plant responses to biotic stress, we wanted to verify whether the same genes could be induced upon contact with GSE from *C. trifolii*, a pathogenic fungus. This experiment allowed to show that most of the genes were still activated but with a completely different pattern of expression: a gradual overexpression over time as opposed to a dramatic down-regulation happening after AM fungal GSE treatment (Figure 2). It would be challenging to demonstrate that this expression pattern could mirror the action of effectors probably contained in the GSE. The genome sequence of *R. irregularis* has revealed the presence of hundreds of small secreted proteins (Tisserant et al., 2013). The fungus we used is *G. margarita* which is, among Glomeromycota, phylogenetically quite far but we expect biological tools necessary to form a symbiosis to be well conserved among AM fungi.

As a further step we wondered whether we could correlate the activity of some chitin oligosaccharides, demonstrated to be present in the GSE, to the different genes activated and, eventually, discriminate between symbiotic and defense-like responses. Genre et al. (2013) showed that CO4 and CO5 are responsible of calcium spiking in transformed roots of Medicago, in contrast with longer chitin oligosaccharides such as CO8. Therefore our hypothesis was that long chitin oligosaccharides could be more connected with plant defense response whereas shorter oligomers could be able to trigger mostly genes related to symbiotic pathway or eventually counteract plant immunity. However, irrespectively, of such differences, among the activated genes, irrespectively, of being traditionally associated to defense-responses. By contrast, CO8 activate AM symbiotic-related genes, irrespectively, of being traditionally associated to defense-responses. However, irrespectively, of such differences, among the activated genes, *LjMATE1* results to be expressed at the plant epidermis providing new tools to investigate the molecular dialog between fungi and their host plants.

| Defence-related genes | G. m. GSE | C. t. GSE | CO5 | CO8 |
|-----------------------|-----------|-----------|-----|-----|
| Symbiosis-related genes | + | n. d. | + | + |
| Ca²⁺ spiking | Genre et al., 2013 |

**Materials and Methods**

**Plant and Fungal Materials**

*Lotus japonicus* (Regel) K. Larsen seeds (MG20, WT) were scarified and surface-sterilized for 5 min in concentrated sulfuric acid and washed three times with sterile water. In a second step the seeds were incubated for 3 min in 1:3 diluted commercial bleach with 1:1000 Triton-X. After washing three times with sterile water, the seeds were germinated on water-agar (0.6%) in Petri dishes. For the microarray experiment, three seedlings were left in a 1.5 mL eppendorf tube containing 1 mL of 10X...
GSE (Genre et al., 2013) or water for 24 and 48 h. C. trifolii race 2 strain MUT 3930 (Richard O’Connell, BIOGER-CPP, 78850 Thiverval-Grignon, France) conidia were produced after 7 days at 23°C on a modified Mathur’s medium (0.1% yeast extract; 0.1% BactoPeptone; 1% sucrose; 0.25% MgSO₄·7H₂O; 0.27% KH₂PO₄; 2% agar in 1 l of sterile distilled water). Spores were prepared as previously described (Torregrosa et al., 2004). A total of 10⁷ spores were diluted in 100 ml of sterile H₂O. After 24 h incubation at 24°C, the germinated spores were pelleted by centrifugation at 5000 g for 15 min and the GSE was recovered for analysis. C. trifolii GSEs were lyophilized and suspended in 1 ml of H₂O.

RNA Isolation and Microarray Hybridization

Roots were harvested and immediately frozen in liquid nitrogen in a 2 ml reaction tube. Two clean metal balls were added into every tube and frozen again. Plant material was then ground using a Retsch® ball mill for 2 min, at least three biological replicates per each condition. RNA was extracted using a modified ‘pine-tree-method’ (Guether et al., 2009). Integrity of RNA samples was checked using an Agilent 2100 Bioanalyzer. RNA purity was determined by ensuring spectrophotometric ratios of A260nm/A280nm ~ 2 and A260nm/A230nm ≥ 2. Removal of genomic DNA was done using the Turbo DNA-free™ reagent (Ambion, Austin, TX, USA) following the manufacturer’s instructions. Absence of genomic DNA was verified by RT-PCR with intron-specific primer for tubulin β-5 (TM0371b.4/TC18284). For each sample, 1 μg of total RNA was sent to AtlasBiolabs1 to perform the microarray experiment. cRNA was hybridized to the Genechip® Lotus1a520343 and scanned, according to the manufacturer's instructions.

Data Analysis

Microarray data were analyzed using the bioconductor software package for the R programming language (Gentleman et al., 2004). Data quality was assessed using the AffyPLM packages (Gautier et al., 2004), and expression estimates were obtained using the RMA algorithm (Irizarry et al., 2003). Control and bacterial probe-sets were removed, and only genes assigned as present (P < 0.05) using the MAS5 present/absent algorithm were retained. Statistical testing for differential expression was performed using mixed models with the LIMMA bioconductor package (Smyth, 2004). Comparison of the obtained data sets to the previous published microarray studies were based on TBLASTX. For the L. japonicus data sets (Deguchi et al., 2007) an e-value threshold of 1e–50 was applied. Furthermore, microarray data are available in the ArrayExpress database2 under accession number E-MTAB-3119.

cDNA Synthesis and Real Time RT-PCR

Real-time experiments were carried out on material derived from root. cDNA synthesis was performed using SuperScriptIII® Reverse Transcriptase and 1 μg of total RNA, following the protocol of the supplier (Invitrogen Ltd, Paisley, UK). Oligonucleotide sequences of all the primers are listed in Table 2. Quantitative RT-PCR was carried out with an iCycler apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Each PCR reaction was carried out in a total volume of 15 μl. The following PCR program was used: 95°C for 90 s, 40 cycles of 95°C for 15 s, 60°C for 30 s. A melting curve (54–70 steps with a heating rate of 0.5°C per 10 s and a continuous fluorescence measurement) was recorded at the end of every run to exclude primers generating non-specific PCR products (Ririe et al., 1997). All reactions were performed for at least three biological and two technical replicates. Baseline range and CT values were automatically calculated using the iCycler software. In order to compare data from different PCR runs or cDNA samples, CT values of all genes were normalized to the CT value of UBQ10 (chr1.TM0487.4) as previously described (Guether et al., 2009). Table 3 indicates the list of primers used in this experiment. Handling small sample size, statistical differences for QPCR analysis were calculated by Kruskal–Wallis

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### Table 3 | List of primers used in this study.

| Gene locus       | Gene name | Primer forward                  | Primer reverse                   |
|------------------|-----------|---------------------------------|---------------------------------|
| Ljwgs_074986.1   | LjPR10    | CTAAAGGTGATGCTAAACCCCTTTGAGC   | GCAAGCAGCGCTAGAAGAAACCTTTGAGC   |
| Ljwgs_044810.1   | LjLeuc    | CCAAGGTTGTGTCAGATGTTGGAAGTT   | GACTTACGTTAACAGTACATGTTGGAAGTT   |
| chr2.CM2001.55   | LjQOX     | GCCAATCTACTAAAGAAAGTTGGAAGTT   | CAATTACGTTAACAGTACATGTTGGAAGTT   |
| chr5.CM2009.44   | LjGST     | GCCATTTCTACGGTACGGTTGGAAGTT   | ACATCAAGAAGCAAGAAGAAACCTTTGAGC   |
| Ljwgs_075692.1.1 | LjHydr    | GAAATGCCGCAAAAGAATTTGGAAGTT   | CATAAAAGCTTGCCTACATGTTGGAAGTT   |
| TC11890          | LjPI      | GAAGTGATGCAAAAGAATTTGGAAGTT   | ATAAAGCTGCTGCTACATGTTGGAAGTT   |
| B39X8            | LjERF19   | TAGACGCTTACGGTACGGTTGGAAGTT   | ACAAGCAGCGCTAGAAGAAACCTTTGAGC   |
| chr1.CM1409.130.r2.d | LjMATE1 for | GGGGCAAGATGTTGACAAAAGACG   | GGGGCAAGATGTTGACAAAAGACG   |
| chr1.CM1409.130.r2.d | LjMATE1 | AGCGTTTGCTACGTATGTGTATGTGT    | GGGGCAAGATGTTGACAAAAGACG   |
| chr1.CM1409.130.r2.d | LjMATE1 | GTGACAATGTGTCCTGTCACATTG     | TACAAAGGTTGACCAAAACCTTTGAGC   |
| GU441766         | LjCCD7    | GTATGAGAAGTTAAGATGCGGAGTTGCTG   | TAAATGACGTTGCTAGGAGTTGAGTTG   |
| TC14054          | LjUBI     | TCCGATTTGTGCTGTCCTGCTGCGGTT   | ACAACAGAAGCAAGAAGAAACCTTTGAGC   |
| chr1.CM1409.130.r2.a | LjVAPa  | GCTATGAGAAGTTAAGGAGTTGAGTTG   | ACAAGGTTGACCAAAACCTTTGAGC   |
| LjSGA_008026.1   | LjVAPb    | CATGATGAGAAGTTAAGGAGTTGAGTTG   | CTGATCAGCTTGCCTGTCAGCTGTTG   |

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1www.atlas-biolabs.de

2www.ebi.ac.uk/arrayexpress
non-parametric one-way ANOVA followed by Mann–Whitney Post hoc test.

Biochemical Used for Bioassays
Short-chain COs were purchased from Yaizu Suisankagaku Inustry Corporation (Tokio, Japan). CO8 was kindly provided by Dr. Naoto Shibuya from Meiji University, Kawasaki, Japan to Andrea Genre. The preparation of solutions was performed following protocols indicated by Genre et al. (2014).

Histochemical Analysis of Root Tissue
Two thousand two hundred forty three base pairs upstream of LjMATE1 cds, one of the genes up-regulated in the microarray, was fused with the GUS gene in the vector pKGWFS70 in order to verify its activation at root level. The red fluorescent marker DsRED, under the control of the constitutive Ubiquitin10 promoter (P\textsubscript{Ubq10}; Limpens et al., 2005), was inserted. \textit{L. japonicus} composite plants carrying transformed roots were treated with water or long chitooligosaccharides. Root fragments, showing DsRED fluorescence were selected under a stereomicroscope. After the treatment, the root segments were covered with freshly prepared GUS buffer [0.1 mM sodium phosphate buffer, pH 7, 0.5 mm K\textsubscript{4}Fe(CN)\textsubscript{6}, 5 mM K\textsubscript{2}Fe(CN)\textsubscript{6}, 0.3% Triton X, 0.3% X–Glc]. Samples were incubated at 37°C for 16 h in the dark, washed with distilled water and observed under an optical microscope (Eclipse E400; Nikon).

Author Contributions
MG participated in the design of the experiment, carried out most of the experimental part and drafted the manuscript. AM produced exudates from \textit{C. trifolii} and perform part of the qPCR and the GUS assay; MN followed the plant cultures, the spore germinating exudates production and the sampling for the microarray experiment; PB conceived the study, participated in its design and coordination and wrote the manuscript. All authors read and approved the final manuscript.

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Supplementary Material
The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.00480

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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