Glucosinolates profile of *Arabidopsis thaliana* modified root colonization of *Trichoderma* species

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Abstract

Trichoderma is a genus of filamentous fungi with highly beneficial species for use in agriculture due to its ability to combat disease, increase growth, tolerance to abiotic stresses and resistance to biotic stresses in plants. To do this, *Trichoderma* must effectively colonize the roots of the plant, being able to overcome the existing plant defenses. In this sense, glucosinolates (GSLs) are a group of secondary metabolites present in cruciferous dishes such as *Arabidopsis thaliana* with great antimicrobial capacity. In order to determine the effect of the *A. thaliana*-roots GSLs profile in *Trichoderma* colonization, we have used different *A. thaliana* mutants (*cyp*: absent in indolic GSLs; *myb*: absent in aliphatic GSLs; *QKO*: absent in indolic and aliphatic GSLs; and *atr1D*: which hyperacumulates indolic GSLs) and inoculated them with the species *Trichoderma harzianum* and *T. parareesei*. The results showed how in the roots of *A. thaliana* mutants absent from IGSLs an increase occurs in the levels of root colonization of *Trichoderma*, without observing differences in the rest of the mutants. This increased root colonization by *Trichoderma* results in an increase in the formation of silques by *A. thaliana* and an increase in the systemic resistance against *Botrytis cinerea* mediated by jasmonic acid, being more significant with *T. harzianum*; in addition to an increase in tolerance to abiotic stresses such as salinity and drought, being more significant with *T. parareesei*. Therefore, the presence of IGSLs in the

roots of cruciferous plants such as *A. thaliana* inhibits *Trichoderma*'s ability to root colonization, reducing the fungus's ability to increase plant productivity, tolerance to abiotic stresses and resistance to biotic stresses.

Keywords: glucosinolates; *Arabidopsis*, *Trichoderma harzianum, Trichoderma parareesei*, systemic resistance, root colonization.

1. Introduction

The genus of filamentous fungi *Trichoderma* includes various species widely used as biological control agents in agriculture due to different mechanisms of action and their ability to grow naturally in very diverse habitats and climatic zones (Harman, 2006; Vinale et al., 2008). Some species are able to mycoparasitize pathogenic fungi in crops, such as *T. harzianum* on *Rhizoctonia solani* (Kullnig et al., 2000), to produce chemical compounds that inhibit their growth and development, a mechanism known as antibiosis (Patil et al., 2016), or to compete efficiently for space and nutrients in the rhizosphere (Segarra et al., 2010). In addition, *Trichoderma* will be able to promote plant growth by enhanced production of hormones (Zhang et al., 2013), and to increase the tolerance of plants against abiotic stresses such as drought and salinity (Poveda, 2020a). Therefore, the root application of *Trichoderma* represents an increase in plant productivity, as has already been observed in the model plant *Arabidopsis thaliana* and in crops such as rapeseed (Poveda et al., 2019a).

Trichoderma-roots colonization involves an ability to recognize and adhere to roots, penetrate the plant and withstand toxic metabolites produced by the plant in response to invasion. In this sense, the plant may perceive *Trichoderma* as hostile and the activation of the plant's defences may limit penetration by the fungus to the root's outermost cell layers (Poveda et al., 2020a). Transcriptomic studies have shown that during the first few hours of interaction between the fungus and the plant, a broad genetic reprogramming occurs. This is preceded by a transient decrease in the plant's immune response during the first 24 hours,

which is probably what allows colonization of the root to take place (Morán-Diez et al., 2012).

In this connection, the successful *Trichoderma*-roots colonization supposes the activation of a systemic plant resistance against the possible attack of pathogens or pests. This defense occurs via induced systemic resistance (ISR), systemic acquired resistance (SAR) or both of these (Mendoza-Mendoza et al., 2018), may indicate the presence of alternative induced resistance mechanisms and a more complicated signaling network that connects the SAR and ISR defense response pathways (Brotman et al., 2012; Nawrocka and Małolepsza, 2013). This variation depends on the species of plant, the strain of *Trichoderma* and the pathogen against which the defense response is directed (Martínez-Medina et al., 2013; Poveda et al., 2020b).

On the other hand, glucosinolates (GSLs) are a group of hydrophilic secondary metabolites formed by a side chain and a sulphur-linked D-glucopyranose moiety (Rosa and Rodrigues, 2001), found in members of the *Brassicaceae* and close families (Buxdorf et al., 2013) such as broccoli, cauliflower, cabbage or the model plant *Arabidopsis thaliana*, and also in mustard or rapeseed (Halkier and Gershenzon, 2006). GSLs are produced in many tissues of cruciferous plants, but differences in quantity and type of GSLs occur between different parts and organs (Touw et al., 2020), having constitutive GSLs concentrations generally higher in roots compared to shoots (van Dam et al., 2009).

The ability of plants in the Brassicaceae family to defend them against different plant-pathogens has been widely demonstrated thanks to the presence in their tissues of GSLs (Vig et al., 2009; Poveda et al., 2020c). Against fungi, it was demonstrated in 1937 how the antifungal activity of mustard oils and of cruciferous plant extracts was due to the presence of GSLs as secondary defense metabolites (Walker et al., 1937).

The antifungal capacity of the GSLs present in different cruciferous species, after their hydrolysis (GSL hydrolysis products: GHPs), has been extensively studied (Bednarek et al., 2009; Sánchez-Pujante et al., 2017; Poveda et al., 2020c). In *A. thaliana* has been observed how indol-3-ylmethyl GSLs are constitutively produced in the phloem and transported to epidermal cells for storage and to be activated to provide broad-spectrum defense against

fungi. Moreover, these authors observed that accumulation of the GSLs biosynthetic enzymes is induced in epidermal cells attacked by the non-adapted barley powdery mildew *Blumeria graminis* f.sp. *hordei*, while this biosynthesis is attenuated during interaction with the virulent powdery mildew *Golovinomyces orontii*, concluding that epidermal *de novo* synthesis of indol-3-ylmethyl GSLs contributes to broad-spectrum antifungal resistance and that adapted powdery mildews may target this process (Hunziker et al., 2019).On the other hand, in disease resistant and susceptible cabbage lines were observed how the major infection with the causal agent of the blackleg (*Leptosphaeria maculans*) in susceptible lines was consequence of a no accumulation of the AGSL glucoiberverin (GIV) and the IGSLs glucobrassicin (GBS) and neoglucobrassicin (NGBS) (Robin et *al.*, 2017). Similarly, in *Sinapis alba*, *Brassica napus*, and *B. juncea* have been observed how against the pathogenic fungus *F. oxysporum* occur an increase of p-hydroxybenzyl GSL content, which does not happen after inoculation with non-pathogenic fungi *Rhizopus oryzae* and *Fusarium graminearum* (Andini et al., 2019). Despite this, little is known about the role that these defense compounds have in beneficial plant-microorganism interaction, as is the case of the fungal genus *Trichoderma*.

The main objective of this work is to demonstrate how the profile of root GSLs in crucifers, such as *A. thaliana*, is capable of modifying the ability of root colonization by *Trichoderma* and, therefore, the benefits that this interaction implies for the plant.

2. Materials and methods

2.1. Plant and fungi

The *A. thaliana* ecotype Col-0, along with different mutants with different glucosinolate profiles, all of them kindly provided by Dr. Vicent Arbona from the Jaume I University, were the plants used in this study. The following have been used: the double knockouts *cyp79b2/cyp79b3* (called in this document as *cyp*) absent in indolic GSLs (IGSLs) (Zhao et al., 2002); double knockouts *myb28/myb29* (called in this document as *myb*) absent in aliphatic GSLs (AGSLs) (Sønderby et al., 2007); the quadruple mutant resulting from their crossing, which knocks out both pathways (called in this document as *QKO*) (Sønderby et

al., 2007); and the *atr1D* mutant which hyperacumulates IGSLs by overexpression of IGSLs biosynthetic genes *CYP79B2*, *CYP79B3* and *CYP83B1* (Bender and Fink, 1998).

Arabidopis seeds were surface-sterilized by vigorous sequential shaking in 70% ethanol and 5% sodium hypochlorite solutions for 10 min each and then washed thoroughly four times in sterile distilled water.

T. harzianum CECT 2424 (referred from here as strain Th) and *T. parareesei* CECT 20106 (referred from here as the Tp strain) (Spanish Type Culture Collection, Valencia, Spain) were used throughout this study. Strains were routinely grown on potato-dextrose-agar (PDA, Sigma-Aldrich, Madrid, Spain) in the dark at 28°C and the spores were stored at −80°C in a 20% glycerol solution. Spores were harvested from 7-day-old PDA dishes as previously described Poveda et al. (2019b).

2.2. Plant growth and *Trichoderma* inoculation

Seeds were grown on Murashige and Skoog (MS) (Duchefa, Haarlem, Netherlands) solid medium (agar 1%) with sucrose (1%) in a growth chamber at 22°C, 40% relative humidity (RH), and a 16 h light/8 h dark photoperiod at 80–100 μE m^{-2} s^{-1}, for 10 days. 20 *Arabidopsis* seedlings (per condition) were individually transferred to 0.2 L-pots, containing a mixture of peat/vermiculite (3:1) sterilized by autoclave (twice, 24 hours apart) and maintained in a greenhouse at 22 ± 2°C.

Each plant was inoculated by *Trichoderma* with 1 mL of a conidial suspension containing 2×10^7 spore mL^{-1}, determined using a haemocytometer, one week after the seedlings had been transplanted.

2.3. Abiotic stress and productivity quantification

In order to analyze the response of the different *A. thaliana* plants inoculated with *Trichoderma*, their productivity was analyzed under normal conditions and under saline stress.

Regarding saline stress, *Arabidopsis* plants were watered with a 200 mM NaCl solution every two days, starting from the development of the third true leaf (two-week-old plants)

until the end of its development cycle (11 weeks). The drought stress was developed due to a progressive reduction in watering from the development of the third true leaf (two-week-old plants). The pots were irrigated twice a week in weeks 3 and 4, once a week in weeks 5 and 6, and once every two weeks until the end of the life cycle (weeks 7–11).

The siliques from 15 *Arabidopsis* plants per condition were collected at the end of the life cycle and counted (11 weeks). Three biological replicates were made, with 15 plants per replicate and condition.

2.4. Quantification of *Trichoderma*-root colonization

In order to quantify the differences in root colonization by *Trichoderma* according to the glycosylate profile of the host plant, its quantification was carried out by qPCR.

For the analyses of *Trichoderma*-root colonization, for each *Arabidopsis* mutant and *Trichoderma* inoculant, roots from five plants per each treatment were pooled and root pools from three independent assays were considered. Roots were collected in 5-week-old plants. All root material was washed with water until there was no remaining substrate, immediately frozen with liquid nitrogen, and pulverized with a mortar.

Following the methodology described by Poveda et al. (2019b), DNA was extracted using the cetyl-trimethyl-ammonium bromide (CTAB) extraction method. A mix was prepared in a 10 µl volume using 5 µl of Brilliant SYBR Green QPCR Master Mix (Roche), 10 ng of DNA, the forward and reverse primers at a final concentration of 100 nM, and nuclease-free PCR-grade water to adjust the final volume. The *Actin* genes of *Trichoderma* and *Arabidopsis* were used as reference genes for the calculation of host plant and fungus DNA, and their corresponding primer pairs are indicated in Table 1. Amplifications were performed in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) programmed for 40 cycles under the following conditions: denaturation, 95°C for 15 s; annealing, 60°C for 1 min; extension, 72°C for 1 min. Each PCR was performed in triplicate by using the DNA extracted from the roots collected. Cycle threshold values served to calculate the amount of fungal DNA using standard

curves. Values of *Trichoderma* DNA were referred to the amount of *Arabidopsis* DNA in every corresponding sample, normalizing to 1 the value of Col-0+Th.

2.5. Biotic stress test

In order to analyze the possible differences in resistance to biotic stresses as a consequence of the different levels of root colonization by *Trichoderma* observed, foliar inoculation with a pathogen was carried out.

For the biotic stress test, the phytopathogenic fungal strain *Botrytis cinerea* CECT 20973 (Spanish Type Culture Collection, Valencia, Spain) was used, following the methodology described by Poveda et al. (2019b). The strain was maintained in the same way as in the case of *Trichoderma* strains.

The tests of infection with the necrotrophic fungus *B. cinerea* were performed on leaves of the *A. thaliana* wild-type ecotype Col-0 and the GSLs-mutants, keeping plants in transparent plastic compartments with high humidity (close to 90%). Three leaves of 5 plants were inoculated per condition. The inoculation of this pathogen was carried out by positioning a drop of 5 µl of a germination solution containing 1000 spores of *B. cinerea* (20 mM glucose, 20 mM KH_2PO_4, pH 6.5 adjusted by KOH) in the center of each leaf. The compartments were placed in a light chamber (Fitotron AGP-1400-HR, Radiber SA, Barcelona, Spain) with a photoperiod of 16 h of light (80–100 E/m^2/s) and 8 h of darkness at a temperature of 22°C and a relative humidity of 40%–50%. Data were obtained from three biological replicates with five plants per replicate for each condition, and three leaves per plant.

2.6. Defense-genes expression studies

In order to analyze the defensive responses involved in the results obtained by infection with *B. cinerea*, the expression analysis of defense genes was performed by RT-qPCR.

For gene expression studies, the pooled roots and pools of 2 leaves per plant of 5 plants per condition and repetition were used for RNA extraction with the TRI reagent (Ambion, Austin, TX, USA), following the manufacturer's instructions. cDNA was synthesized from

2 µg of RNA, which was treated with DNase RQ1 (Promega Biotech Ibérica, Alcobendas, Spain), and then used for reverse transcription with an oligo(dT) primer with the Transcriptor First Strand cDNA Synthesis Kit (Takara Bio, Inc., Tokyo, Japan), following the manufacturer's protocol. Gene expression was analyzed by RT-qPCR, using an ABI PRISM 7000 Sequence Detection System with Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA, USA). All PCR reactions were performed in triplicate in a total volume of 10 µL for 40 cycles under the following conditions: denaturation, 95 ◦C, 30 s; annealing, 60 ◦C, 1 min; extension, 72 ◦C, 1 min. Threshold cycles (CT) were determined using the 7000 SDS System Software (Applied Biosystems, Foster City, CA, USA), and CT values were calculated using the *Arabidopsis Actin* gene as an endogenous control. The primers used are given in Table 1: genes of the isochorismate synthase 1 (*ICS1*), pathogenesis-related protein 1 (*PR-1*), lipoxygenase 1 (*LOX1*), and vegetative storage protein (*VSP2*). Data were expressed using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The choice of these defense genes is based on previous studies in *Arabidopsis* (Poveda et al., 2019a, 2019b).

2.7. Vitality test and indirect quantification of ROS in tissues

In order to analyze and quantify the damage produced by the pathogen *B. cinerea* on the leaves of *A. thaliana* in other ways, the viability test was carried out and the ROS produced were quantified indirectly, following the methodology described by Poveda (2020b).

Reduction of triphenyltetrazolium chloride (TTC) by tissue to the red-colored insoluble triphenylformazan (TF) is directly linked to the activity of the mitochondrial respiratory chain. Thus, only living tissues should reduce TTC to TF. From pools formed from 1 leaf per plant and 5 plants per condition and repetition, 100 mg were transferred to 1 mL of 1% TTC in triplicate and incubated for 48 h at 37 °C. After incubation, 100 mg of Ballotini Glass Balls 0.15–0.25 mm diameter and others 100 mg of 1 mm diameter were added to each sample in 1,5 mL Eppendorf tubes, shaking vigorously by a pulse of 20 s in Silamat S6 (Ivoclar Vivadent, Madrid, Spain). After centrifuging the samples for 15 min at 10,000 rpm, the supernatant was removed and 1 mL of isopropanol was added per tube. The samples were again agitated in Silamat and centrifuged in the same way, and the

supernatant was used to quantify its absorbance at 620 nm, being an indirect measure of the vitality of the *A. thaliana*-tissues.

The indirect quantification of reactive oxygen species (ROS) in *A. thaliana*-tissues was carried out by measuring electrolyte leakage, which really measures cellular oxidative damage related to the production of ROS. From each pools formed from 1 leaf per plant and 5 plants per condition and repetition, 1 cm^2 of fresh tissue was briefly mixed with water and floated on 5 mL of double-distilled water for 6 h at room temperature. The conductivity of the water was measured using a Crison™ Conductimeter GLP31 (Crison, Barcelona, Spain). This represented the electrolyte leakage from the tissues (Reading 1). Then, samples were boiled for 20 min at 90 °C. After the liquid cooled down, the conductivity of the water was measured again. This represented the total ions present in the tissues (Reading 2). Electrolyte leakage, an indirect measurement of ROS, was represented as the percentage of total ions released [(Reading 1/Reading 2) × 100].

2.8. Statistical analysis

The statistical analysis of the data was carried out with the Statistix 8.0 software. One-way ANOVA using Tukey's multiple range test at $P < 0.05$ was used for pairwise comparisons (Tukey, 1949); different letters indicate significant differences ($P < 0.05$).

3. Results

3.1. *Arabidopsis* productivity

In plants without stress, inoculation with Th significantly increased the formation of siliques per plant, not observed with the application of Tp in Col-0. No significant differences between Col-0 and the different GSLs-mutants of *A. thaliana* were quantified without the application of *Trichoderma*. In the *cyp*, *myb* and *QKO* mutants, the application of Th also suggested a significant increase compared to non-inoculated plants, without finding differences again in the case of Tp. Furthermore, the increase in productivity quantified in *cyp* and *QKO* mutants with Th is significantly greater than in Col-0 and *myb* with Th. On the other hand, inoculation with Th and Tp did not lead to significant

differences in the number of siliques formed per plant in the case of the *atr1D* mutant (Fig. 1A).

Under salinity stress, *cyp* and *QKO* mutants show a significant decrease in the number of siliques formed per plant compared to Col-0, while *myb* mutant does not show significant differences, and *atr1D* mutant significantly increases them. The application of Th in Col-0 supposes a significant increase in its productivity, being even more significant with the application of Tp. The same results were observed in *myb*, *cyp* and *QKO* mutants, with the latter two being a more significant increase compared to non-inoculated plants. In *atr1D* mutant, the application of *Trichoderma* does not suppose significant differences in the number of siliques that each plant forms (Fig. 1B).

As drought stress is concerned, only *myb* mutant has significantly less silique formation per plant compared to the other mutants. The application of Th in Col-0 significantly increases its productivity, being even greater with the application of Tp, similar results to those obtained with *QKO* mutant. In *cyp* mutant, the application of Th and Tp also supposes an increase in the number of siliques per plant, being significantly greater with Tp, and being in both cases significantly greater than in the case of Col-0. This significant increase after the application of Th is also observed in *myb* mutant, still remaining below the value of Col-0 without inoculating, but equaling it when the fungus applied in *myb* is Tp. *atr1D* mutant does not show significant differences when *Trichoderma* is applied (Fig. 1C).

Trichoderma-roots colonization

Regarding the root colonization of *A. thaliana* by *Trichoderma*, both in Th and Tp (Figure 2A and 2B, respectively) there is a significant increase in the levels of colonization of the *cyp* and *QKO* mutants with respect to Col- 0. On the contrary, in the *atriD* mutant a significant decrease in colonization levels in Th and Tp, with respect to Col-0, is quantified. In the case of the *myb* mutant, there are no significant differences compared to Col-0 in Th and Tp colonization.

Effect of _Trichoderma_ in _Arabidopsis_-roots defense

The RT-qPCR analysis of the expression levels of different genes related to plant defense showed how there are no significant differences between the different mutants of *A. thaliana* in the expression levels of the four genes studied. In Col-0 with both *Trichoderma* species the expression of the genes of synthesis and response to SA (*ICS1* and *PR-1*) increases significantly, and the expression of the genes involved in synthesis and response to JA (*LOX1* and *VSP2*) decreases significantly. Same results were obtained for all the mutants studied, except for the *atr1D* mutant, where no significant differences were observed after the fungal application. Furthermore, in the case of the *cyp* and *QKO* mutants, the increase in the expression of the genes related to SA is even more significant (Fig. 3).

Effect of *Trichoderma* in *Arabidopsis*-leaves defense against biotic stress

Regarding the systemic expression of defense genes in the different mutants of *A. thaliana* inoculated with *Trichoderma*, we could see how there are no significant differences in the expression of the different genes studied between the different mutants. In Col-0, root inoculation with *Trichoderma* significantly increases the expression of JA-related genes (*LOX1* and *VSP2*), this increase being significantly greater with Th than with Tp, in addition, the expression of SA-related genes (*ICS1* and *PR-1*) is significantly reduced. The application of *Trichoderma* supposes a significant increase in the expression of the genes related to JA also in *cyp*, *myb* and *QKO* mutants, being also significantly higher with Th than with Tp, and in the case of *cyp* and *QKO* mutants being significantly higher than in the case of *myb* mutant. With respect to SA, the application of *Trichoderma* also supposes a decrease in the expression of its genes in *cyp* and *QKO* mutants, with no differences between the inoculated and non-inoculated plants in the case of *myb* and *atr1D* mutants. Furthermore, *atr1D* mutant also does not significantly changed the expression of its JA-related genes after *Trichoderma* inoculation.

After foliar infection of *A. thaliana* with the necrotrophic fungus *B. cinerea* we were able to observe how *cyp* and *QKO* mutants were significantly more affected by the pathogen compared to Col-0, while *myb* mutant was similarly affected, and *atr1D* mutant was significantly less affected than Col-0. In Col-0 plants inoculated with Th and Tp, a significant reduction in the diameter of the lesions was observed compared to the plants without inoculation, being even more significant in the case of Th. The same significant

306 increases with the application of *Trichoderma* werere observed in the rest of the *A. thaliana*
307 mutants, observing the same differences between Th and Tp, being significantly higher than
308 those observed in Col-0 in the case of *cyp* and *QKO* mutants, and significantly less in the
309 case of *myb* and *atr1D* mutants (Fig. 5 and Fig. S1).

310 **DISCUSSION**

311 The hypothesis of the fundamental role of glucosinolates in the root colonization of
312 cruciferous plants by endophytic fungi is currently being studied in depth (Poveda et al.,
313 2020d). The ability of *T. harzianum* to colonize the roots of crucifers such as *A. thaliana* or
314 *B. napus* had already been related to its ability to tolerate the presence of GSLs in the roots,
315 thanks to a KELCH-type protein (Poveda et al., 2019b), without deepen the importance of
316 the profile GSLs or *Trichoderma* species used. In our study, we have verified how IGSLs
317 are mainly involved in controlling root colonization by *Trichoderma* in *A. thaliana* roots,
318 since in mutants unable to accumulate them, such as *cyp* and *QKO*, the fungal presence in
319 the roots increases significantly, while in hyperaccumulator mutant (*atr1D*) this is greatly
320 reduced. While the absence of changes in the levels of root colonization in AGSLs-absent
321 mutant (*myb*) suggests that the amount of these does not modify the colonizer-root capacity
322 of *Trichoderma*. These data agree with other studies in which the role of IGSLs as a root-
323 antifungal was highlighted, for example, against the hemibiotrophic *Colletotrichum*
324 *gloeosporioides* (Hiruma et al., 2013) or the necrotrophic *Plectosphaerella cucumerina*
325 (Frerigmann et al., 2016), and against oomycetes as *Phytophthora brassicae* (Schlaeppi et
326 al., 2010). It has even been verified in the *cyp* mutant that the absence of IGSLs causes
327 beneficial fungi, promoters of plant growth in *A. thaliana* such as *Colletotrichum tofieldiae*
328 and *Serendipita indica*, to behave as pathogens as there are no plant defenses that limit the
329 colonization of plant tissues (Hiruma et al., 2018). On the contrary, the ability of IGSLs to
330 promote fungal growth has also been reported in the ectomycorrhizal fungi *Paxillus*
331 *involutus* and *Pisolithus tinctorius* (Zeng et al., 2003). Furthermore, it has been verified
332 how the absence of IGSLs in roots of *A. thaliana* does not modify its defensive capacity
333 against *Fusarium oxysporum* (Kidd et al., 2011).

334 The first effect that we could verify as a consequence of the increased root colonization by
335 *T. harzianum* in *A. thaliana* was a significant increase in the formation of siliques,

therefore, in its productivity, something that did not happen with *T. parareesei*. Exactly these same results were obtained in rapeseed plants, where it was found that *T. parareesei* increases the weight of seeds produced by each plant, to a lesser extent than *T. harzianum* is capable of (Poveda, 2020a). Under abiotic stress conditions such as salinity and drought, we have been able to verify how the mutants incapable of producing IGSLs (*cyp* and *QKO*) presented a lower tolerance to salinity stress, the hyperaccumulative mutant (*atr1D*) being the most tolerant, while the most tolerant to drought situation were the mutants unable to accumulate AGSLc (*myb*). These results agree with those obtained in *Brassica rapa*, where it has been possible to verify how there is a direct relationship between the content of IGSLs and tolerance to salt stress (Steinbrenner et al., 2012) and the content of AGSLs and tolerance to drought (Zhang et al., 2008). Furthermore, after applying *Trichoderma*, we were able to verify that the mutants with the lowest content of IGSLs and, as we had seen, greater capacity to be colonized by the fungus, presented a greater tolerance to both abiotic stresses. Similarly, an increase in the salt-tolerance was observed with the inoculation of tomatoes with *T. harzianum* (Mastouri et al., 2010) and *T. parareesei* (Rubio et al., 2014), and in drought-tolerance with the inoculation of tomato with *T. harzianum* (Mona et al., 2017). Contrary to what happened in a situation without abiotic stress, we observed how *T. parareesei* improved tolerance to both stresses in a significantly better way than *T. harzianum* did. As it could be observed in rapeseed plants, this is due to the presence in *T. parareesei* of a chorismate mutase that improves its root colonization capacity under salinity and drought stresses, and increases the expression of genes related to the hormonal pathways of abscisic acid (ABA) under drought stress, and ethylene (ET) under salt stress (Poveda, 2020a).

At the root level, we have been able to verify how SA is the main defensive hormonal route involved in the control of colonization by *Trichoderma*, as determined by Alonso-Ramírez et al. (2014), reporting how, in the absence of SA, *Trichoderma* colonizes the roots of *A. thaliana* in a massive and uncontrolled way up to the vascular bundles, behaving as a systemic pathogen. In this sense, we quantified a higher response mediated by SA in those plants where colonization by *Trichoderma* was greater, in the same way as Poveda et al. (2019a) determined how the increase in root colonization by *Trichoderma* is linked to an increase in the response by SA to control it.

After foliar infection with the necrotrophic pathogen *B. cinerea*, we verified the importance of IGSLs in the defense of *A. thaliana* against the fungus, since the mutants unable to synthesize them (*cyp* and *QKO*) were the most affected, while the mutant that overdrive (*atr1D*) was the strongest. In this sense, it has been verified how *A. thaliana* susceptibility to *B. cinerea* is increased by reducing the biosynthesis of IGSLs (Cargnel et al., 2014), and how the overexpression of the glucosinolate biosynthesis gene *BnUGT74B1* in *Brassica napus* enhanced resistance to *S. sclerotiorum* and *Botrytis cinerea*, thanks to an increase on IGSLs (Zhang et al., 2015).

After the application of *Trichoderma* we have been able to verify how the harmful effect of the pathogen decreases in all mutants, there being a direct relationship between root colonization by *Trichoderma* and the reduction of the disease. This result would be due to a systemic increase in the expression of JA-related genes as root colonization increases, as other authors had related the plant defensive capacity against *B. cinerea* mediated by JA in *A. thaliana* (Yang et al., 2007; Méndez-Bravo et al., 2011). Furthermore, we observe how *T. harzianum* is more effective than *T. parareesei* in activating systemic resistance against *B. cinerea* in *A. thaliana*. This activation of systemic resistance against *B. cinerea* had previously been verified in *A. thaliana* with *T. harzianum* (De Meyer et al., 1998) and in tomato with *T. parareesei* (Pérez et al., 2015), making in this study the first comparison of activation of systemic resistance between both species of *Trichoderma* on the same plant.

In summary, we have been able to determine how IGSLs are indirectly involved in reducing the ability of *Trichoderma* to colonize the roots of cruciferous plants such as *A. thaliana*, due to the formation of hydrolysis products with broad-spectrum antifungal activity. In this sense, we have been able to relate the levels of root colonization with the ability of *Trichoderma* to promote plant productivity, tolerance to abiotic stresses and resistance to biotic stresses trough JA pathway. Furthermore, determining how *T. parareesei* works better than *T. harzianum* in increasing plant tolerance, but it does worse in the rest of the studied capacities.

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581 **Tab. 1.** Oligonucleotides used in this work.

Code	Sequence (5'-3')	Use
Act-T-F	ATGGTATGGGTCAGAAGGA	Endogenous *Trichoderma* gene
Act-T-R	ATGTCAACACGAGCAATGG	
Act-At-F	CTCCCGCTATGTATGTCGCC	Endogenous *Arabidopsis* gene
Act-At-R	TTGGCACAGTGTGAGACACAC	
ICS1-At-F	GATCTAGCTAACGAGAACGG	Synthesis gene of SA in *Arabidopsis*
ICS1-At-R	CATTAAACTCAACCTGAGGGAC	
PR-1-At-F	GGCTAACTACAACTACGCTG	Response gene to SA in *Arabidopsis*
PR-1-At-R	GGCTTCTCGTTCACATAATTC	
LOX1-At-F	GTAAGCTCTGATGTTACTGATTC	Synthesis gene of JA in *Arabidopsis*
LOX1-At-R	CTGCGGTTAACGACGTGATTG	
VSP2-At-F	GTTAGGGACCGGAGCATCAA	Response gene to JA in *Arabidopsis*
VSP2-At-R	TCAATCCCGAGCTCTATGATGTT	

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584 **Fig. 1**. *A. thaliana* productivity. The number of siliques per plant collected from the
585 different *A. thaliana* lines (Col-0, *cyp*, *myb*, *QKO* and *atr1D*) and the different *Trichoderma*
586 inoculations: *T. harzianum* (+Th) and *T. parareesei* (+Tp), without stress (A), under salt
587 stress (NaCl 200mM) (B) and under drought stress (C).

588 **Fig.2.** Measurements of *Arabidopsis*-root colonization by *T. harzianum* (+Th) (A) and *T.*
589 *parareesei* (+Tp) (B). To quantify *Arabidopsis*-root colonization, the DNA of the fungus
590 was quantified by qPCR from radicular samples using the *Actin* genes from both the plants

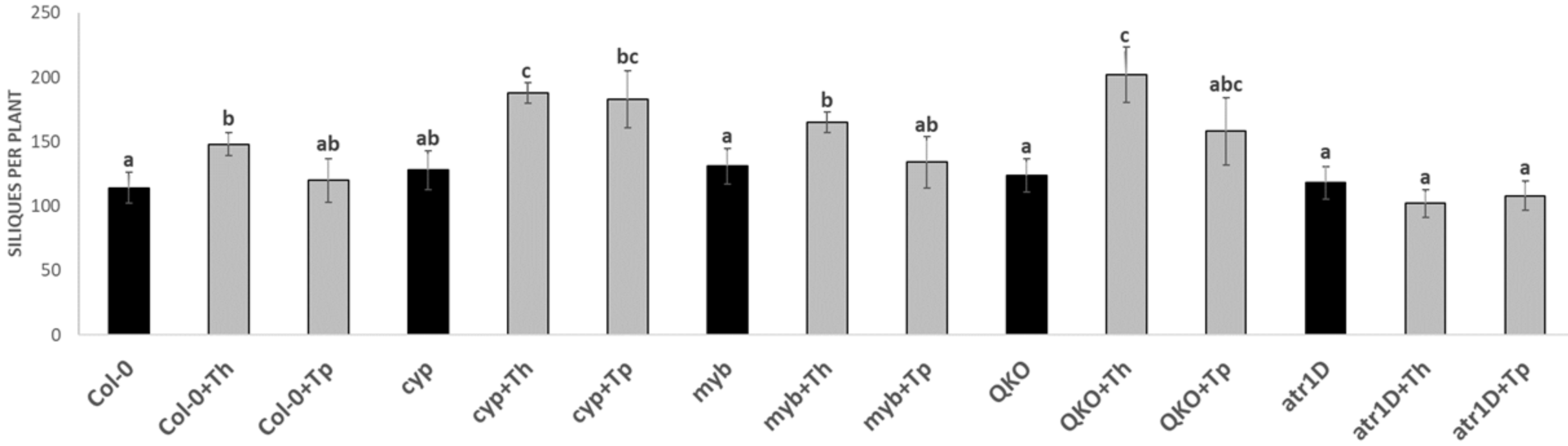
591 and the fungus. Fungal DNA/plant DNA ratio was normalized to 1 in the case of Col-0 and
592 was calculated based on this data for the rest of the lines.

593 **Fig. 3.** Quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis of
594 the expression of some defense genes in the roots of *A. thaliana* lines (Col-0, *cyp*, *myb*,
595 *QKO* and *atr1D*) colonized by *T. harzianum* (+Th) and *T. parareesei* (+Tp). Genes of the
596 isochorismate synthase 1 (*ICS1*), pathogenesis-related protein 1 (*PR-1*), lipoxygenase 1
597 (*LOX1*), and vegetative storage protein (*VSP2*). Values correspond to relative
598 measurements against Col-0 ($2^{-\Delta\Delta Ct} = 1$).

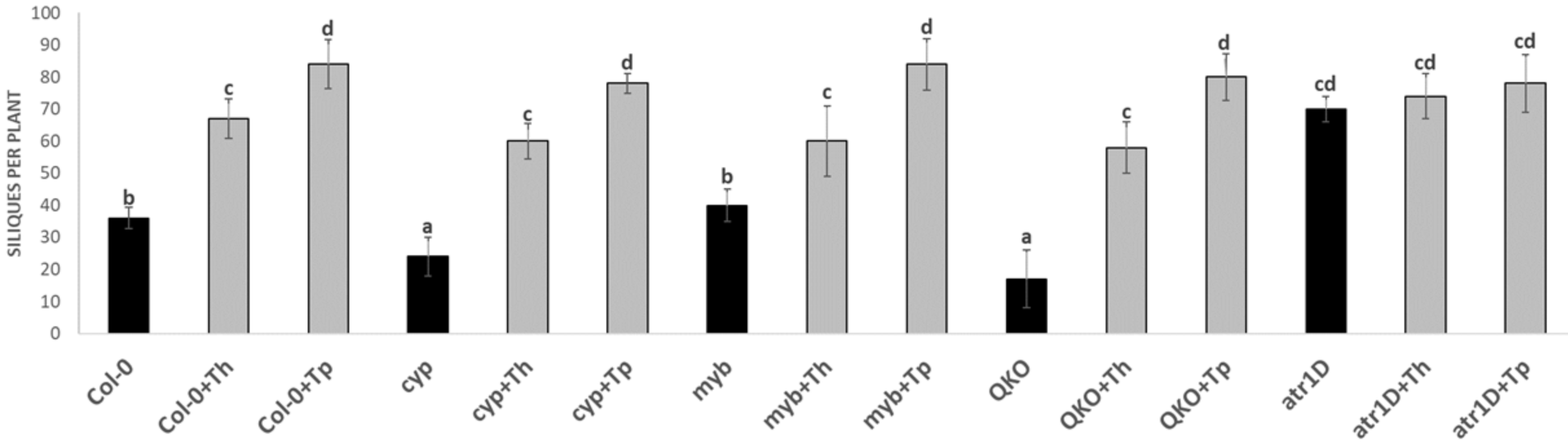
599 **Fig. 4.** Quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis of
600 the expression of some defense genes in the leaves of *A. thaliana* lines (Col-0, *cyp*, *myb*,
601 *QKO* and *atr1D*) root-colonized by *T. harzianum* (+Th) and *T. parareesei* (+Tp). Genes of
602 the isochorismate synthase 1 (*ICS1*), pathogenesis-related protein 1 (*PR-1*), lipoxygenase 1
603 (*LOX1*), and vegetative storage protein (*VSP2*). Values correspond to relative
604 measurements against Col-0 ($2^{-\Delta\Delta Ct} = 1$).

605 **Fig. 5.** *A. thaliana* leaf lesions caused by *B. cinerea*. *In planta* assay performed on *A.*
606 *thaliana* lines (Col-0, *cyp*, *myb*, *QKO* and *atr1D*) which roots are colonized by *T.*
607 *harzianum* (+Th) and *T. parareesei* (+Tp), at 120 h after pathogen inoculation (A). The
608 quantification of the fungal lesions (B) is represented by columns that show the percentages
609 of the diameter (mm) lesion groups. Different letters represent significant differences at $P \leq$
610 0.05 using the non-parametric Friedman's test.

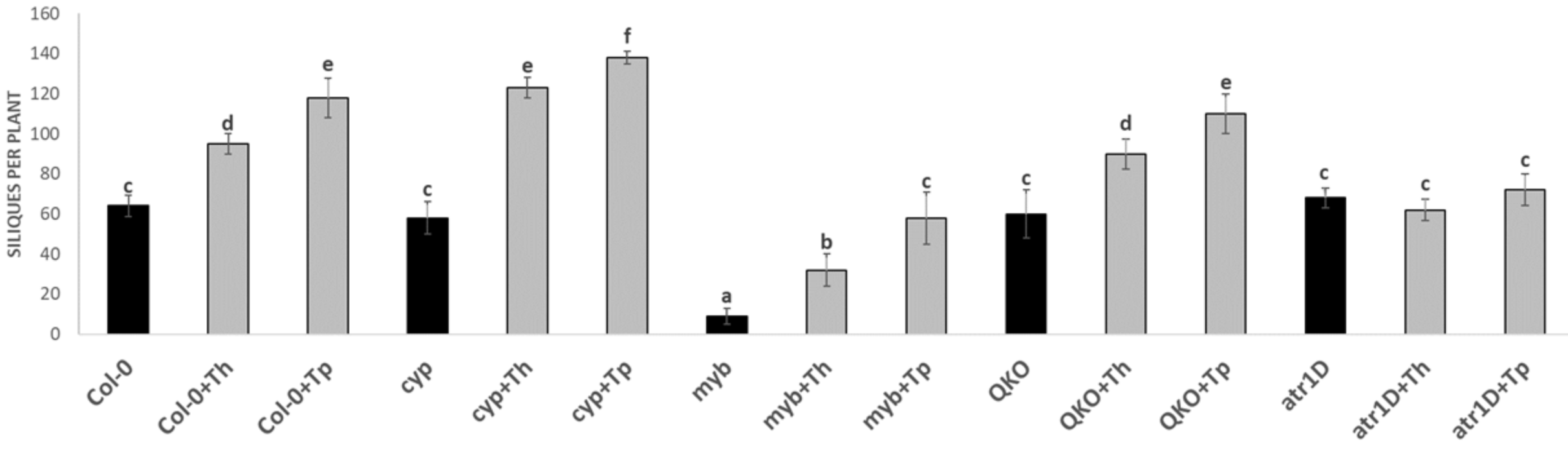
A)

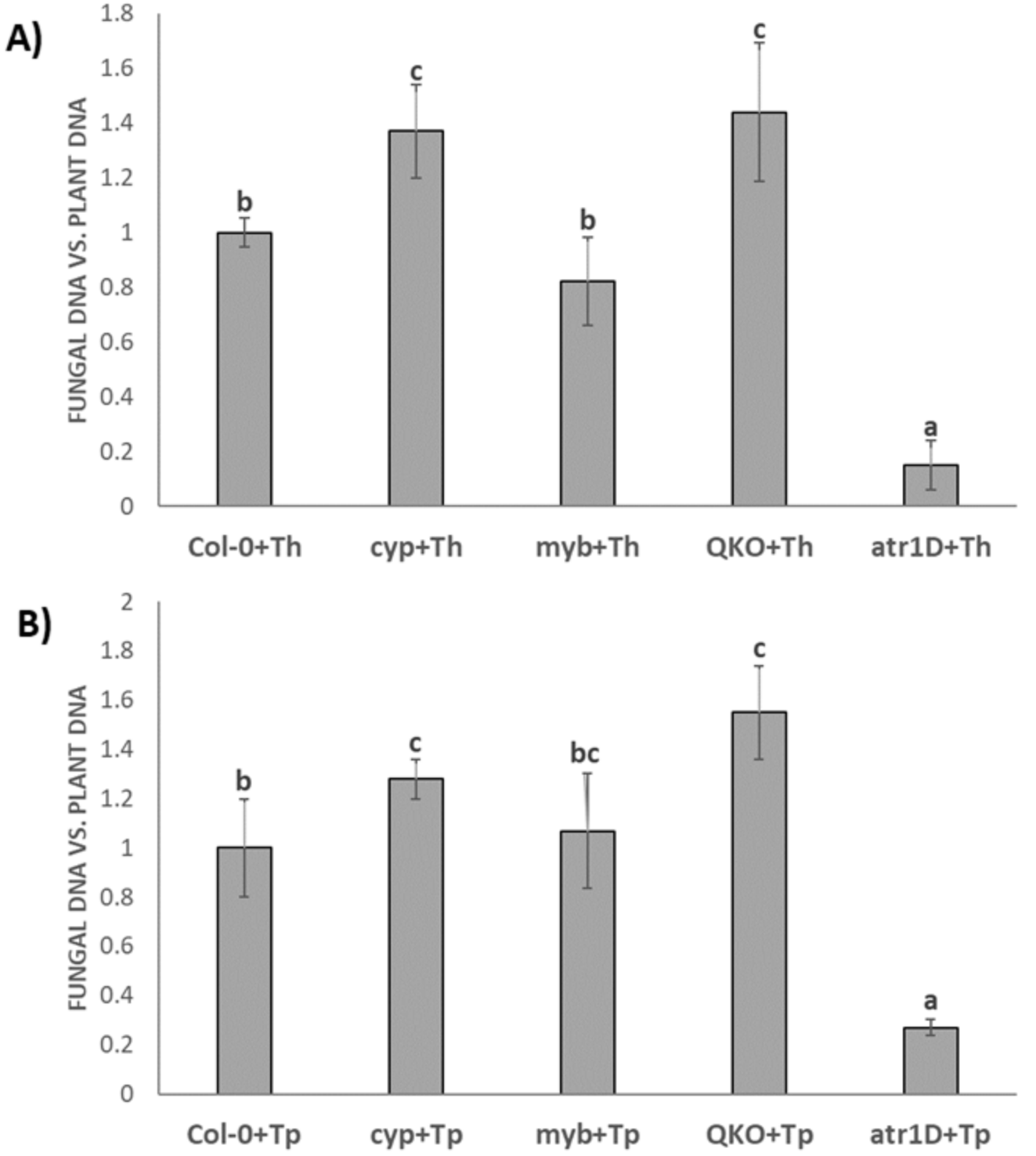

SILIQUES PER PLANT
250
200
150
100
50
0
a
b
ab
ab
c
bc
a
b
ab
a
c
abc
a
a
a
Col-0
Col-0+Th
Col-0+Tp
cyp
cyp+Th
cyp+Tp
myb
myb+Th
myb+Tp
QKO
QKO+Th
QKO+Tp
atr1D
atr1D+Th
atr1D+Tp

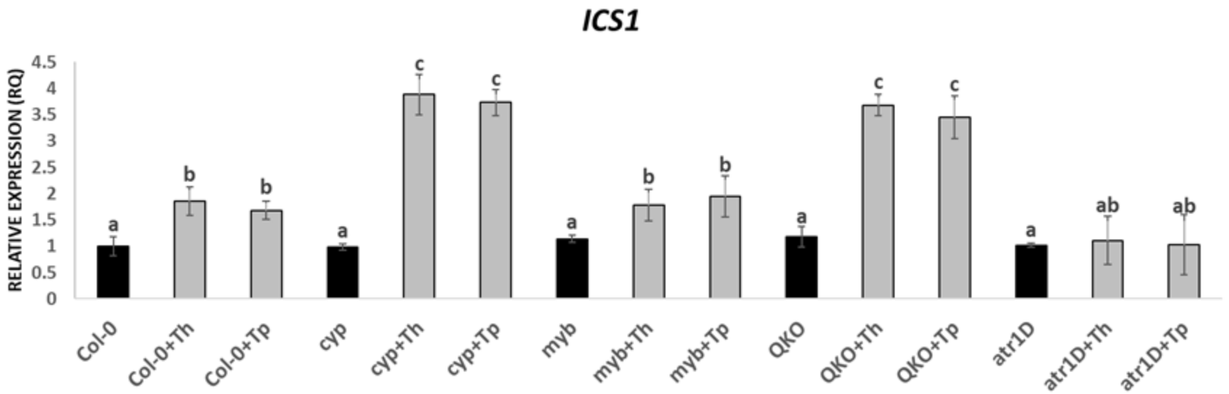
B)

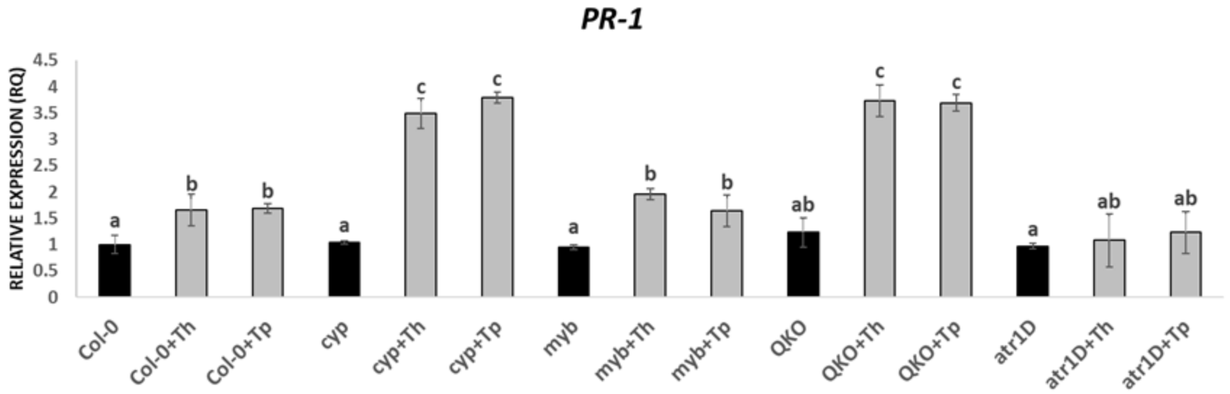

SILIQUES PER PLANT
100
90
80
70
60
50
40
30
20
10
0
b
c
d
c
a
c
d
b
c
d
a
c
d
cd
cd
cd
Col-0
Col-0+Th
Col-0+Tp
cyp
cyp+Th
cyp+Tp
myb
myb+Th
myb+Tp
QKO
QKO+Th
QKO+Tp
atr1D
atr1D+Th
atr1D+Tp

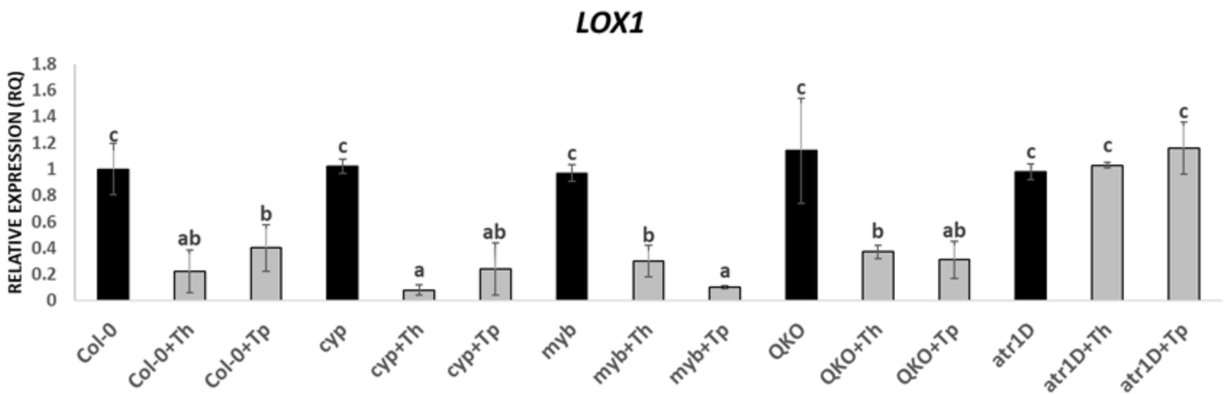
C)

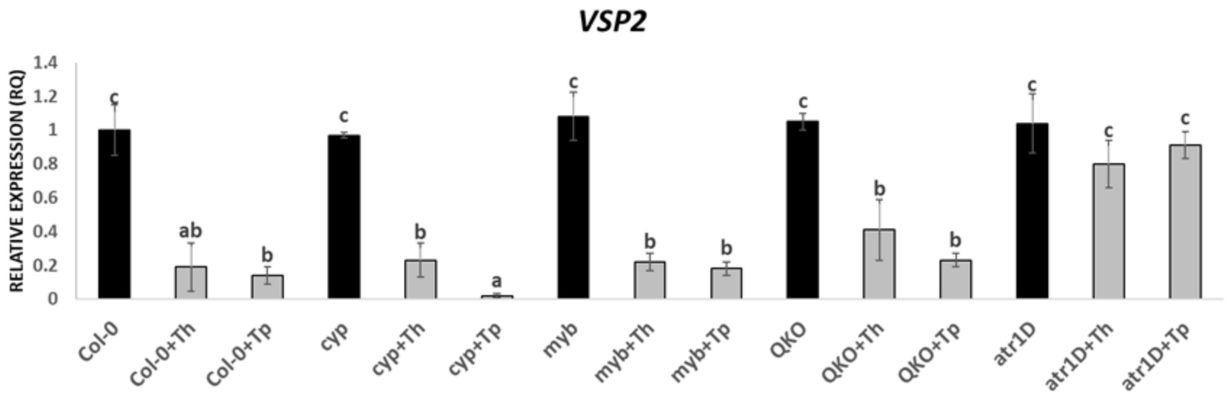

SILIQUES PER PLANT
160
140
120
100
80
60
40
20
0
c
d
e
c
e
f
a
b
c
c
d
e
c
c
c
Col-0
Col-0+Th
Col-0+Tp
cyp
cyp+Th
cyp+Tp
myb
myb+Th
myb+Tp
QKO
QKO+Th
QKO+Tp
atr1D
atr1D+Th
atr1D+Tp

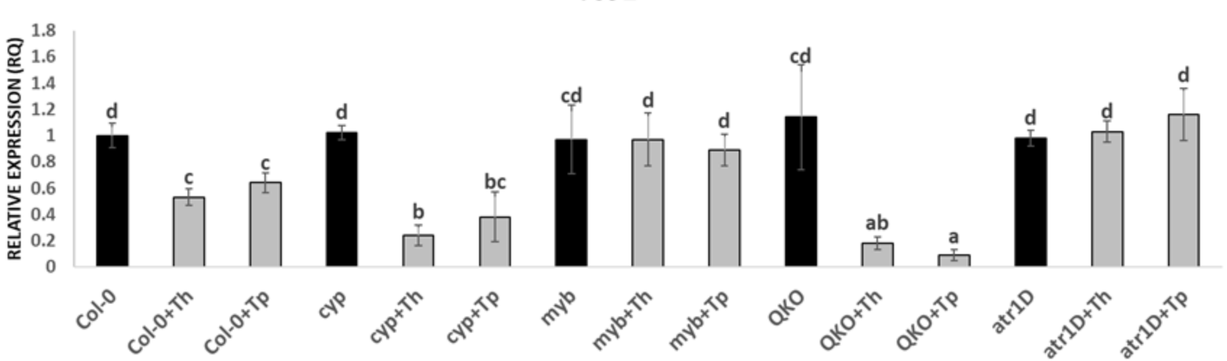

A)
FUNGAL DNA VS. PLANT DNA
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
b
c
b
c
a
Col-0+Th
cyp+Th
myb+Th
QKO+Th
atr1D+Th
B)
FUNGAL DNA VS. PLANT DNA
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
b
c
bc
c
a
Col-0+Tp
cyp+Tp
myb+Tp
QKO+Tp
atr1D+Tp

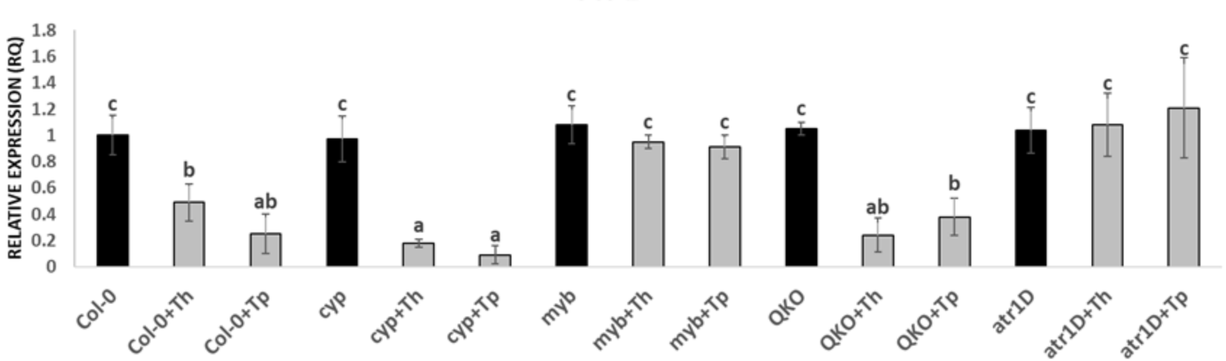

ICS1
RELATIVE EXPRESSION (RQ)
Col-0
Col-0+Th
Col-0+Tp
cyp
cyp+Th
cyp+Tp
myb
myb+Th
myb+Tp
QKO
QKO+Th
QKO+Tp
atr1D
atr1D+Th
atr1D+Tp

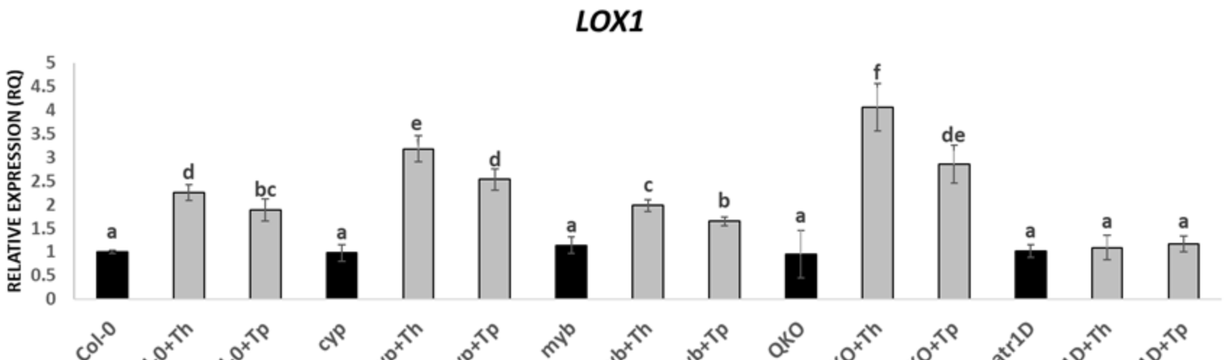

PR-1
RELATIVE EXPRESSION (RQ)
Col-0
Col-0+Th
Col-0+Tp
cyp
cyp+Th
cyp+Tp
myb
myb+Th
myb+Tp
QKO
QKO+Th
QKO+Tp
atr1D
atr1D+Th
atr1D+Tp

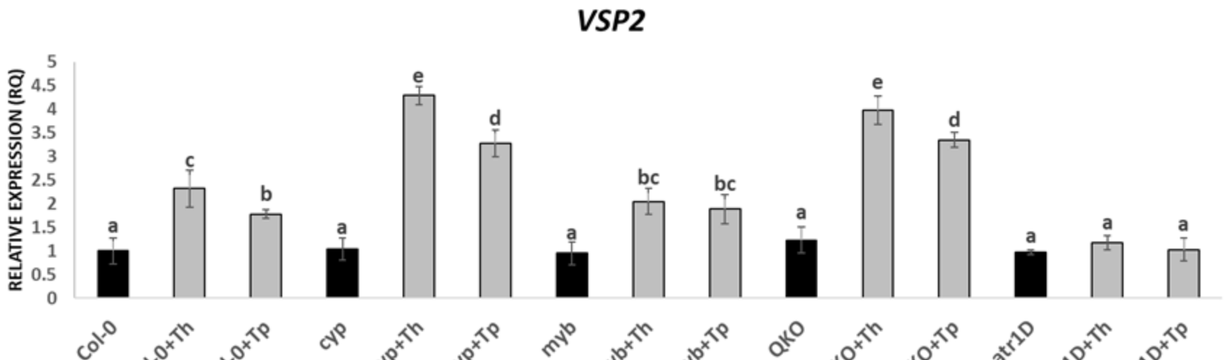

LOX1
RELATIVE EXPRESSION (RQ)
Col-0
Col-0+Th
Col-0+Tp
cyp
cyp+Th
cyp+Tp
myb
myb+Th
myb+Tp
QKO
QKO+Th
QKO+Tp
atr1D
atr1D+Th
atr1D+Tp


VSP2
RELATIVE EXPRESSION (RQ)
Col-0
Col-0+Th
Col-0+Tp
cyp
cyp+Th
cyp+Tp
myb
myb+Th
myb+Tp
QKO
QKO+Th
QKO+Tp
atr1D
atr1D+Th
atr1D+Tp

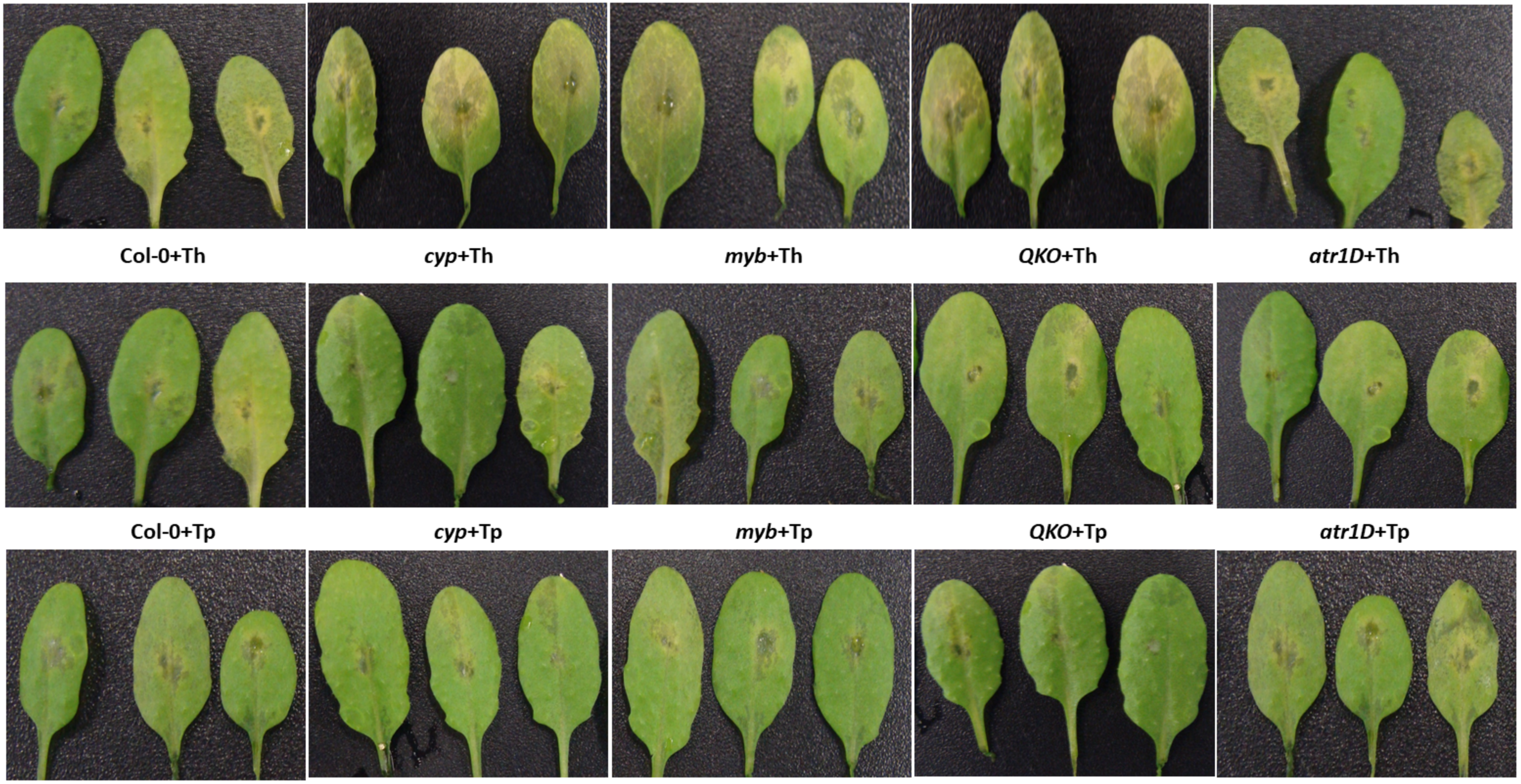

ICS1
RELATIVE EXPRESSION (RQ)
Col-0
Col-0+Th
Col-0+Tp
cyp
cyp+Th
cyp+Tp
myb
myb+Th
myb+Tp
QKO
QKO+Th
QKO+Tp
atr1D
atr1D+Th
atr1D+Tp


PR-1
RELATIVE EXPRESSION (RQ)
Col-0
Col-0+Th
Col-0+Tp
cyp
cyp+Th
cyp+Tp
myb
myb+Th
myb+Tp
QKO
QKO+Th
QKO+Tp
atr1D
atr1D+Th
atr1D+Tp

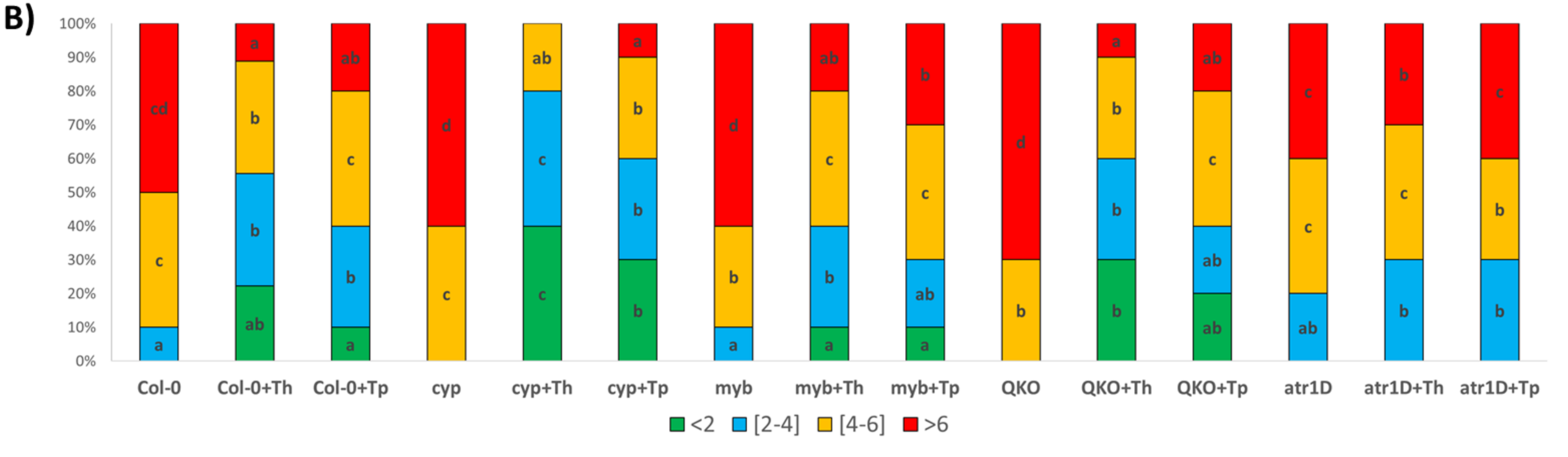

LOX1
RELATIVE EXPRESSION (RQ)
Col-0
Col-0+Th
Col-0+Tp
cyp
cyp+Th
cyp+Tp
myb
myb+Th
myb+Tp
QKO
QKO+Th
QKO+Tp
atr1D
atr1D+Th
atr1D+Tp


VSP2
RELATIVE EXPRESSION (RQ)
Col-0
Col-0+Th
Col-0+Tp
cyp
cyp+Th
cyp+Tp
myb
myb+Th
myb+Tp
QKO
QKO+Th
QKO+Tp
atr1D
atr1D+Th
atr1D+Tp

A)


Col-0
cyp
myb
QKO
atr1D
Col-0+Th
cyp+Th
myb+Th
QKO+Th
atr1D+Th
Col-0+Tp
cyp+Tp
myb+Tp
QKO+Tp
atr1D+Tp

B)


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Col-0
Col-0+Th
Col-0+Tp
cyp
cyp+Th
cyp+Tp
myb
myb+Th
myb+Tp
QKO
QKO+Th
QKO+Tp
atr1D
atr1D+Th
atr1D+Tp
<2
[2-4]
[4-6]
>6