Strigolactone profiles in Sorghum bicolor: in relation to arbuscular mycorrhizal fungal symbioses

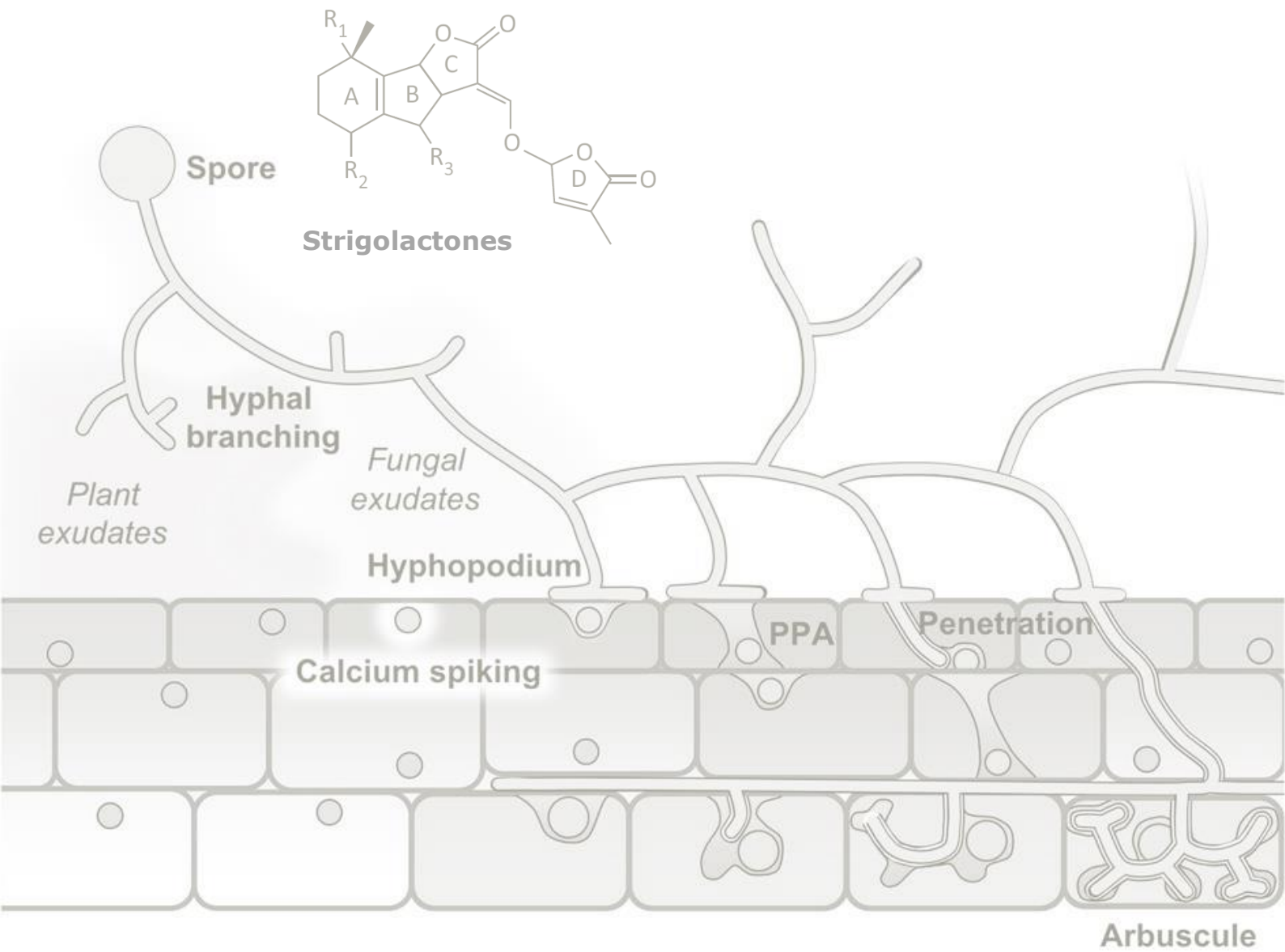

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Table of Contents

1. Summary

The interaction between arbuscular mycorrhizal (AM) fungi and strigolactone profiles of Sorghum bicolor was studied in two genotypes of sorghum that are resistant (SRN 39) and susceptible (Shanqui red) to Striga spp. Both sorghum genotypes, with different strigolactone profiles, were capable of inducing AM symbiosis with similar efficiency. Colonization by Glomus intraradices was higher than Glomus custos and Glomus clarum and no significant differences in symbiosis were found between single and mixed inoculations, so no competition between AM fungal strains was shown. More pronounced differences in colonization level, plant height and plant biomass above- and belowground, between treatments are expected after growing plants for a longer time.
Secondly we searched for the homolog of the Petunia strigolactone transporter gene PDR1 in the two sorghum genotypes to explore whether allelic differences are responsible for the differences in the observed strigolactone exudation profiles. The two most likely candidates were identified but other putative candidates still have to be ruled out before functionality testing.

2. Introduction

2.1 Parasitic plants in Sorghum bicolor

A small portion of all plant species has the ability to parasitize on other plants. Parasitic plants can be divided into facultative and obligate parasites. *Orobanche* spp. and *Striga* spp. are two examples of the latter, and can thus only survive if they parasitize on a host plant [4]. The seeds of both parasites are generally dormant and are viable in the soil for 15-20 years before germinating upon host stimuli [5, 6]. The parasitic plants rely on the host plant to obtain water, assimilates and nutrients necessary for growth and development [4].
While *Orobanche* spp. does parasitize crops like legumes, crucifers, tomato and sunflower in the more temperate regions, *Striga* spp. mainly infests cereal crops in Africa. In West Africa more than 60% of the cereal growing areas are infested [3] and *Striga* spp. may cause yield losses in cereal crops up to 100% [6]. *Sorghum bicolor* is one of those infested cereal crops. It is a widely grown crop, particularly in the semi-arid tropics of Africa and India, which are characterized by high temperatures and low rainfall [7]. As *Striga* spp. has most of its life cycle underground it is difficult to diagnose infestation before emergence, at which point damage has already been done [8]. Many different sorghum genotypes have been screened and differences in susceptibility against Striga were found. Some genotypes such as Shanqui red are highly susceptible while others as the SRN39 cultivar are resistant in the field [9, 10].

Due to its nature as an obligate parasite, *Striga* spp. seeds predominantly germinate upon sensing host stimuli. Strigolactones were found to be the compounds increasing germination in the seeds of parasitic plants [11]. Strigolactones are a group of carotenoid-derived compounds. They typically consist out of four rings (Figure 1.), A-D, where the C-D part is highly conserved due to the biological activity of the enol ether bridge [2, 11].

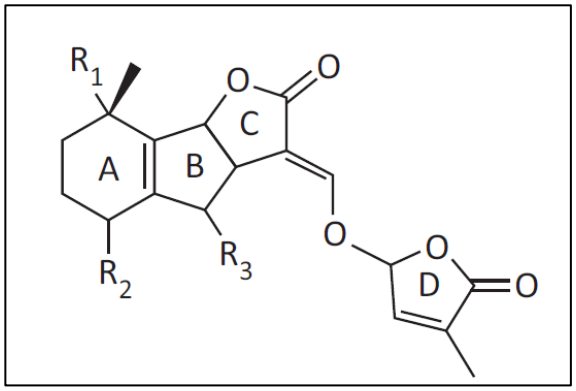

Figure 1. General structure for strigolactones. From: Ruyter-Spira *et al.*, 2013 [2].

It is this enol ether bridge that plays an important role in the germination of *Striga spp.* seeds [11]. Besides their important role in the germination of striga seeds, strigolactones also have other functions that are more advantageous for the plant. For example, the main reason for producing and exuding strigolactones is to attract and induce root colonization by arbuscular mycorrhizal (AM) fungi [12], which provide the plant with nutrients such as phosphorous or nitrogen under limiting conditions.

Next to AM fungi, nodulation by symbiotic rhizobia is positively affected as well by strigolactones, although not via a direct effect on the bacteria [1]. Furthermore strigolactones are a 'new' class of hormones regulating above and below ground architecture by inhibiting branching of roots and shoots [13-15]. All those advantages, could be an explanation for the fact that plants did not lose the strigolactone biosynthesis via evolution even though there is a risk of getting infected by parasitic plants.

2.2 Strigolactone biosynthesis, downstream signaling and transportation

Many steps are involved in the biosynthesis of strigolactones, first of all the 2C-methyl-D-erythritol 4-phosphate (MEP) pathway. This pathway, active in the plastids, is responsible for the production of IPP and DMAPP, the substrates for isopropene, monoterpenes, diterpenes and tetraterpenes (carotenoids) [16]. Three units of IPP with DMAPP provide geranyl-geranyl diphosphate (GGPP) with the help of GGPP synthase [17, 18]. Then via multiple enzymes all-*trans*-β-carotene is formed. Via D27 this is subsequently converted to 9-*cis*-β-carotene, from which the intermediate carlactone is formed by carotenoid cleavage dioxygenases (CCDs). By a Cytochrome P450 (Max1) and perhaps some other enzymes, carlactone is then converted into 5-deoxystrigol [2] the precursor of all other strigolactones (Figure 2. and 3.). Nowadays 15 different natural strigolactones have been structurally characterized in plants, however several new strigolactones are currently being isolated and characterized [2].

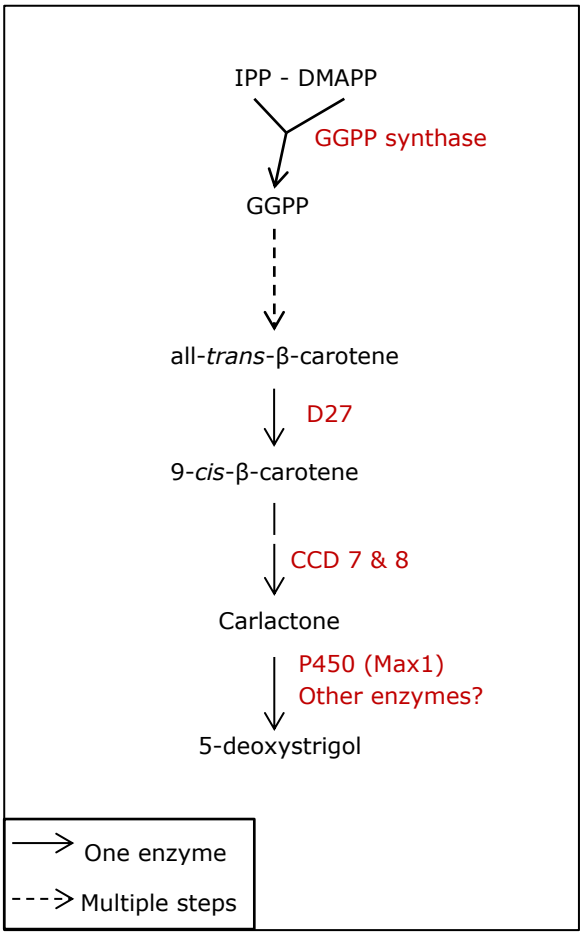

Figure 2. Strigolactone biosynthesis pathway. The arrows represent the enzymatic steps. Names of enzymes are shown in red. Adjusted from Bouwmeester *et al.*, 2007 and Ruyter-spira *et al.* 2013 [2, 3].

After biosynthesis the strigolactones are transported throughout the plant and a part of it is then exuded with the help of PDR1 transporters from the roots to the rhizosphere. The PDR1 transporter is part of the ATP-binding cassette transporter family, which is known for phytohormone translocation, and was first found to be involved in strigolactone transport in *Petunia* and to be up regulated during phosphate deficiency, application of synthetic strigolactone GR24 and/or colonization by AM fungi [19].

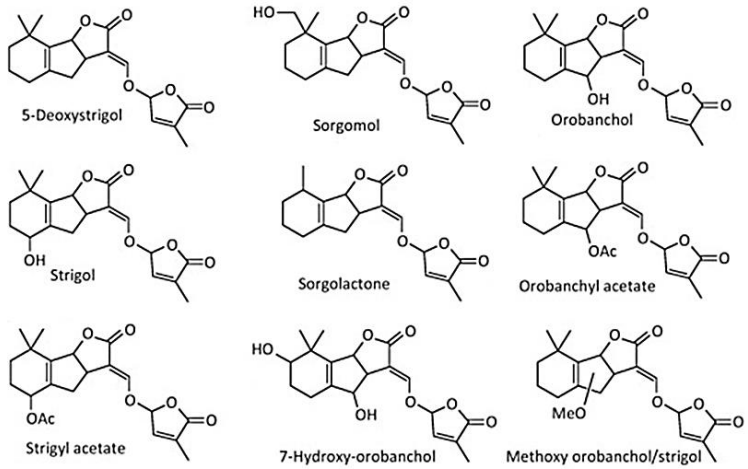

Figure 3. Structure of 5-deoxystrigol and eight of its derived strigolactones.
Adjusted from Ruyter-spira et al., 2013 [2]

Once the strigolactones enter the rhizosphere multiple things can happen, they encounter spores of AM fungi (1) or Striga spp. seeds (2) and induce their germination or they are degraded (3) before encountering either the seeds or the spores. It is in this frame that the study will take place, with the focus on the interaction between strigolactones and AM fungi.

AM fungi are obligate biotrophs, meaning that when no host is sensed nearby during their germination the fungi will retract their cytoplasm and become dormant until they restart their germination [20]. The fungi will provide the plant with water, phosphate and nitrogen in return for carbon [12, 21]. In a P- (and N-) deficient environment plants start to exude more strigolactones to attract AM fungi for symbiosis[8]. Upon detection of the exuded strigolactones a pre-symbiotic stage is induced in the fungus, which is characterized by hyphal branching of the germinated spores. In addition to hyphal branching strigolactones may stimulate spore germination or act as a chemo attractant to direct the AM hyphae towards the roots [1]. Once activated AM fungi produce Myc factors, that help induce the common symbiotic pathway in plants, stimulating AM establishment [1, 20]. AM fungi select the location for root penetration upon which they form a hypopodium/appressorium. The hyphae then enters the cell and forms highly branched exchange structures called arbuscules. A perifungal membrane (secreted by the plant) follows the surface of the arbuscule branches to keep plant cell integrity and creates a site for nutrient exchange. The arbuscules have a lifespan of 4-5 days until they shrink and disappear from the cell and colonization of new cells takes place [20]. Inorganic Phosphorous is taken up by the hyphae of the AM fungi and quickly accumulates in the vacuole where it is converted to polyphosphates (Figure 4.). The polyphosphates are then transported from the extraradical to the intraradical hyphae, where subsequently they are hydrolized for P release to the host plant [20, 21].

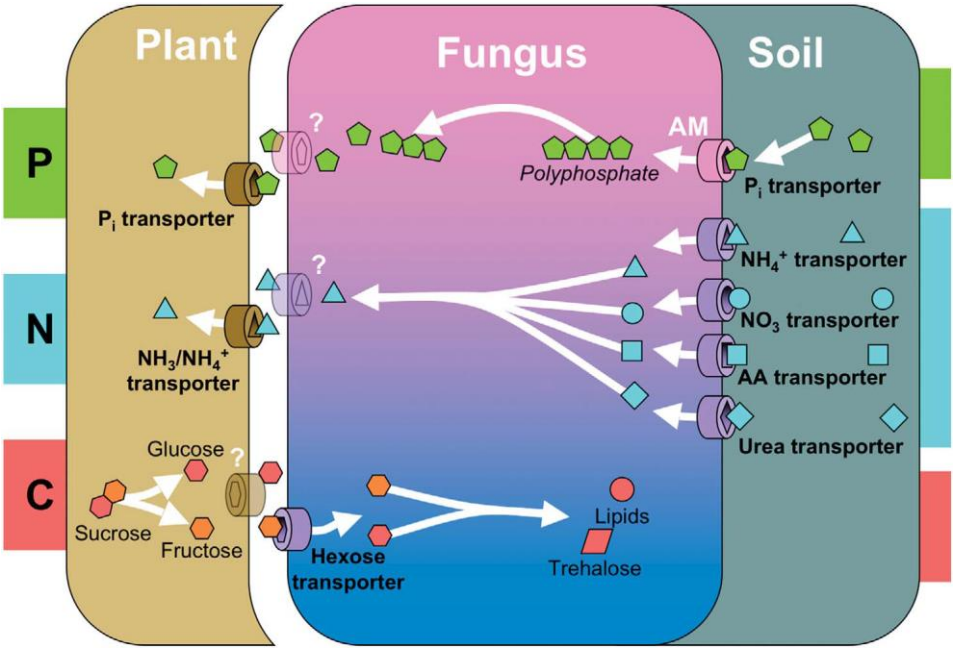

Figure 4. Inorganic P, mineral forms of N and amino acids are taken up at the Soil-fungus interface. Phosphorus is transported by the fungus as polyphosphates and is imported as Pi from the symbiotic interface to the plant cells through selective transporters. In return Carbon is transported from the plant to the fungus in the form of sugars. From: Bonfante and Genre, 2010 [20]

As it is costly for the plant or AM fungi to provide the other with nutrients it has been suggested by Kiers *et al.* (2011) that one of them might try to deflect from providing nutrition. It was indeed found that *Glomus custo*s takes carbon from its host but gives less P in return than the cooperative strain *Rhizophagus irregularis* (formerly known as *Glomus intraradices*). According to the theory of a 'mutualism market' both the host plant and fungi sense where resources are supplied and adjust their own supply accordingly. This was confirmed in *Medicago Truncatula* by a 85% decrease in *G. custos* abundancy when inoculated together with *G. intraradices* [22]. Due to this mechanism of sensing supplies and having multiple partners at a time, none of the partners is enslaved by the other, creating a mutualistic balance.

Not only differences in fungal cooperation but also differences in strigolactone exudation profiles between sorghum genotypes may influence whether and how well symbiosis is established. The differences in strigolactone exudation profiles could be either qualitative (type of strigolactone) and/or quantitative (amount of strigolactones exuded) differences. The difference in exudation profile between multiple sorghum genotypes is most likely due to different biosynthesis profiles and as we hypothesize due to a difference in functionality and specificity of the PDR1 transporters for certain strigolactones. The differences in exudation profile are thought to explain the difference in susceptibility to *striga* spp. As mentioned before sorghum genotypes such as Shanqui red are highly susceptible while others such as SRN39 are resistant to *Striga* spp. [9, 10, 23]. In earlier research (unpublished), Shanqui red was found to mainly exude 5-deoxystrigol, while SRN39 exuded the strigolactone orobanchol. Satish *et al.*(2012) stated that the difference in susceptibility towards *striga* spp. is due to the fact that low stimulant activity accessions such as SRN39, contain mostly unstable hydroxy-strigolactones like orobanchol [23]. Strigolactones are found to be instable in watery solutions and the presence of a hydroxyl group makes them less stable than other strigolactones like 5-

deoxystrigol. As strigolactones are exuded in the nano/pico range, hydroxyl-strigolactones may be degraded and lose their biological activity before encountering and thereby inducing germination of *Striga* seed in the field [8, 23].
A second explanation for the difference in susceptibility of SRN39 and Shanqui red to *Striga* spp. is the biological specificity of strigolactone perception by *Striga* spp.
It could very well be that *Striga* spp. does not recognize or misses the downstream signaling for orobanchol while it does react to 5-deoxystrigol, thus explaining the difference between the genotypes in susceptibility.

2.3 Research objective

Strigolactones play an important role in the infestation and colonization by parasitic plants and AM fungi (Figure 5.). The strigolactone 5-deoxystrigol is one of the strigolactones found to be responsible for the increase in hyphal branching of AM fungi in a petri dish (Figure S1)[24]. However, 5-deoxystrigol is also found to be present at higher levels in the *Sorghum* genotypes that are more susceptible to *Striga* spp. than in the resistant genotypes[23], therefore the question arises whether the resistant genotypes, with different strigolactone exudation profiles, are still capable of inducing colonization by AM fungi. Differences in type and amount of exuded strigolactones may influence efficiency of AM fungal colonization. By inoculating both the highly susceptible line shanqui red and the resistant line SRN39 with *G. intraradices* (cooperative), *G. custos* (less cooperative), *G. clarum* and a mixture of the first two and all three, this question will be answered. The type and/or amount of strigolactones are expected to be different for the two lines and might thus have a differential effect on symbiosis by the AM fungi.

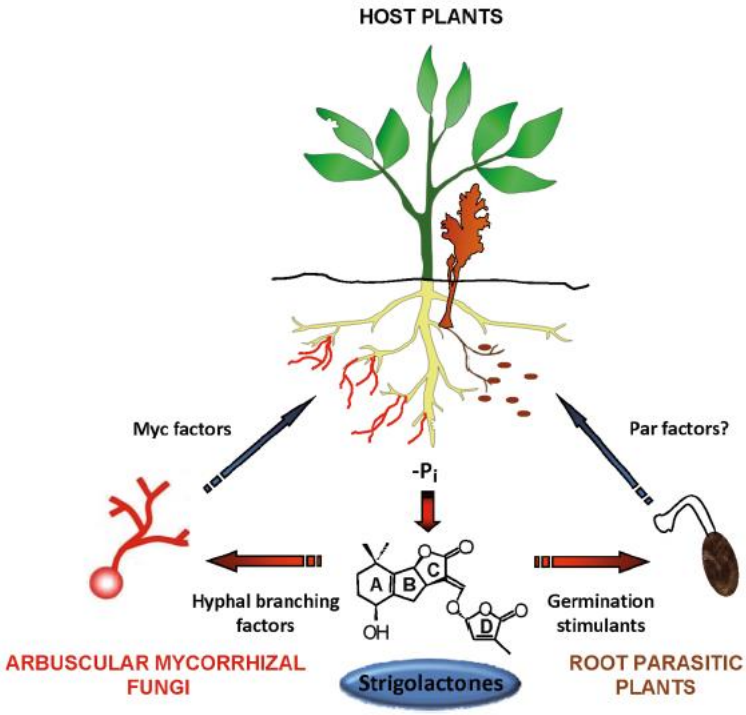

Figure 5. Rhizosphere communication between plants, AM fungi and root parasitic plants. Strigolactones stimulate germination in root parasitic plants and induce hyphal branching in AM fungi. From: López-ráez *et al.*, 2011 [1]

Subsequently the question whether a putative allelic difference in PDR1 transporter, between SRN39 and Shanqui red, is responsible for the previously found differences in strigolactone exudation profiles (mainly orobanchol and 5-deoxystrigol respectively) or not, will be dealt with. It has been argued (personal communication) that SRN 39 has a mutation which allows it to make orobanchol instead of producing mainly 5-deoxystrigol.

This would have necessitated a change in the specificity of the PDR1 transporter. The PDR1 gene from both shanqui red and SRN39 will be identified. After this it will have to be cloned and expressed, together with all genes belonging to the strigolactone pathway, in *N. benthamiana,* to produce the respective strigolactones and to see whether the PDR1 transporter alleles are causing differential strigolactone exudation patterns.

3. Materials and methods

3.1 AM fungi

In order to test the influences of strigolactone profiles in *Sorghum bicolor* on AM fungi symbiosis, the two different *S. bicolor* lines shanqui red and SRN39 were grown at low Phosphate concentration (100mM) in the presence of *G. intraradices*, *G. custos, G. clarum* or a mixture thereof (1500 spores in total).
Five week old plants were harvested and analyzed for their strigolactone profile, AM fungal colonization and to detect aberrations in growth due to a lack of phosphate. Biomass of roots and shoots was determined to see whether plants fall behind in growth due to Phosphate limitations and to investigate the effect that AM fungal symbiosis has on this. Roots were studied under a microscope to detect the arbuscular, vesicular and total colonization percentage by AM fungi. To determine which AM fungi have colonized the roots and in which amount/ratio they are present a qPCR experiment was performed. The strigolactone profile was analyzed for both the roots and the exudates by LC-MS to re-evaluate the previously observed difference in strigolactone biosynthesis and exudation between the two sorghum lines.

3.1.1 Growing and harvesting of Sorghum plants

Seeds of multiple sorghum genotypes were surface sterilized with bleach for ten minutes under occasional turning. The sterilized seeds were then washed and incubated on a moist filter paper for 48 hours in the dark at 25℃ to germinate. Subsequently the plants were grown under an eleven hour light regime at a temperature of 28 ℃ and a humidity of 56%.
In order to compare the influence of multiple AM fungal treatments on the strigolactone exudation profile and the effect of these profiles on AM fungal symbiosis both SRN39 and Shanqui red seedlings were grown in silversand, which was autoclaved to avoid contamination by soil fungi. The seedlings are watered with half-strength Hoagland solution (full phosphate, 1.96mM) for ten days and were then transferred to a bigger pot (14 cm ø, 1 seedling per pot) and inoculated with 1500 spores per plant of *G. intraradices, G. custos, G. clarum,* a combination of *G. intraradices* and *G. custos* or a mix of all three AM fungal strains. Control plants were mock treated by dripping 2 ml solution without spores on the roots with a syringe. In the combined treatments the total number of spores was kept constant and for each treatment ten replicates were taken and 5 for each control (no AM fungi). To rule out a place effect in the climate chamber a complete randomized block design was used.
The plants were then grown for five weeks and watered with ½ Hoagland solution with 100 µM phosphate. Fourty-eight hours before harvesting pots were flushed with 1 L nutrient solution to wash away all exuded strigolactones from the soil. Upon harvesting pots were again flushed but this time exudates were collected. Shoot height and shoot and root biomass were determined. Roots were cut in 1-2cm long pieces and approximately half of it was distributed over 50% ethanol (microscopy) or frozen in liquid nitrogen (qPCR and LC-MS). For the remaining roots and the shoot the dry weight was determined by drying 48 hours at 80 °C.

3.1.2 LC-MS

Liquid chromatography- mass spectrometry (LC-MS) was used to determine the strigolactone profile for both Sorghum root exudates and root extracts. Five replicates per genotype*treatment combination were used.

3.1.2.1 Extraction and purification of sorghum root exudates and extracts

In order to measure strigolactones on the LC-MS they first need to be extracted from the plant. Exudates are already in the soil and can be easily captured from the soil by flushing with 1 Liter of nutrient solution. The strigolactones were concentrated over a C18 column (500mg/3ml; Grace Davison Discovery Sciences) with a vacuum pump, simultaneously getting rid of the very polar compounds, and eluted in 4ml 100% acetone. To two ml of those samples the internal standard D6-5-deoxystrigol was added to get a concentration of 100pmol after final purification. The samples were then dried with a speedvac (Thermo Scientific) and dissolved in 50µl ethyl acetate with subsequent addition of 4ml hexane. To extract the strigolactones still trapped in the roots, 2ml of ethyl acetate containing D6-5-deoxystrigol was added to 500 mg of ground root material and sonicated for 15 minutes. The organic phase was carefully transferred and the roots were re-extracted with another 2ml of ethyl acetate. The total four ml of collected extracts were then dried with a speedvac and dissolved in 50µl ethyl acetate and 4ml of hexane. Both extracts and exudates were then purified over a Silica column (200mg/3ml; Grace Davison Discovery Sciences) to get rid of the very a-polar compounds. Elution was done in two fractions of 2ml Hexane:Ethyl acetate (10:90) and 2ml Methanol:Ethyl acetate (10:90). The fraction eluted in Hexane:Ethyl acetate (10:90) was then dried with a speedvac and dissolved in 200 µl of 25% acetonitrile in water and filtered with a 0.45µm minisart syringe filter (Sartorius).

3.1.2.2 Strigolactone analysis using Liquid Chromatography- tandem Mass Spectrometry

Characterization and quantification of strigolactones in sorghum root extracts and exudates was done with ultra-performance liquid chromotography coupled to tandem Quadrupole mass spectrometry (UPLC-MS/MS). Analyses were performed using an Acquity UPLC system (Waters), coupled to an Xevo TQ MS mass spectrometer (Waters). Chromatographic separation was achieved using an Acquity UPLC BEH C18 column (1.7 µm, 100 x 2.1 mm; Waters) applying a water-acetonitrile gradient. Separation started at 5% acetonitrile for 40 seconds, followed by a 8 min. gradient to 90% acetonitrile, which was then maintained for 0.5 min, followed by a 0.2 min gradient back to 5% acetonitrile. The column was equilibrated at this solvent composition for 2.5 min before the next run. The flow rate was 0.5 ml/min (sample injection volume of 20 µl) and the mass spectrometer was operated in positive ionization mode. The collision energy and cone voltage were optimized for each compound. An overview of all MRM channels measured and their corresponding cone and collision voltage can be found in table S3. Data acquisition and analysis were performed using masslynx 4.1 software (Waters).

3.1.2.3 Spiking

In order to see whether losses in IS concentration are due to low recovery or ion suppression a spiking experiment was performed. The principle of this method is to measure the quantity of a hormone, in this case 5-Deoxystrigol, Orobanchol and Strigol, in three different samples; external standard plus solvent, matrix plus solvent and matrix plus external standard. In case the measured peak area of the last sample is the sum of the first two samples there are no problems with ion suppression and all losses of IS are due to recovery of the sample after extraction. The discrepancy between the sum in peak area of standard and matrix together and the combined sample is a measure for the amount of ion suppression or instability of the compound in a certain matrix.

3.1.3 Microscopy

To determine the colonization percentage of the AM fungi in the roots, the 1-2 cm long root pieces were stained and then scored under the microscope. The staining procedure is adapted from Brundrett *et al.* (1996) [25], where the root pieces were first cleared with 10% KOH at 90 ℃ for 30 minutes. The roots were then carefully rinsed with water in a very fine sieve and incubated 30 minutes at 90 ℃ in staining solution. Subsequently the roots were rinsed again and stored in 50% glycerol.

Staining solution: 5% (v/v) black ink, 5% (v/v) acetic acid

To make sure the scoring was done as objective as possible the method first described by mcGonicle *et al.* (1990) [26] was used. First of all samples were randomized during the staining procedure to prevent biased scoring. In total 7 replicates were scored for the presence of; hyphae only, arbuscles, vesicles or nothing at all in 100 views. This was done under a microscope (Zeiss, axioskop) at 400x magnification. To have a more accurate measurement of colonization percentage and thus the density of the vesicles and arbuscles, not the entire view but only the thin line of intersection between the root and the crosshair of the microscope eyepiece was scored (Figure 6). To pick the views as random as possible the slides were moved by a constant distance for each view.

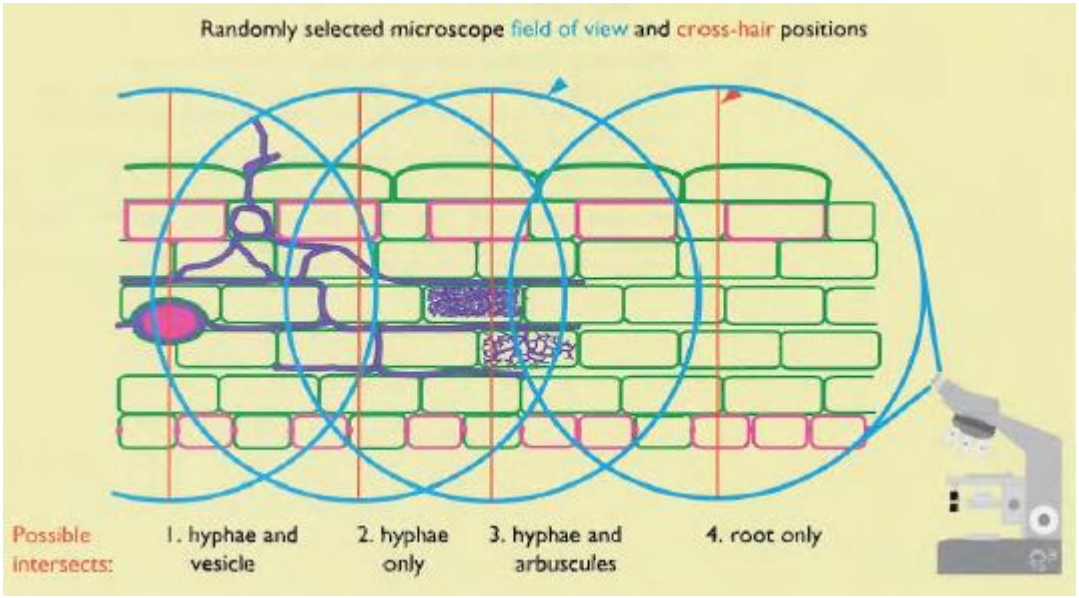

Figure 6. Overview of the microscopic examination of root colonization. Roots are scored for the presence of arbuscules, vesicles, hyphae only or nothing at all. Scoring is done at the line of intersection between the cross-hair of the microscope eyepiece and the root. When both an arbuscule and vesicle are present they are both scored but total score count only increases by 1. From: Brundrett *et al* 1996 [25]

After scoring all replicates the colonization percentage was calculated by dividing the number of views with arbuscles (arbuscular colonization), vesicles (vesicular colonization) or hyphae (total colonization) by 100. The number of views of the hyphae is the total number of views minus root only views.

3.1.4 Isolation of plasmid and genomic DNA

For the isolation of plasmid DNA, 1ml of E. coli culture was centrifuged (4 min., max. speed) and DNA was isolated from the pellet via the QIAprep Spin Miniprep kit (Qiagen). The concentration and purity of the obtained DNA was tested with the use of spectrometry (nanodrop), where the concentration is measured at 280nm. The copy number of the plasmid was determined with the dsDNA copy number calculator from the URI genomics and sequencing center (Rhode Island)[27].

Total DNA isolation (plant plus fungal DNA) for the symbiosis analysis was performed with a protocol from Xu et al (2005) with minor modifications. After weighing exactly 70mg of already ground root material the samples were ground an extra time with the Retsch MM400 grinder cooled with liquid nitrogen. Each sample was mixed with 400µl of extraction buffer B. Subsequently 400µl of phenol-chloroform-isoamylalcohol (25:24:1) was added and centrifuged (20 min. 12,000 rpm) to separate the different phases. DNA is then precipitated with isopropanol and washed with 70% ethanol before dissolving in 30µl MQ.
The RNA still present in the samples was destroyed by a 30 min. incubation step at 37 °C with 0.1µl RNase A (100µg/µl).

Buffer A:	0.6 M NaCl, 0.1 M Tris (pH 7.5), 40 mM EDTA (pH 8.0), 4% (w/v) sarkosyl, 1% (w/v) SDS
Extraction buffer B:	1 volume buffer A, 1 volume 10M urea, 5% Phenol (equilibrated with Tris)

3.1.5 PCR

PCR (polymerase chain reaction) was done for multiple purposes; to check for positive colonies and to preliminary check the presence of *G. intraradices* and *G. custos* in sorghum roots.
A PCR is a procedure to amplify DNA fragments. Two oligonucleotides (primers) bind to the complementary template DNA, enabling exponential amplification by a polymerase over multiple cycles (Table 3). The components that were needed to perform this reaction are shown in table 2. Primers against the plasmid and the two AM fungal strains were designed by Kiers *et al.* 2011 [22] (Table S1). The strain specific primers were designed against the mitochondrial large ribosomal subunit (mtLSU) to avoid putative introns.

Table 1. PCR components

Components	End Concentration
Template DNA	10-50 ng
Firepol -polymerase	1-2 U
10x PCR buffer B	1x
$MgCl_2$	2.5 mM
dNTP's	0.2 mM
Forward-primer	2 pmol
Reverse-primer	2 pmol

Table 2. PCR parameters

	Temperature (°C)	Time	Cycles
Initiation step	95	3 minutes	1x
Denaturation step	95	30 seconds	35x
Annealing step	60	20 seconds	
Elongation step	72	20 seconds	
Final elongation step	72	8 minutes	1x
Storage	4	∞	

3.1.5.1 qPCR

QPCR (quantitative PCR) is a special PCR technique to determine the gene expression (RNA) or copy number (DNA) of the gene in question. A PCR is run in a 96 wells plate, including three technical replicates for each biological replicate. SYBR green is a fluorescent dye, which is added to the PCR reaction where it binds to the DNA. Its signal is detected by the qPCR machine, and once it passes a set threshold, is expressed as a CT value corresponding to the number of cycles after which it was detected. A housekeeping gene is taken along to correct for deviations in template concentration as it is found to be present at a constant level for all different treatments.

For the analysis of the copy number of different AM fungal strains present in sorghum roots a qPCR was done against *G. intraradices* and *G. custos*. A plasmid containing a cassava mosaic virus was added in a concentration of 1*10^9 copies during DNA extraction, immediately after addition of the extraction buffer. The plasmid was provided by Kiers *et al.* 2011 [22]. The cassava mosaic virus does not normally occur in our environment, so the plasmid could be used as an internal standard (IS) to correct for dilutions and losses during extraction. Ubiquitin is taken along as a housekeeping gene to correct for extraction efficiency as the IS does not correct for the efficiency of breaking plant and fungal cells to make the DNA available for extraction. The qPCR was performed as described in table 3 and 4 with the Bio rad CFX manager system as this machine can handle small volumes, thus less template was needed. Obtained ct values were corrected as described above and then the cop number was calculated with the help of a calibration curve of the internal standard ranging from 10 to 1*10^10 copies.

Table 3. qPCR components for copy number analysis.

Components	End Concentration
Template DNA	100ng
2x iQ SYBR mix	1x
Forward-primer	5 pmol
Reverse-primer	5 pmol
Reaction volume	10 µl

Table 4. qPCR parameters for copy number analysis.

	Temperature (°C)	Time	Cycles
Initiation step	95	3 minutes	1x
Denaturation step	95	15 seconds	40x
Amplification step	60	30 seconds	
Melting curves	95	10 seconds	1x
	65	5 seconds	
Storage	4	∞	

3.2 PDR1

To study whether the PDR1 transporter is responsible for the differential strigolactone exudation patterns, the gene candidates were first to be identified, which was done in both Shanqui red and SRN39. This was done by blasting the petunia PDR1 cDNA sequence against the sorghum bicolor project database[28], aligning the sequences and look for the most likely candidates based on a phylogenetic tree and conserved domains. All candidates were tested in PCR for the criteria given by Kretzschmar *et al*. (2012) [19], which are up regulation in minus phosphate treatment or upon addition of GR24. A third criterion is up regulation at the presence of AM fungi. The candidates with a positive result (up regulated) were then tested in a qPCR experiment to check if the expression level is significantly higher when compared to the untreated control.
Besides identifying the PDR1 candidates for SRN39 and Shanqui red, the strigolactone exudation profile (as described in 3.1.2) was analyzed for 11 different genotypes to see their general exudation profile. Those genotypes included the African lines; SRN39, Sudan Z. Z. and P90950, the Chinese lines; Shanqui red, HUI 39B and Is4 225 and five more lines that are crosses from the S*triga* spp. susceptible Shanqui red and the resistant SRN39. The aim of this experiment was to see which other genotypes would be good to include in the PDR1 specificity screening.

3.2.1 Searching for putative PDR1 candidate genes

In order to find putative candidate PDR1 genes, the functionally proven PDR1 genes from *P. hybrida* and *P. axillaris* were blasted against the sorghum bicolor project database [25]. Those with an E-value of 0 were selected and examined to see whether they belong to the same gene family as *Arabidopsis thaliana* PDR1 or PDR12. Brule and Smart (2002) have shown that from all 15 PDR proteins, AtPDR1 and AtPDR12 are in distant clusters[29]. By checking for both gene families the putative PDR candidates could be selected while the chance of excluding a PDR gene from all putative candidates is minimalized. Also AtPDR12 is found to be closely related to the petunia genes in phylogenetic analysis[19].
Subsequently the putative candidates were aligned with the software MEGA 5.1 together with the PDR1 genes from *Petunia, Oryza sativa* and *Arabidopsis* and candidate genes for *Solanum lycopersicum*. Based on the alignment in combination with the derived tree, gene specific primers were designed (Table S1). The putative candidates were examined for functional domains in the pfam [30] and InterPro [31] database. The combined data of the two databases was used to compare the domains of the candidate genes with those of the *P. axillaris* and *P. hybrida* genes. The candidates left after this pre-screening were tested for their gene expression in multiple genotype*treatment combinations.

3.2.2 Growing and harvesting of Sorghum plants

Plants were grown in a similar way as the plants for the AM fungi experiment (see 3.1.1). However unlike with the AM experiment only three replicates were used for the 11 genotypes and plants were grown in the presence of phosphate for four weeks. After these four weeks the treatment shifted to ½ Hoagland solution minus phosphate for one additional week. Upon harvesting plants were again flushed in advance and 1 Liter of exudates is collected after 48 hours. Roots were now only harvested for LC-MS analysis. For testing PDR1 candidate upregulation upon GR24 addition a hydroponics system was used. Tubes (50ml) were covered in aluminium foil to mimic the dark conditions of the soil for the roots. The germinated seeds were placed inside and the shoot was emerging from the tube through a hole in the lid, which was covered by a piece of cotton to prevent evaporation. The seedlings were grown for two weeks in this system, watered by ½ hoagland solution (full phosphate). After those two weeks synthetic strigolactone GR24

was added to the solution for half of the plants in a concentration of 10µM. After 24 hour incubation with GR24, root material was harvested and three plants were pooled together as 1 biological replicate while grinding.

3.2.3 Isolation of RNA

RNA extraction was performed on already grounded root material with the FavorPrep ™ Tissue total RNA purification mini kit (Favorgen). Extraction was done on root samples of both SRN39 and Shanqui red grown under +/- P and those +/- GR24 to enable analysis of gene expression for the different PDR1 candidates. Sixty mg of root material was used and ground a second time before following the protocol as recommended by the company. A DNase (Qiagen) treatment was performed to get rid of the DNA present. The concentration and purity of the obtained RNA was tested with the use of spectrometry (nanodrop). The concentration is measured at 260 nm and the purity is assessed at the two ratios of 260/280 and 260/230 nm. Integrity of the RNA is tested on gel, a good integrity can be seen in the presence of two clear bands for the 28S and 18S rRNA, where the intensity is in a 2:1 ratio respectively.
One µg of RNA was converted to cDNA with the reverse transcriptase from the iScript cDNA kit (Bio rad) in a total reaction volume of 20µl.

3.2.4 PCR

Before doing a qPCR to determine whether gene expression of putative candidates is upregulated under –P or +GR24 treatment a normal PCR was performed. This semi-quantitative analysis was done to pre-screen the putative candidates, eliminating some candidates from the qPCR work. A PCR was done as described in paragraph 3.1.5 and run on a 1% agarose gel, comparing the gene expression for minus versus plus Phosphate and for with/without addition of GR24. Those which seemed upregulated on the gel were then tested in a qPCR reaction.
Primers were designed in the program Primer3Plus [32] against the gene specifiec sequences found during alingnment and blasted back in the NCBI sorghum database to check for specificity (Table S1).

3.2.4.1 qPCR

For the gene expression analysis of the putative PDR1 candidates a qPCR (Bio rad, IQ 5 system) was performed on cDNA and ubiquitin was taken along as a housekeeping gene (Table 5. and 6.). Samples were normalized with this housekeeping gene to correct for deviations in template cDNA concentrations. From this data a relative expression compared to the control treatment was calculated.

Table 5. qPCR components for gene expression analysis.

Components	**End Concentration**
Template cDNA	2x dilution (2µl)
2x iQ SYBR mix	1x
Forward-primer	10 pmol
Reverse-primer	10 pmol
Reaction volume	20 µl

Table 6. qPCR parameters for gene expression analysis.

	Temperature (°C)	Time	Cycles
Initiation step	95	5 minutes	1x
Denaturation step	95	15 seconds	40x
Amplification step	60	1 minute	
Melting curves	95	1 minute	1x
	55	1 minute	
Cooling	55	10 seconds	

4. Results

4.1 AM fungi

4.1.1 Strigolactone profile

Strigolactone profiles were determined by LC-MS analysis for the different genotype*fungal strain combinations both in exudates and extracts (Figure 7). For the exudates in control plants mainly orobanchol is found back for SRN 39 while for Shanqui

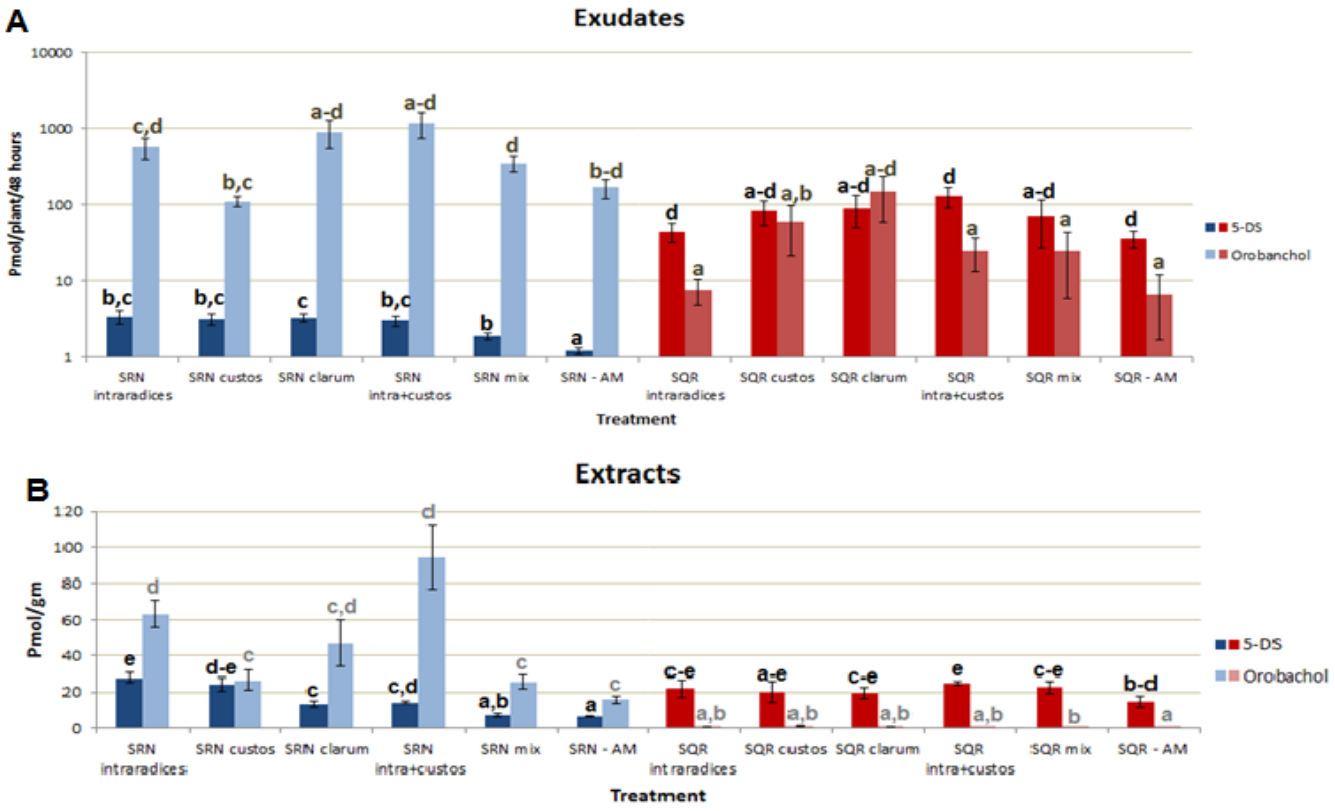

Figure 7. Strigolactone profiles in sorghum of SRN 39(blue) and Shanqui red (red) for multiple AM fungal strain combinations. A) Strigolactones found to be exuded into the soil in pmol/plant/48 hours. Y-axis, logarithmic scale. SRN 39 exudes mainly orobanchol while Shanqui red exudes both 5-DS and orobanchol but more of the first. SRN custos and SRN intraradices are not significantly different for their orobanchol level even though the fold difference is 10x. B) Strigolactones found in root extract, depicted in pmol/gram. Levels of orobanchol still present in the roots are higher for SRN 39 (around 20 pmol/gram) compared to those of Shanqui red (around 0). The levels of 5-DS are around 10 to 20 pmol in both genotypes.

red both 5-deoxystrigol (5-DS) and orobanchol are found. In SRN the exudation of 5-DS is significantly upregulated in all treatments compared to the control without AM fungi. For orobanchol the same trend seems to be present, except for the custos treatment, however this difference is not significant. For Shanqui red there are no significant differences between treatments in both 5-DS and orobanchol exudation. However there is again a trend in which plants inoculated with AM fungi, with the exception of *G. intraradices* alone, seem to exude more strigolactones than the control. For the extracts all SRN plants, except for those inoculated with all three AM fungal strains, have a significant increase of 5-DS present in the roots compared to the control. For Shanqui red only the combination of intraradices and custos produces a significantly higher amount of 5-DS than the control. Hardly any orobanchol is found back in the roots of Shanqui red

while around 20pmol/gram is found back in SRN 39. Here the intraradices and the intraradices plus custos treatment both have a significantly higher orobanchol level than the control.

4.1.2 Plant growth

Upon harvesting, the height and biomass of all plants was measured. No significant difference or a clear trend was found between treatments for the height, underground biomass and total biomass. There was a significant difference in aboveground biomass for SRN inoculated with intraradices compared to the non-inoculated control (Figure 8A). For Shanqui red again no differences can be found (Figure 8B). The ratio between root and shoot biomass is also similar for all treatments in Shanqui red. For SRN 39, plants inoculated with intraradices have a similar ratio as the control of ~1, while all other treatments have a lower ratio, thus relatively larger shoot than root biomass (Figure 8C).

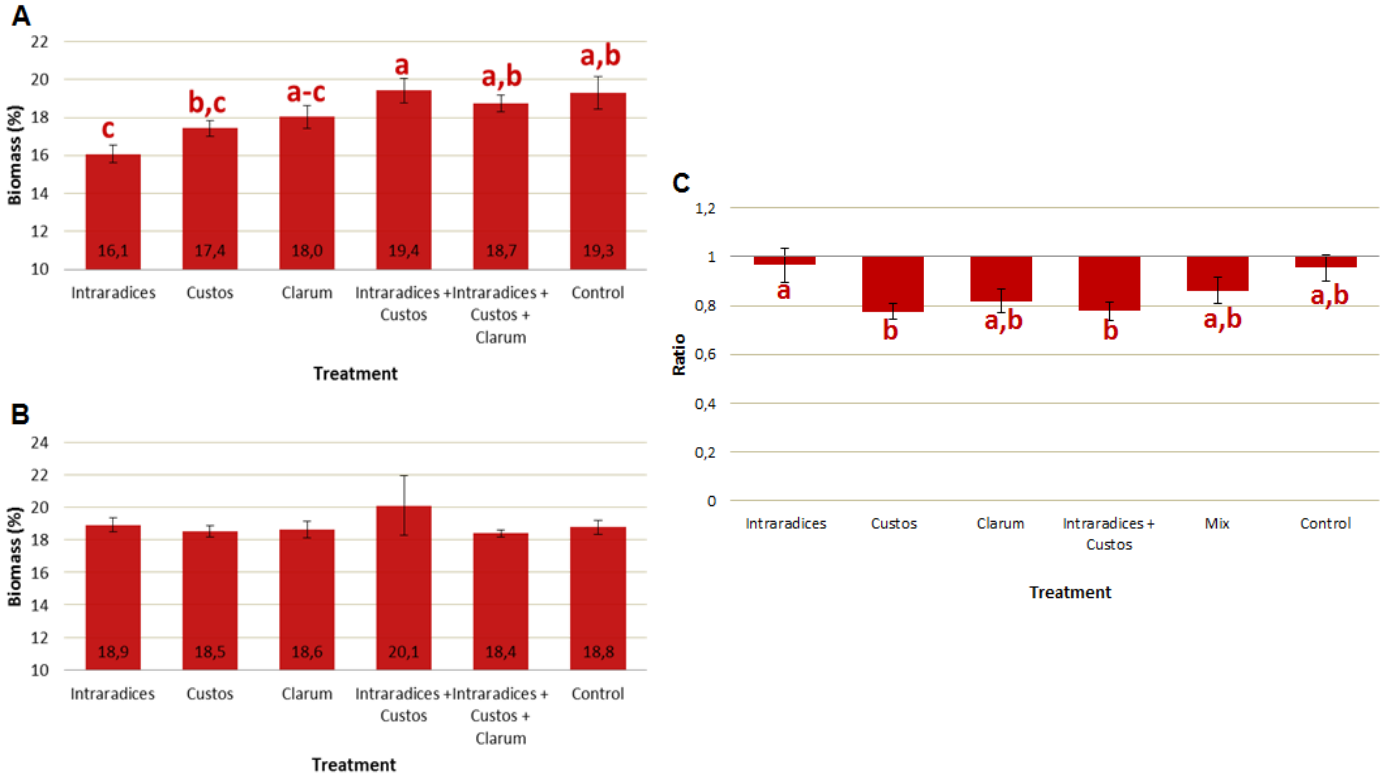

Figure 8. Aboveground biomass percentage for SRN 39 (A) and Shanqui red (B) under different AM fungi treatments. For SRN 39 the plants inoculated with *G. intraradices* have a lower shoot biomass, while the other treatments do not significantly differ from the control. For Shanqui red no significant differences can be found. The root/shoot biomass ratio is shown for SRN 39 (C) where intraradices has a similar ratio as the control of ~1, while all other treatments (although not significant) seem to have a lower ratio thus a relatively larger shoot than root biomass.

4.1.3 Colonization percentage

With the help of microscopy the colonization percentage could be calculated for arbuscules, vesicles and total colonization (arbuscules, vesicles and hyphae). In figure 9 you can see that arbuscular and total colonization follow the same pattern for all treatments. The same holds true for the vesicular colonization, however the ratio between the treatments is different. Colonization percentage is high for intraradices in both SRN 39 and Shanqui red. Also the mix of intraradices and custos and the mix of all three strains are high in colonization percentage, although not always significantly different from the non-inoculated control. Inoculation with Clarum follows a similar pattern as the colonization with Custos although clarum seems to do better in SRN while custos seems to be slightly better in Shanqui red.

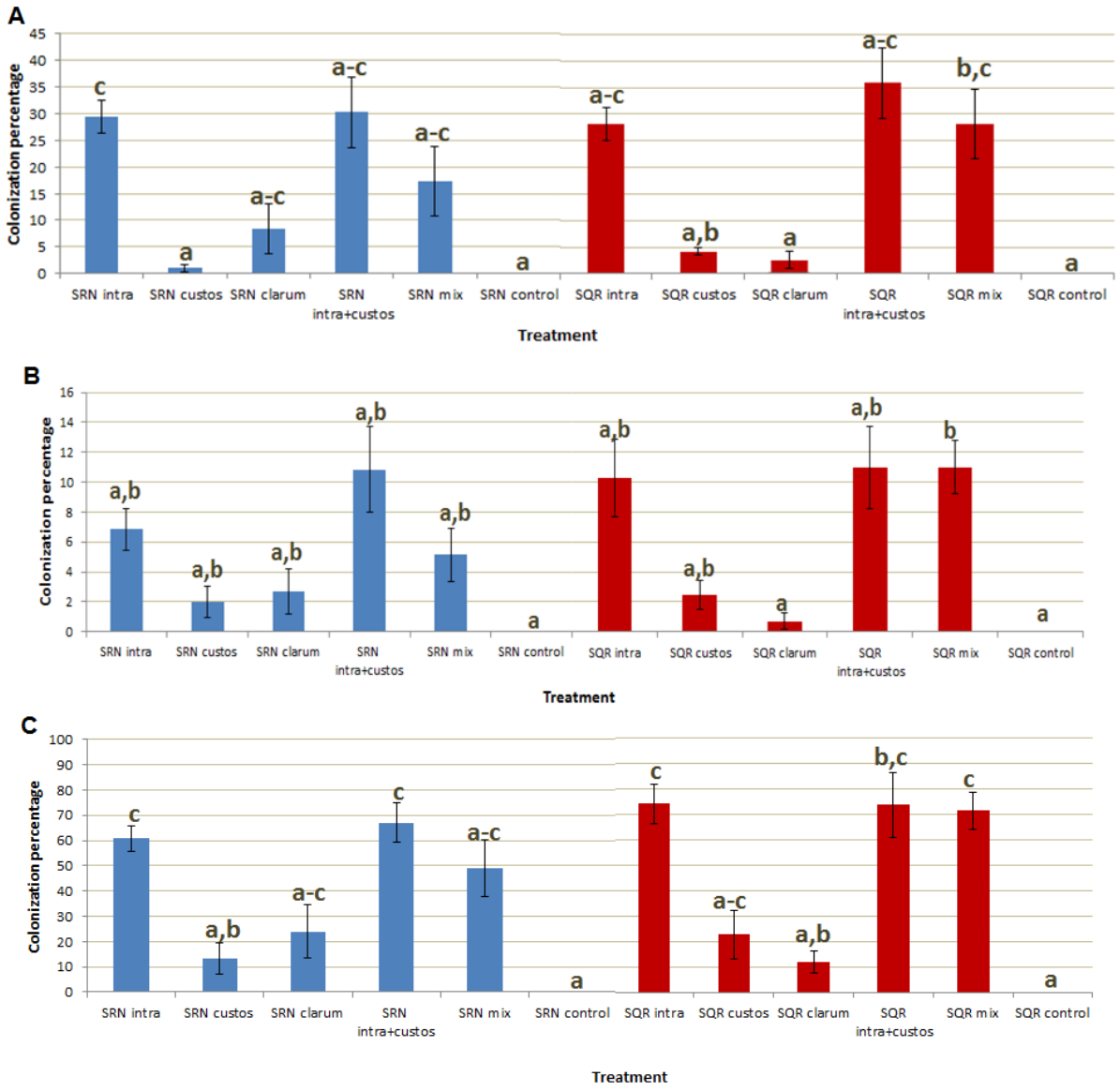

Figure 9. Colonization percentage in sorghum roots of SRN 39(blue) and Shanqui red (red). A) Arbuscular colonization, B) vesicular colonization and C) hyphal or total colonization percentage of AM fungal symbiosis for multiple inoculation treatments.

For the vesicular colonization, although there seems to be a similar trend again, only the mix of all three strains in Shanqui red is significantly higher than the non-inoculated control.

4.1.4 Gene expression

With qPCR the copy number of *G. intraradices* and *G. custos* for 70mg of root material was determined. The results are shown in figure 10. In single inoculation, custos has a lower copy number than intraradices, with a factor 100. Also in the plants inoculated with both intraradices and custos this is the case but now with a factor difference of 10. No significant difference is found between the two strains in SRN inoculated with all strains and for Shanqui red there is no difference between intraradices and custos for all different treatments. For each treatment no significant difference was found between the two sorghum genotypes.

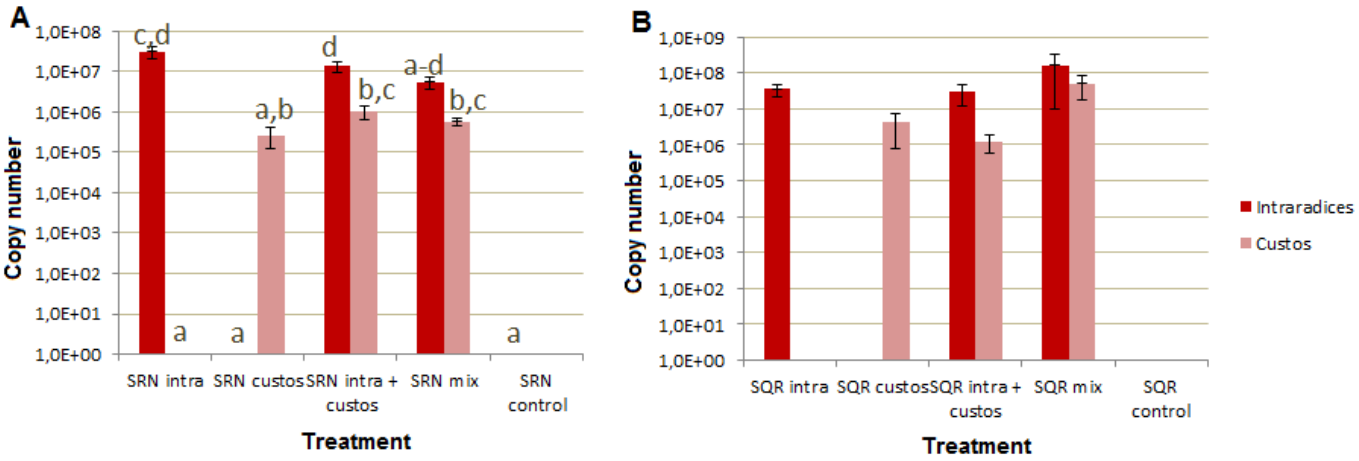

Figure 10. *G. intraradices* and *G. custos* copy number. Quantitative PCR for *G. intraradices* and *G.* custos on SRN 39 (A) or Shanqui red (B) roots: grown after inoculation with different AM fungal strain combinations. Y-axis, logarithmic scale. For Shanqui red no significant differences were found for the two AM fungal strains between treatments or compared to each other. Neither were there significant differences found between SRN39 and Shanqui red.

Both for intraradices and custos there is no significant difference in copy number between their single and the mixed inoculations in either SRN39 or Shanqui red.

4.2 PDR1

4.2.1 Strigolactone profile

Eleven different genotypes were tested for their strigolactone profile, to see whether they had different biosynthesis/exudation ratios (Figure 11). The amount of strigolactones in the exudates and extracts cannot be directly compared as they are in different units, however the patterns can be compared. No strigol is found back for the crosses of SRN 39 and Shanqui red (SSD#3-177 to SSD#3-767) while different biosynthesis/exudation ratios were found back for those genotypes in earlier experiments (Figure S2).

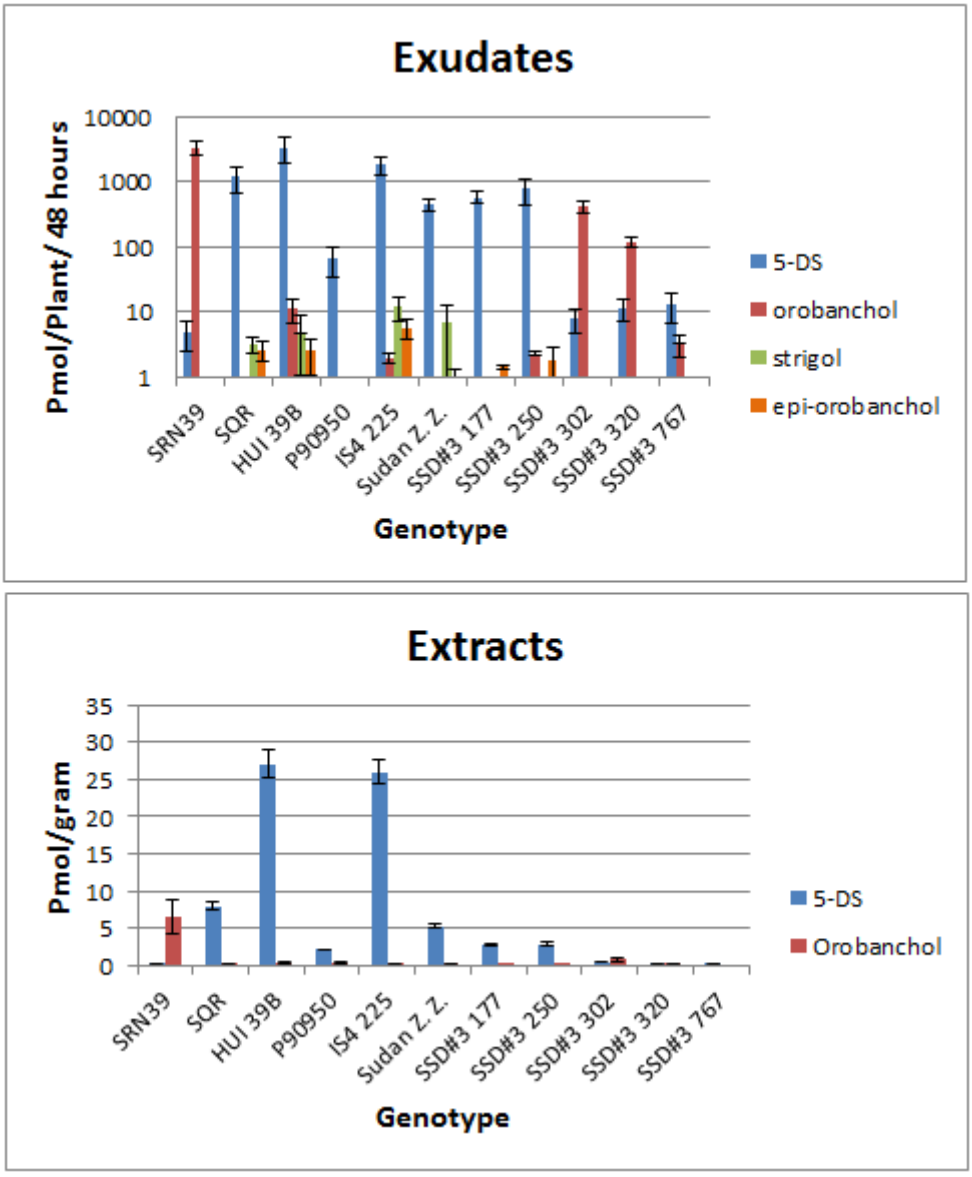

Figure 11. Strigolactone profile for eleven different sorghum genotypes. A) Strigolactones found to be exuded into the soil in pmol/plant/48 hours. Y-axis, logarithmic scale. For SRN39 the strigolactones found are similar to the AM experiment. For shanqui red, this time only 5-DS and no orobanchol is found back. The pattern for all other lines B) Strigolactones found in root extracts, depicted in pmol/gram. Strigol and epi-orobanchol are not shown for the extracts as they were not found.
Absolute strigolactone values are shown in Table S5.

For the extracts only small amounts of 5-DS and orobanchol are found except for Hui39B and IS4 225 where concentrations of approximately 25 pmol/gram of 5-DS are found. Although they are also higher in exudation than the other genotypes, with exception of

Shanqui red, the shift is not directly proportional meaning that HUI 39B and IS4 225 do not exude almost all 5-DS produced while the other genotypes do. The data are deviating in biosynthesis/exudation pattern of 5-DS from earlier research (Table S4). In the current experiment P90950 and Shanqui red appear to exude almost all 5-DS produced while in earlier findings they only exuded a small amount of the 5-DS produced. For orobanchol all genotypes exude almost all of the synthesized strigolactone. Absolute values for the current experiment are depicted in table S5.

4.2.2 PDR1 candidate selection

Nineteen putative PDR1 candidates with an E-value of 0 were selected after blasting the cDNA of the functionally proven PDR1 genes from *P. hybrida* (Genbank accession number JQ292813) and *P. axillaris* (Genbank accession number JQ292812) against the sorghum bicolor project database [25]. After checking whether their gene family included *A. thaliana* PDR1 or PDR12 genes 17 candidates were left (see methods 3.2.1). Based on the alignment made from the amino acid sequences in combination with the derived phylogenetic tree (Figure 12.) primers were designed (Table S1). Due to the fact that some groups of genes shared almost 100% homology except for a few point mutations and one or two small gaps, even in the cDNA sequence, candidate specific primers could only be designed for 12 candidates (Figure 12).

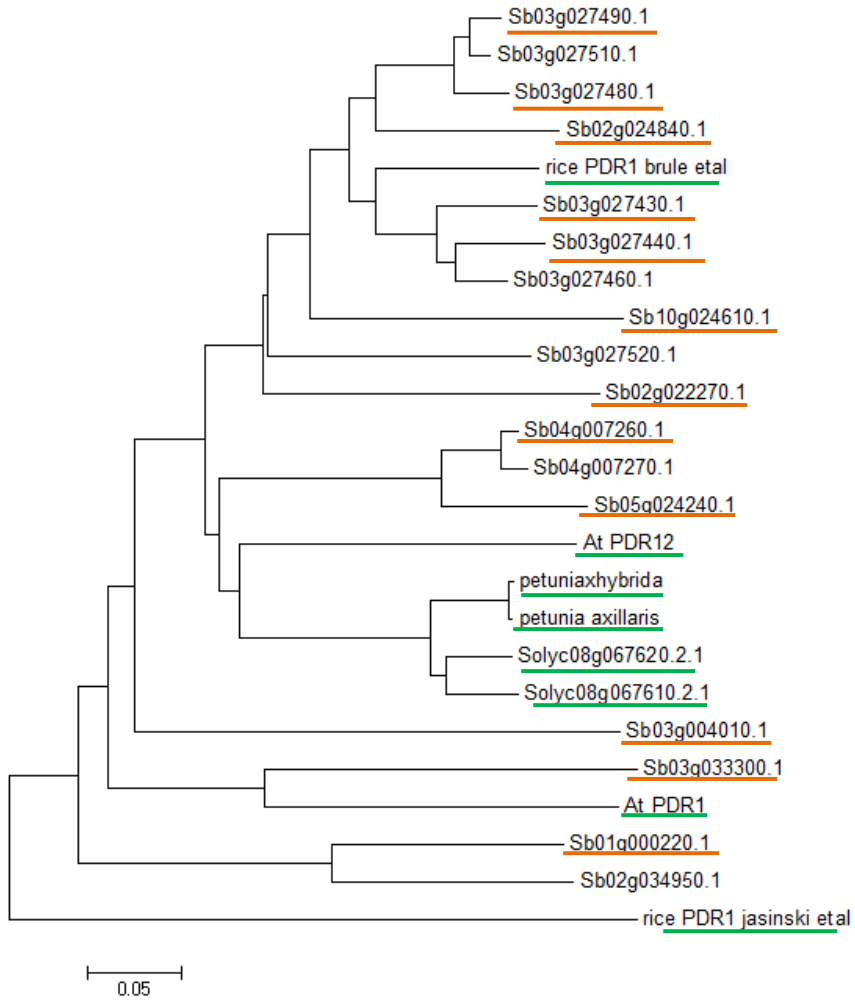

Figure 12. Phylogenetic tree of putative PDR1 homologs in *S. bicolor.* Reference genes (*P. hybrid, P. axillaris, O. sativa, S. lycopersicum* and *A. thaliana*) are underlined in green and candidates tested are indicated with orange.

The functional domains of the PDR1 candidates found in the pfam [30] and InterPro [31] database were compared to those of *P. axillaris* and *P. hybrida*. The PDR1 gene contains four larger domains (Figure 13.) and a few small domains. The candidate genes mainly deviate from petunia in the small domains where they almost all have an extra copy of one domain or have an additional domain, which is not present in the petunia PDR1. However some candidates also miss a domain; Sb03g027480 and Sb03g027490 (not tested further) miss one of the ABC transporter domains, while Sb10g024610 misses the ABC-transporter extracellular N-terminal. Sb03g004010 and Sb03g033300 both have a gap in one of the domains. The putative candidate Sb04g007260 is the only candidate with the exact same domains as the petunia PDR1 gene.

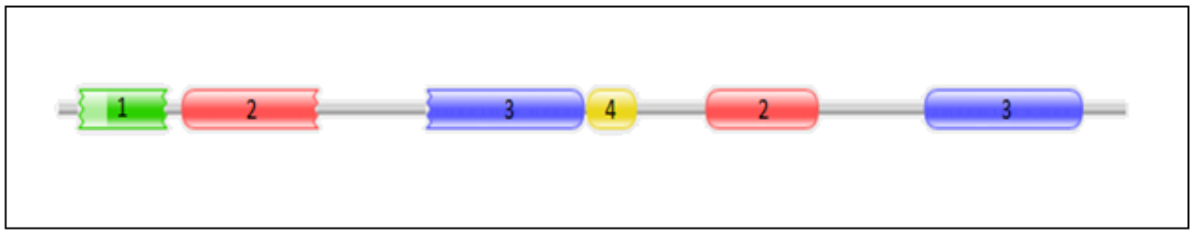

Figure 13. Overview of the main domains of PDR1 in *P. axillaris* and *P. hybrida*. The gene contains an ABC-transporter extracellular N-terminal 1), two ABC transporters 2), two ABC-2 type transporters 3) and a plant PDR ABC transporter associated domain 4).

The putative candidates missing a domain, which are more distant from the petunia PDR1 in the phylogenetic tree, are not taken along for further testing. As the petunia PDR1 is up regulated in plants grown under low phosphate conditions, upon presence of AM fungi and in the presence of strigolactones, the candidate genes were first tested for up regulation under phosphate starvation. PDR1 cDNA was amplified using a routine PCR procedure (conditions as described in paragraph 3.2.4, primers are shown in Table S1) and the product was analyzed on an agarose gel (Figure 14). Only a few gene candidates were found to be up regulated in both genotypes (Figure 14A). Those that were seemingly up regulated in both Shanqui red and SRN 39 (Sb01g000220 and Sb03g027430) were further analyzed using qPCR to confirm the results. The positive control (ubiquitin) in the PCR with Sb04g007260 and Sb05g024240 gave a strange result (Figure 14B). As Sb04g007260 seemed to be present at very high levels though, it was tested in a qPCR together with Sb01g000220 and Sb03g027430.

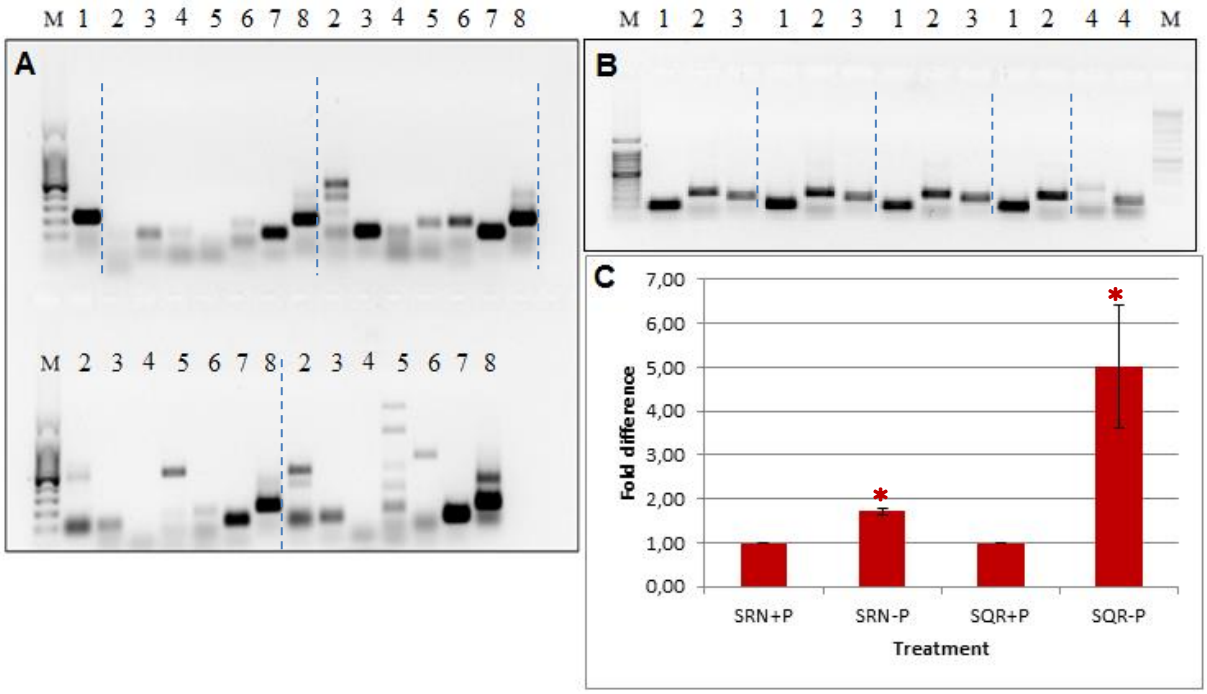

Figure 14 PDR1 upregulation under phosphate deficiency. A+B) Results of semi-quantitative PCR in both SRN 39 and Shanqui red. A) The first gene shown is the ubiquitin control and then Sb03g027430 (set1, [2]), Sb03g017430 (set2, [3]), Sb03g027440 ([4]), Sb10g024610 ([5]), Sb02g024840 ([6]), Sb01g000220 ([7]) and Sb02g022270 ([8]) are shown in SRN+P, SRN-P, Shanqui red (SQR)+P and SQR-P respectively. B) The ubiquitin control ([4]) is tested for SRN+P and SRN-P respectively where in both cases two bands can be seen while it should appear as 1 clear band (+/- 250 bp) as depicted in figure A lane 1. Then Sb04g007260 ([1]), Sb05g024240 (set1, [2]) and Sb05g024240 (set2, [3]) are tested in SRN+P, SRN-P, SQR+P and SQR-P respectively. C). Results of the qPCR experiment for Sb04g007260, the gene is significantly upregulated under phosphate deficiency in both SRN39 (P=0.001) and Shanqui red (P=0.046).

Of those tested in qPCR only Sb04g007260 was significantly up regulated under phosphate deficiency in both SRN (P-value 0.001) and Shanqui red (P-value 0.046). This gene and the closely related Sb05g024240 gene were then tested for up regulation upon addition of GR24. For Sb05g024240 there was something wrong with the water control and the gene was not found back at all in either treatment, while it was visible on gel (Figure 16). The Sb04g007260 putative candidate was significantly up regulated by GR24 in Shanqui red (P= 0.011) but not in SRN 39 due to one deviating technical replicate (Figure 15.).

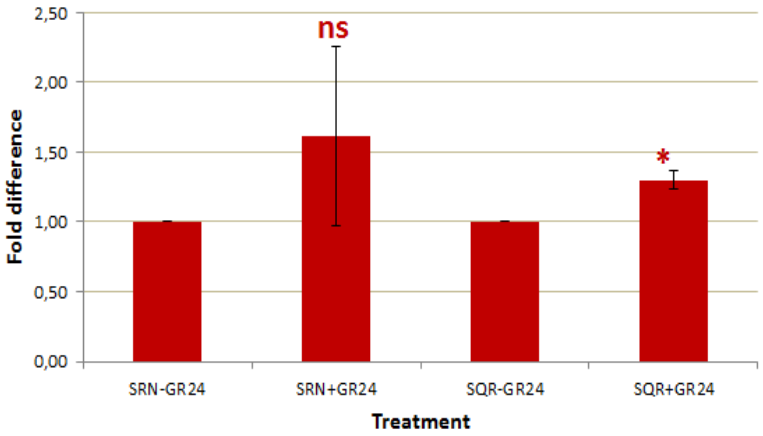

Figure 15. qPCR on Sb04g007260 in addition to GR24 treatment. The gene is significantly up regulated under phosphate deficieny in Shanqui red (P=0.011) but not in SRN 39 (P=0.218) due to a deviating technical replicate.

A normal PCR was performed on the root material grown with/without GR24 and tested against the Sb04g007260 and Sb05g024240 gene to see whether upregulation is to be expected (Figure 16). Also the genes Sb03g027430, Sb01g000220, Sb02g022270 and Sb02g024840 are taken along in this PCR as they seemed upregulated in at least one of the sorghum genotypes grown without phosphate. From this gel it can be seen that Sb04g007260 and Sb05g024240 indeed seem to be upregulated and Sb03g027430 only for Shanqui red. Sb02g022270 is unclear on this gel as well and the other putative candidates are not upregulated under GR24 addition.

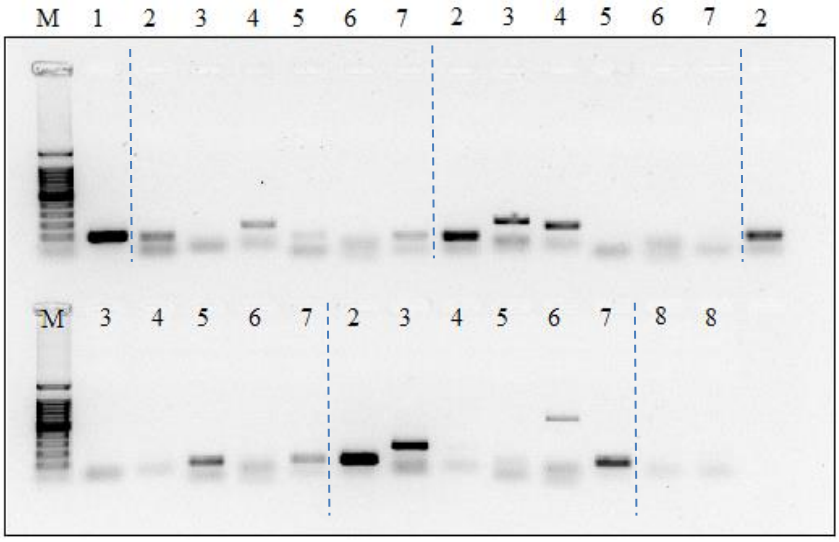

Figure 16. Results of semi-quantitative PCR on SRN 39 and Shanqui red with or without GR24 addition. Lane 1 is the ubiquitin control and then Sb04g007260 ([2]), Sb05g024240 (set 1, [3]), Sb05g024240 (set2, [4]), Sb01g000220 ([5]), Sb02g024840 ([6]) and Sb03g024840 ([7]) are tested in SRN+P, SRN-P, SQR+P and SQR-P respectively. Sb02g022270 ([8]) was tested in SRN+P and SRN-P respectively.

5. Discussion

Strigolactones play an important role in the colonization of parasitic plants and AM fungi. Therefor it was questioned whether a difference in strigolactone profile between the *Striga* spp. resistant sorghum genotype SRN 39 and the susceptible Shanqui red would influence colonization by AM fungi as it does for *Striga* spp. It is hypothesized that a mutation in SRN39 makes it convert 5-DS to orobanchol while this does not occur/at a lower level in Shanqui red (unpublished). In a watery environment hydroxyl-strigolactones such as orobanchol are short lived and thus show little or no germination stimulant activity for *Striga* spp. [23]. It was indeed found, not only in the current research but also in previous experiments, that SRN 39 exudes mainly orobanchol while in Shanqui red the level of 5-DS is higher.
Inoculation of sorghum plants with *G. intraradices* was found to give a high colonization percentage. At the same time a reduction in aboveground biomass percentage is observed in SRN 39 when compared to the non-inoculated control plants. This was not the case for Shanqui red. As the plant height and underground biomass do not deviate from the other treatments, the difference is probably caused by a differential carbon allocation. This implies that instead of investing in shoot biomass the plant might give carbon to the AM fungi in return for the phosphate they get. Carbon allocation in the roots is most likely kept stable in order to have enough roots suitable for symbiosis. A differential carbon allocation, in the form of lower shoot biomass, could imply a lower yield for farmers when no striga is present. In the presence of striga however, this small decrease in shoot biomass, to keep the symbiosis, might be preferable as AM fungi were previously found to reduce the amount of emerged striga plants and their amount of damage done to the sorghum plants by a decline and simplification of their exudates[7] and simultaneously provide the plant with phosphate and is thus preferred over no AM fungi at all. Upon inoculation with AM fungi however we see an increase in strigolactone level instead of a decline or simplification of the exudation profile. Also it is expected that shoot biomass would show an increase in the presence of AM fungi compared to the non-inoculated control as those plants would not be suffering that much from a shortage in phosphate. Therefor it can be argued that the phosphate level is not optimal (too high) to observe the beneficial effects of AM fungi.

The high level of symbiosis of *G. intraradices*, measured by both copy number and colonization percentage, is similar between SRN 39 and Shanqui red (Figure 9 and 10). Besides that it was found that SRN 39 exudes mainly orobanchol while Shanqui red exudes 5-DS. These data correspond with the data of Akiyama *et al.* 2010 [24] where they found that both orobanchol and 5-DS have a very strong hyphal-branching inducing activity in *G. margarita*. The *Striga* spp. resistant genotype SRN 39 is, like the susceptible genotype Shanqui red, thus capable of forming a symbiotic relationship with AM fungi. However, it can be questioned whether it is still resistant to striga in this case as the exudation of 5-DS is slightly (1,35-5,4 fold) but significantly increased when AM fungi are present (Figure 7A). This would however contradict earlier research where it was found that AM fungi reduce the number of attached and emerged *Striga harmonthica* plants in *S. bicolor* through a decline and simplification of root exudates [7].
Although *G. custos* has a lower copy number than *G. intraradices*, there is no difference between the single or mixed inoculations (Figure 10). The same might hold true for the clarum strain, which is comparable to custos in colonization percentage under the microscope, but as a species specific primer for qPCR was not available yet it will need

confirmation. The fact that there is no significant difference between the single or mixed inoculations for *G. custos* implies that the presence of *G. intraradices* in the mix does not influence the symbiosis between custos and the plant. In medicago, on the contrary, Kiers *et al.* [22] found that the colonization of *G. custos* decreased by 85% when *G. intraradices* was present. The fact that our data do not show a decrease in colonization by *G. custos* could be due to multiple explanations. First of all, it could be that the total number of spores used is not optimal for sorghum and the two fungal strains might be present in a too low concentration to interfere with each other during colonization. We found copy numbers of 1*10^5 to 1*10^7 per 70mg roots (fresh weight) corresponding to 60 to 1,2*10^6 copies per 100ng DNA while Kiers *et al.* [22] finds values of 5*10^7 to 4*10^8 copies per 500ng RNA. Our values are thus lower than those found in *Medicago Truncatula* which could explain the lack of interference between AM fungal strains found in our results. A second explanation could be that the phosphate level is again not optimal for efficient symbiosis and the difference in phosphate gained by the plant between the two AM fungal strains is too small to develop a preference for one of them. Another explanation could be that the time point of harvesting is too early for the plant to express its possible preference for one of the strains. This could also explain why no significant difference or even trend was observed between treatments for other parameters checked such as the plant height and root biomass profiles. In order to confirm this explanation, plants that have been grown for eight weeks after inoculation with custos and intraradices plus custos are available for analysis and may provide a better representation of the competition between the two different AM fungal strains.

PDR1 is the ABC transporter responsible for transporting strigolactones from the root to the surrounding soil. As it was found before (unpublished work, Table S4) African lines differ in their strigolactone exudation profile from Chinese lines while their biosynthesis is not so different. The logical question to be answered is whether this differential exudation profile is caused by genotypic differences in the PDR1 transporter. Putative PDR1 candidates were screened, however still need to be confirmed and subsequently tested for their ability to exude different strigolactones in a benthamina system where all genes for strigolactone biosynthesis are incorporated.
It can be said that, from all putative candidates (17 candidates of which 12 can be distinguished from each other by gene-specific primers), the two most likely PDR1 candidates are Sb04g007260 and Sb05g024240 due to their homology to the petunia PDR1 and as they both appear to be upregulated under phosphate deficiency and upon the addition of GR24 as shown by semiquantitative PCR (Figure 14). For Sb04g007260 upregulation during phosphate starvation was also confirmed by qPCR (Figure 14) where a 1,7 (SRN 39) and 5 fold (Shanqui red) increase in gene expression were found compared to a 2-3 fold increase in petunia [19]. The qPCR performed on material from roots grown in the presence of GR24 (Figure 15) was not conclusive due to a deviating technical replicate and should thus be repeated. For Shanqui red it was significantly increased in gene expression by 1,3 times against 2-4 (1 or 10 µM GR24 respectively) times in petunia [19]. The magnitude of induction of the gene is thus lower than expected; however as it is a different plant more time points or a higher GR24 doses might be needed for an optimal induction. As a quite high dose of GR24 was used during the experiment, measuring in different time points may be preferred.
For the putative PDR1 candidate Sb03g027430 it is questionable whether it could be a PDR1 gene, responsible for the exudation of strigolactones, as even though semiquantitative PCR shows that it may be upregulated under both phosphate deficiency and GR24 addition (SQR only) this was not significant in the qPCR experiment where the response to phosphate deficiency was tested. However, just to make sure a qPCR on the

GR24 treated plant material should be done. For Sb02g022270 a qPCR might need to be done as well as it was unclear on gel whether the gene was upregulated or not. Those that are not upregulated under phosphate deficiency should nevertheless be checked under GR24 addition on PCR as well, before ruling them out completely. However, as upregulation under phosphate deficiency is found to be one of the criteria for the PDR1 gene in petunia [19], it could still be said that Sb04g007260 and Sb05g024240 are the two most likely PDR1 candidates.

From the eleven genotypes tested for their different biosynthesis/exudation profiles the difference in strigolactone exudation seems to be dependent on biosynthesis only as almost all of the synthesized strigolactones are exuded from the roots. IS4 225 and HUI39B are an exception in this as they only partially exude the produced 5-DS. However in earlier experiments (Table S4 and Figure S2 respectively) the 5-DS and strigol exudation patterns were different. There P90950 only partially exuded its 5-DS and HUI39B and IS4225 exuded almost all of the synthesized 5-DS. Secondly the SSD#3 genotypes differ in their strigol exudation as they all produce strigol however, SSD#3-177, SSD#3-320 and SSD#3-767 exude part of the strigol, SSD#3-302 only exudes a small amount and SSD#3-250 exudes nothing at all. The discrepancy between these and our own data might be caused by differential growing conditions such as a different time of the year and new lights in the climate cell. However the absolute strigolactone levels found in the current experiment do not differ so much from the previous experiments (Table S4 and S5). The level of orobanchol is slightly lower compared to the previous data, but 5-DS levels are higher than before, with the exception of P90950. Total strigolactone production is thus not the cause for the discrepancy in exudation patterns between the two experiments. In the end it cannot be concluded yet which genotypes are deviating most in their biosynthesis/exudation ratio and would thus be good to check for their PDR1 efficiency or specificity.

6. References

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7. Supplementary data

Table S1. Overview of primers used with their purpose.

Primer name	Sequence (5'-3')	Purpose
M0050 (Ubiquitin)	GGAAACATAGGGACGCTTCA	qPCR/PCR
M0051 (Ubiquitin)	AAGGAGTCCACCCTTCACCT	qPCR/PCR
MK1003CR3-027430-FWP1	CTGCAGGATTGGCATGTTTA	qPCR/PCR
MK1004CR3-027430-RVP1	CACCATCGTGTCAGCACATA	qPCR/PCR
MK1005CR3-027430-FWP2	AGCCAGCATGACCTTCATATT	qPCR/PCR
MK1006CR3-027430-RV2	TGTTCAGAGTCATACCGAAACG	qPCR/PCR
MK1007CR3-027440-FW	GGACCTTGGTGGTAAAATGTAA	qPCR/PCR
MK1008CR3-027440-RV	CCTGGTAGGTACAGAATCATAAGC	qPCR/PCR
MK1009CR10-024610-FW	CCTCAGTGGAACAGCAGTCA	qPCR/PCR
MK1010CR10-024610-RV	GTCAGGGCATCTGAACCCTA	qPCR/PCR
MK1011CR2-024840-FW	AACCGAGCAGCGTAAGAGAC	qPCR/PCR
MK1012CR2-024840-RV	GATCGTGCAAACCACAGTTC	qPCR/PCR
MK1013CR1-000220-FW	CCAAACAATGACACGACTATTGA	qPCR/PCR
MK1014CR1-000220-RV	TCCGATGGATAGCCAAAATC	qPCR/PCR
MK1015CR2-022270-FW	CTGCCTCACATTCCTCAAGC	qPCR/PCR
MK1016CR2-022270-RV	CTGTCGCTTCATCATTGCTC	qPCR/PCR
MK1017Cr4-007260-FW	GTTTTCCGACCAGTTTGGCC	qPCR/PCR
MK1018Cr4-007260-RV	ATTTAATGGGAGATCTTACATTGAAGT	qPCR/PCR
MK1019Cr5-024240-FW1	TGTTTCTGACAAGGGACTGAGA	qPCR/PCR
MK1020Cr5-024240-RV1	TCCTCTCTTCTTGGTGTCTGC	qPCR/PCR
MK1021Cr5-024240-FW2	TGGCTTTGTATTTGGAACCAT	qPCR/PCR

Primer name	Sequence (5'-3')	Purpose
MK1022Cr5-024240-RV2	CAATTAAAAGAACCGCAGCA	qPCR/PCR
M169 (Internal standard)	CGAACCTGGACTGTTATGATG	qPCR
M170 (Internal standard)	AATAAACAATCCCCTGTATTTCAC	qPCR
A (*G. intraradices*)	TTTTAGCGATAGCGTAACAGC	qPCR
B (*G. intraradices*)	TACATCTAGGACAGGGTTTCG	qPCR
64 (*G. custos*)	TCTAACCCCAGAAATGTATAG	qPCR
65 (*G. custos*)	AAGGACTGCCTTGTGTTC	qPCR

Table S2. Overview of used machines and software.

Company/Institute	Machine/software name	Purpose
Bio rad	C1000 ™ Thermal cycler	PCR
Bio rad	iCycler, MyiQ™/bio-rad iQ5	qPCR
Bio rad	CFX manager	qPCR
Bio rad	Image lab	Gel imaging
Branson	Branson 3510 ultrasonic bath	Sonication
Eppendorf	Centrifuge 5810 R	Centrifuge (96 well plate)
MSE	Centaur 2	Centifuge (10ml)
Retsch	MM400	Grinding
Thermo Scientific	Heraeus Fresco 17 centrifuge	Centrifuge (eppendorf)
Thermo Scientific	Savant SPD121P Speedvac Concentrator + Refrig. Vapor trap	Drying samples for LC-MS
Wageningen University	Primer3Plus	Primer design
Waters	Acquity UPLC system coupled to Xevo TQ MS mass spectrometer	LC-MS
Waters	Masslynx 4.1	LC-MS analysis
Zeiss	Axioskop	Microscopy

Table S3. Measured MRM channels of LC-MS analysis.

Compound	Parent (m/z)	Daughter (m/z)	Dwell time	Cone (V)	Collision (V)
Sorgolactone	317.16	96.96	0.017	20	20
Sorgolactone	317.16	133.09	0.017	20	18
Strigol	329.14	96.96	0.017	18	20
Strigol	329.14	215.1	0.017	18	12
5-Deoxystrigol	331.2	97	0.017	15	22
5-Deoxystrigol	331.2	216.15	0.017	15	15
5-Deoxystrigol	331.2	234.15	0.017	15	10
D6-5-DS	337.1596	97.0165	0.017	16	22
D6-2'-epi-5-DS	337.1596	240.1896	0.017	16	10
Orobanchol	347.2	97	0.017	18	22
Orobanchol	347.2	205.2	0.017	18	18
Orobanchol	347.2	233.15	0.017	18	12
Sorgumol	347.2	317.2	0.017	18	8
C13-epistrigol	349.0958	217.1148	0.017	12	12
C13-strigolNa+	371.0958	274.1483	0.017	26	18

Table S4. Unpublished work on the strigolactone 5-DS and orobanchol exudation and root extract levels for African and Chinese sorghum lines determined by LC-MS.

		Plant material							
		Root exudates (pmol/plant/24h)				Root extracts (pmol/gram FW)			
		5-DS		orobanchol		5-DS		orobanchol	
Sorghum lines		pmol	s.e.	pmol	s.e.	pmol	s.e.	pmol	s.e.
African	P90950	161.6	5.0	5.1	1.2	417.0	48.4	0.0	0.0
	Sudan Z. Z.	107.4	31.8	1.8	0.6	286.1	13.7	1.5	0.4
	SRN39	0.2	0.1	2285.3	780.1	0.5	0.2	37.9	9.9
Chinese	Hui 39B	433.5	22.3	12.1	2.9	21.4	2.5	1.3	0.3
	Shanqui Red	157.5	42.5	6.9	1.0	91.9	14.0	0.5	0.1
	IS 4225	666.7	187.3	4.8	0.9	8.5	0.1	0.0	0.0

Table S5. Absolute strigolactone levels found in exudates and extracts for the current experiment determined for African and Chinese sorghum lines and five crosses of SRN39 and Shanqui red by LC-MS.

		Plant material											
		Root exudates (pmol/plant/48hours)						Root extracts (pmol/gram FW)					
		5-DS		Orobanchol		Strigol		5-DS		Orobanchol		Strigol	
Sorghum lines		pmol	s.e.	pmol	s.e.	pmol	s.e.	pmol	s.e.	pmol	s.e.	pmol	s.e.
African	P90950	65,7	32,7	0,3	0,2	0,0	0,0	2,1	0,7	0,3	0,2	0,0	0,0
	Sudan Z. Z.	449,9	89,8	0,8	0,2	6,6	6,1	5,2	2,1	0,1	0,0	0,0	0,0
	SRN 39	4,8	2,4	3346,6	820,5	0,0	0,0	0,3	0,1	6,4	3,2	0,0	0,0
Chinese	HUI 39B	3302,4	1386,2	11,3	4,4	4,8	3,8	27,1	13,7	0,4	0,2	0,0	0,0
	Shanqui red	1191,6	510,1	0,5	0,3	3,2	1,0	7,9	4,1	0,2	0,1	0,0	0,0
	IS4 225	1843,9	597,8	2,0	0,4	11,9	4,6	25,9	11,6	0,2	0,1	0,0	0,0
Crosses	SSD#3 177	569,1	119,5	0,7	0,1	0,0	0,0	2,8	0,8	0,0	0,0	0,0	0,0
	SSD#3 250	762,5	338,6	2,3	0,2	0,3	0,3	2,9	1,2	0,0	0,0	0,0	0,0
	SSD#3 302	7,8	3,2	407,4	91,7	0,0	0,0	0,4	0,1	0,8	0,4	0,0	0,0
	SSD#3 320	11,3	3,9	120,6	20,7	0,0	0,0	0,2	0,1	0,2	0,1	0,0	0,0
	SSD#3 767	12,9	6,1	3,2	1,2	0,0	0,0	0,2	0,1	0,0	0,0	0,0	0,0

Table S6. Overview of test results for putative PDR1 candidates. ***: sequence too similar to previous gene to design gene-specific primers. a) Domains found relative to the *P. hybrida* and *P. axillaris* domains. b) relative distance to petunia PDR1 genes. c) No upregulation (1), upregulation only seen in semi-quantitative PCR (2), upregulation in both semi-quantitative PCR and qPCR (3) and inconclusive results (4).

Putative candidate	Domains a	Homology to petunia PDR1 b	Upregulation under phosphate starvation c	Upregulation in presence of GR24 c	Likely PDR1 candidate
Sb01g000220	Extra	Distant	1	-	no
Sb02g022270	Extra	Intermediate	4	4	-
Sb02g024840	Extra	Distant	4	1	no
Sb02g034950	Gap	Distant	-	-	no
Sb03g004010	Gap	Intermediate	-	-	no
Sb03g027430	Extra	Distant	2	4	no
Sb03g027440	Extra	Distant	1	-	no
Sb03g027460 *	Extra	Distant	-	-	no
Sb03g027480	Missing	Distant	-	-	no
Sb03g027490 *	Missing	Distant	-	-	no
Sb03g027510 *	Missing	Distant	-	-	no
Sb03g027520	Gap	Intermediate	-	-	no
Sb03g033300	Gap	Intermediate	-	-	no
Sb04g007260	Exact	Closely related	3	2,4	yes
Sb04g007270 *	Exact	Closely related	-	-	yes
Sb05g024240	Extra	Closely related	4	2,4	yes
Sb10g024610	Missing	Intermediate	1	-	no

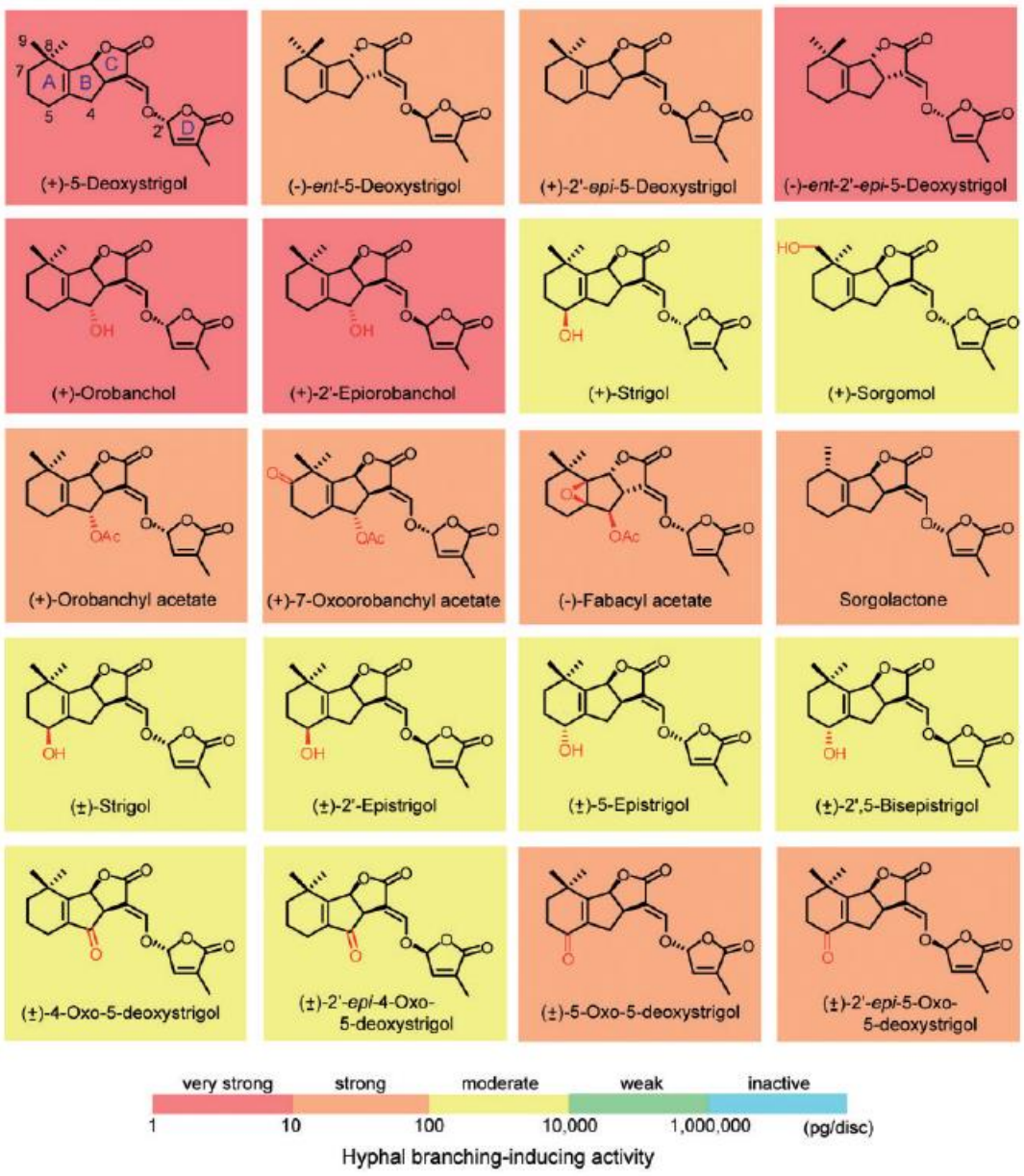

Figure S1. Chemical structures of natural and chemically modified natural strigolactones. Hyphap branching-inducing activities in *Gigaspora margarita* are indicated by a colored background to each chemical structure. From: Akiyama *et al.* (2010)[24]

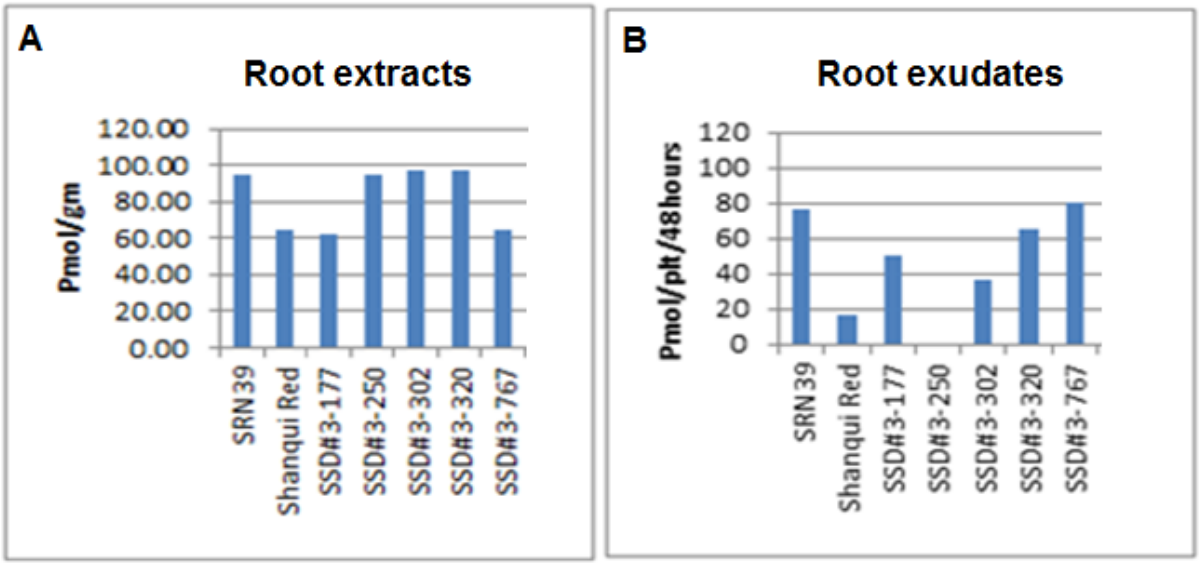

Figure S2. Strigol profile for SRN39, Shanqui red and five crosses of the two for A) root extracts and B) root exudates. Differences can be seen for the ratio of strigol biosynthesis and exudation. Although the amounts of strigol cannot be directly compared to each other for extracts and exudates the patterns can be compared. For example SSD#3-177 exudes some strigol and also has some strigol left in the roots while SSD#3-250 has more strigol in the root extracts but does not exude any of it. From: unpublished work