Novel insights into the early stages of ratoon stunting disease of sugarcane inferred from transcript and protein analysis

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ABSTRACT

Despite of the importance of ratoon stunting disease, little is known on the responses of sugarcane to its causal agent, the vascular bacterial endophyte *Leifsonia xyli* subsp. *xyli*. The transcriptome and proteome of young plants of a susceptible cultivar with no symptoms of stunting but with relative low and high bacterial titers were compared at 30 and 60 days after inoculation. Increased bacterial titers were associated with alterations in the expression of 267 cDNAs and in the abundance of 150 proteins involved in plant growth, hormone metabolism, signal transduction and defense responses. Some alterations are predicted to benefit the pathogen, such as the up-regulation of genes involved in the synthesis of methionine. Also, genes and proteins of the cell division cycle

were all down-regulated in plants with higher titers at both times. It is hypothesized that the negative effects on cell division related to increased bacterial titers is cumulative over time and its modulation by other host and environmental factors results in the stunting symptom.

Additional keywords: *Leifsonia xyli* subsp. *xyli*; plant-pathogen interaction, microarray, 2D-DIGE

INTRODUCTION

Ratoon stunting disease (RSD) of sugarcane occurs worldwide (Young 2016) and because of stunting it can cause a substantial impact on biomass production. Annual losses were estimated at US$ 11 million in Australia (Fegan et al. 1998) and US$ 36 million in Florida (Dean and Davis 1990). In Brazil, a recent study reported an annual loss of US$ 1 million considering only the incidence of RSD in the most planted variety grown in 29% of the cultivated area (Urashima et al. 2017). RSD, caused by the gram-positive Actinobacteria *Leifsonia xyli* subsp. *xyli* (Lxx), is regarded as one of the least tractable diseases of sugarcane because its external symptoms, characterized by reduction in height and in stalk diameter, can be easily mistaken by the effects of other factors that affect plant growth.

The main control method of RSD relies on using healthy cuttings (setts) taken from *in vitro* cultured or heat-treated canes raised in nurseries as planting material. Immersion of setts in hot water (50-52^{o}C) for 30-120 minutes is the most used heat treatment but its efficiency in eradicating the bacterium is variable (Damann Jr and Benda 1983; Carvalho et al. 2016), which contributes to the high incidence of Lxx-infected plants reported in commercial fields (Rago et al. 2004; Ponte et al. 2010; Urashima and Marchetti 2013; Li et al. 2014). Since sugarcane varieties differ in their resistance to Lxx multiplication (Davis et al. 1988; Comstock et al. 1996), breeding and selection for Lxx-restrictive varieties represents a promising control strategy. However, although the disease was first described

more than 70 years ago, there is little information on the molecular and physiological mechanisms of sugarcane involved in the interaction with Lxx.

Stunting is attributed to the plugging of the xylem vessels and resulting water stress by the association of bacterial cells and a matrix of uncertain origin and composition observed in basal internodes of mature stalks (Kao and Damann Jr. 1978) and resistance has been associated to the anatomy of the vessels which are more branched in resistant genotypes thus presumably restricting colonization (Teakle et al. 1978). At the physiological level, aside from impairing the activity of acid invertase in buds (Madan et al. 1986), modulating the activity of antioxidative enzymes and negatively interfering in photosynthetic parameters (Zhang et al. 2016a), it has also been reported that infection with Lxx induces changes in the production of auxin (IAA), gibberellic acid (GA3) and abscisic acid (ABA) (Zhang et al. 2016b). At the gene expression level, a study based on hybridizations of cDNA arrays containing 3,575 expressed sequenced tags (EST) identified 49 genes mostly related to signal transduction with altered expression in response to inoculation with Lxx (Ferro et al. 2007). Due to the poor understanding of RSD and to the lack of cost-effective and high-throughput screening methods to select resistant genotypes at the seedling stage, selection for resistance to this disease has not been the focus of breeding programs (Dal-Bianco et al. 2012). Thus, knowledge on the mechanisms involved in the early stages of the disease may reveal candidate genes that could be used as markers to identify resistant genotypes.

Leifsonia xyli subsp. *xyli* is a peculiar plant pathogen in many aspects. So far, its only described natural host is sugarcane (Mills et al. 2001; Zavaglia et al. 2016), where it systemically colonizes the xylem vessels, the mesophyll and the bundle sheath cells (Bailey 1977; Quecine et al. 2015). Its genome has an unusually high number of pseudogenes, indicating that it has undergone a process of genome decay that could account both for this restricted host range and for its slow and fastidious growing habit (Monteiro-Vitorello et al. 2004). Also, there is a remarkable low level of genetic variability among isolates, suggesting that it comprises a clonal population (Young et al. 2006; Zhang et al. 2016). A recent

review on the origin of RSD proposes that the progenitor species of modern sugarcane cultivars, *Saccharum officinarum* is not the natural host of Lxx but rather that the bacterium was acquired as an endophyte from *S. spontaneum* after artificial interspecific hybridization carried out at the beginning of the last century (Young 2016). As the damage caused by Lxx is directly correlated to its concentration in plant tissues (Bailey 1977; Davis et al. 1988; McFarlane 2002; Zekarias et al. 2012) and it can be detected in plants with no stunting or internal symptoms, this bacterium can be considered an endophyte whose pathogenicity depends on biotic and abiotic factors that control its multiplication in the host.

The objectives of this study were to compare changes in the transcriptome and in the proteome of a susceptible sugarcane variety in response to variations in the concentration of the pathogen within its tissues at the beginning of disease development before the appearance of the stunting symptom. For this, infected plants with low bacterial titers were either mock-inoculated or inoculated with Lxx in this case to create plants with increased bacterial titers, and changes in the abundance of approximately 2,500 proteins and in the expression of 14,522 sugarcane expressed sequenced tags (SAS) were compared at two times after inoculation.

MATERIALS AND METHODS

Plant material and inoculation: Three-bud setts of the susceptible variety SP80-3280 were harvested from Lxx-infected first ratoon plants which are kept in a greenhouse as a source of inoculum for experimental purposes and thermally treated by water immersion at 50.5°C for 2 hours to reduce the population levels of Lxx (Carvalho et al. 2016). The material was cut into one-bud setts using a disinfected cane knife, immersed in a fungicide solution (90 g of 70% thiophanate-methyl L^{-1} water) and planted in 0.5-L pots containing Basaplant substrate (Base). Thirty days after planting, plants were transferred to 22.5-L pots (3 plants/pot) containing a 2:1 mixture of steam-sterilized topsoil and substrate supplemented with 2 g L^{-1} of 15-8-12 Basacote (3M) slow release fertilizer and were inoculated 7

days after transplanting by cutting off the shoots 2 cm above the soil with sterile scalpel blades, perforating the remaining stub with sterile needles (three perforations/stub), and by placing 50 µL of a liquid culture (OD_{600} = 0.8) of Lxx strain CTCB07 grown in M-SC medium (Teakle and Ryan 1992, Monteiro-Vitorello et al. 2004) on the surface of the stub. The experiment consisted of four treatments: two inoculation conditions (inoculated with Lxx or mock-inoculated with sterile culture medium; Lxxl or MI treatments, respectively) x two evaluation times (30 and 60 days after inoculation – DAI). The evaluation times were chosen based on previous data which indicated that 30 DAI was the minimum time required to detect increases in bacterial titers after inoculation and at 60 DAI these increases were more pronounced (G. de Carvalho, unpublished data). Each treatment was represented by three replicates arranged in a randomized block design and each replicate was composed of a pot with three plants. For Lxx quantification and RNA and protein extraction, 1g of leaf whorl tissue including the shoot apex, was collected from each plant of a replicate and pooled. Lxx was quantified by real time PCR (Carvalho et al. 2016).

RNA extraction, microarray hybridization and data analysis

Total RNA was extracted from 100 mg of plant tissue with Trizol (Thermo Fisher Scientific, Waltham, MA, U.S.A.), treated with DNAse I Amplification Grade enzyme (Thermo Fisher Scientific) and purified with RNeasy Mini Kit (Qiagen, Hilden, Germany). The concentration of RNA was determined in a NanoDrop (Thermo Scientific, Waltham, MA, U.S.A) and its quality was evaluated on 1% agarose/formaldehyde gel. Two of the biological replicates were used for microarray hybridizations. An aliquot of the RNA was used as template in a PCR reaction to assess the possibility of DNA contamination. Hybridizations were performed on the customized sugarcane CaneRegNet 4x44K chip (Agilent Technologies, Santa Clara, CA, U.S.A.) consisting of 43,802 probes corresponding to 14,522 Sugarcane Assemble Sequences (SAS) released by the SUCEST - Sugarcane EST project (Vettore et al. 2003; Lembke et al. 2012). Sample preparation, hybridizations and data analysis were performed according to Lembke

et al. (2012). Comparisons were made between LxxI and MI plants within each time after inoculation. Dye swaps were used for each time point analyzed. The normalization was composed of two steps using a non-linear LOWESS normalization and a modified HTself method (Vêncio and Koide 2005; Lembke et al. 2012). A gene was considered as differentially expressed if 95% of confidence was obtained using the modified HTself method. The data are available in the GEO (Gene Expression Omnibus) of the National Center for Biotechnology Information (NCBI) http://www.ncbi.nlm.nih.gov/geo/ under the accession number GSE87826.

Validation of differentially expressed genes by real time quantitative PCR (qPCR)

Differentially expressed genes were selected from each time point for validation by qPCR. Primers for these genes were designed using the software Primer3Plus (Untergasser et al. 2007). The specificity of the primers was checked *in silico* against the SUCEST database (www.sucest-fun.org) using the BlastN tool and by checking for the presence of a unique peak in the melting curve of the qPCR. Primer efficiency was evaluated using the software LinReg PCR (Ramakers et al. 2003). cDNA was synthesized using the SuperScript III First-Strand Synthesis Super Mix (Thermo Fisher Scientific). PCR amplifications were done using Platinum Sybr Green qPCR supermix UDG kit (Thermo Fisher Scientific). The analysis of differentially expressed genes was performed with the REST software (Pfaffl et al. 2002). All qPCR reactions were run in duplicates. The polyubiquitin and 14-3-3 (accession IDs SCCCST2001G02.g and SCCCLR1048F12.g) genes were used as normalizers (Papini-Terzi et al. 2005). The three biological replicates were used for validation. The R software was used to calculate the Pearson´s correlation coefficient between the microarray and the qPCR data.

Extraction and precipitation of leaf proteins: proteins were extracted from 0.5 g of plant tissue macerated in 10 mL of extraction buffer (10% w/v trichloroacetic acid and 0.7% v/v 2-mercaptoethanol in acetone) and precipitated for 2 hours at -20°C. The tubes were centrifuged for 15 minutes at 12,000 rpm and -20°C, the

supernatant was discarded, and the pellet was washed twice with 0.7% 2-mercaptoethanol in acetone, dried at 4°C and resuspended in a solubilizing buffer (33 mg of pellet/1 mL of buffer) containing 7 M urea, 2 M thiourea, 4% CHAPS, 2% Pharmalyte (immobilized pH gradient buffer - IPG) and 0.3% of DTT (Amalraj et al. 2010). An aliquot was used for total protein quantification by the Bradford assay using bovine serum albumin as a standard. The proteins were precipitated in a methanol solution with 0.1 M ammonium acetate at -20°C for 12 hours. A ratio of 1,200 µL of precipitant solution to 300 µL of protein sample was used. The tubes were centrifuged at 12,000 rpm for 30 minutes at 4ºC and the supernatant was discarded. Then, 80% methanol was added to the pellet and these were kept at -20ºC for 1 hour. The tubes were centrifuged at 12,000 rpm for 30 minutes at 4º C and the supernatant was discarded. Acetone (80%) was added to the pellet and this was kept at -20ºC for 1 hour and centrifuged again at 12,000 rpm for 30 minutes at 4ºC. The supernatant was discarded, and the final pellet was dried at 4ºC in a desiccator for 1 day and stored in 100 µL of ethanol.

2D-DIGE analysis: analyses were performed at Applied Biomics (Hayward, CA, USA) according to standardized procedures. Briefly, proteins were solubilized in buffer (30 mMTris-HCl, pH 8.8, containing 7 M urea, 2 M thiourea, and 4% CHAPS) for analysis in 2D gels, and quantified with the Bio-Rad Protein Assay kit (BioRad, Hercules, CA, USA). Proteins of the MI treatments were labeled with Cy3-red and those from the LxxI treatment were labeled with Cy5-blue. Proteins of an internal standard (IS) comprising an equimolar mixture of all samples were labeled with Cy2-yellow. Labeled samples were subjected to isoelectric focusing performed in IPG strips at pH 3–10 (GE Healthcare, Chalfont St. Giles, U.K.) and were separated by size in SDS-PAGE. Twenty-five micrograms of protein per sample/run were used.

Gel images were generated in a Typhoon Trio imager (GE Healthcare) and analyzed using the ImageQuant software (v.6.0, GE Healthcare). The standardization and statistical analysis of protein volumes were carried out using the biological variation analysis (BVA) module of the DeCyder software (v.6.5, GE

Healthcare). Comparisons of normalized protein abundances were made within each time between the LxxI30 *vs* MI30 and the LxxI60 *vs* MI60 treatments as in the transcriptome experiment as well as between times for Lxx-inoculated (LxxI60 *vs* LxxI30) and mock-inoculated (MI60 *vs* MI30) treatments using Student´s *t* test. Proteins with significant variations in abundance ($P < 0.05$) and mean fold changes (r) lower than -1.3 or greater than 1.3 were considered as differentially accumulated and chosen for MS analysis.

Protein sequencing: selected proteins were excised from the gel using an Ettan Spot Picker device (GE Healthcare), dried, hydrated, and digested with trypsin in digestion buffer (Trypsin Gold, Promega, Madison, WI, USA) at 37°C. Peptides were extracted from the gel in TFA buffer under agitation, desalinated using C-18 Zip-Tips (Millipore, Billerica, MA, USA) columns, mixed with CHCA matrix (alpha-cyano-4-hydroxycinnamic acid), and analyzed by MALDI-TOF-MS and TOF/TOF tandem MS/MS in an AB SCIEX TOF/TOFTM 5800 system (AB SCIEX, Framingham, MA, USA). Approximately 10–20 of the most abundant peptides in each sample (excluding autolytic trypsin peptides and other known background ions) were subjected to fragmentation and tandem mass spectrometry analysis.

Peptide spectra were used in searches against the NCBI non-redundant protein database using the GPS Explorer and MASCOT software (www.matrixscience.com) to identify proteins with similar spectra. Searches were performed with no constraints to protein molecular weight or isoelectric point, with variable carbamidomethylation of cysteine and oxidation of methionine residues and allowing one missed cleavage in the parameters. Only hits with confidence intervals > 95% for protein or total ion scores were considered significant and among these the hit with the highest score was reported.

Bioinformatic analyses and data mining: Differentially expressed genes and proteins were assigned to functional categories based on the classification of the SUCEST database (Vettore et al. 2003).

RESULTS

Quantification of bacterial titers and plant growth: Lxx titers differed ($P < 0.05$) between MI and LxxI plants at both times, indicating that the inoculation method was successful in establishing treatments with different bacterial densities (Figure 1). Moreover, the endophytic levels of Lxx increased five-fold in the mock inoculated plants in the lapse of 30 days (7 and 34 Lxx cells/100 ng of plant DNA at 30 and 60 DAI, respectively; $P<0.05$), whereas in the inoculated plants, bacterial titers increased ten-fold (27 and 263 Lxx cells/100 ng of plant DNA at 30 and 60 DAI, respectively; $P<0.05$). These titers are in the same order of magnitude as those reported for 8 months-old first-ratoon plants of the same variety (Carvalho et al. 2016). Notwithstanding the contrasting bacterial titers, LxxI and MI plants presented no visual differences in growth at both times. Also, no symptoms of water stress such as leaf rolling or senescence were observed in any treatment. The mean temperature and relative air humidity registered during the experiment was 25.5°C and 58%, respectively.

Microarray: A total of 267 differentially expressed genes ($P < 0.05$) were identified between the LxxI and the MI treatments (Supplementary Table S1). There were noticeable differences between the two times of analysis since only fourteen genes (5% of total) were differentially expressed at both times (Figure 2). Most genes exclusively detected at 30 DAI were down-regulated (82 down-regulated and 22 up-regulated) whereas most at 60 DAI were up-regulated (110 up-regulated and 39 down-regulated) (Figure 2). Moreover, differences were also found related to gene functional categories, as genes encoding proteins related to cytoskeleton functions were down-regulated only at 30 DAI whereas most of those related to hormone metabolism (ethylene, abscisic acid and IAA), were up-regulated only at 60 DAI except for a lipoxygenase that also was up-regulated at 30 DAI. Another interesting difference relates to the regulation of genes involved in signal transduction, the majority of which were down-regulated at 30 DAI but up-regulated at 60 DAI. Notwithstanding these differences, a strikingly similar response was found between 30 and 60 DAI regarding the expression of genes related to the control of the cell

division cycle, the largest represented category, where most were down-regulated (Figure 3).

Validation of microarray data by real time PCR quantification of gene expression (qPCR): Twenty-nine differentially expressed genes were chosen for validation (Supplementary Table S2), being thirteen exclusives of each DAI and three common to both times. In all cases there was an agreement between the qPCR and the microarray results (Figure 4). The Pearson´s correlation coefficient between the microarray and the qPCR data was 0.82, indicating a good correlation.

2D-DIGE: 2D-DIGE protein analysis provided a satisfactory resolution of approximately 2,500 proteins (Supplementary Figure S1). After image processing and statistical calculations based on standardized volumes of each protein spot, 155 proteins were detected with significant changes in abundance for the comparisons studied ($P<0.05$). Abundance ratios ranged from -4.88 to +2.56.

Variation in protein abundance as a function of variation in bacterial titers and of variation in bacterial titers and time: The effect of different bacterial titers on the abundance of proteins was studied by comparing LxxI and MI plants at each DAI (LxxI 30 *vs* MI30 and LxxI60 *vs* MI60). A second type of comparison was made between DAIs for the LxxI and MI (LxxI60 *vs* LxxI30 and MI60 *vs* MI30) to identify variations related both to differences in bacterial titers and to time. A total of 150 proteins from diverse functional classes were identified in these comparisons (Supplementary Table S3), being 7 in the first, 148 in the second and 5 in both. In the first type of comparison, three were exclusive of each DAI and one (calreticulin 2) common to both (Figure 2), whereas in the second type, 13 were identified exclusively in the mock-inoculated contrast (MI60 *vs* MI30), 66 were identified exclusively in the inoculated (LxxI60 *vs* LxxI30), and 69 were identified in both contrasts (Figure 2). Among the 13 proteins of the (MI60 *vs* MI30) contrast, the largest number (5) belong to the defense class. In the (LxxI60 *vs* LxxI30)

contrast, the carbohydrate metabolism class was the more numerous (25 proteins), followed by protein metabolism (15), and transcription regulation (6). Other classes with lower number of proteins were also detected, including the cell division cycle class. Proteins common to the MI60 *vs* MI30 and LxxI60 *vs* LxxI30 contrasts were grouped into various classes (Supplementary Table S3).

DISCUSSION

Increased bacterial titers induce discrete changes in gene expression and protein accumulation

Of the 14,522 SAS analyzed, significant changes were found in the expression levels of a small proportion of genes (1.9%) in response to increased bacterial titers considering the two times of analysis. This agrees with a previous study which analyzed the expression of 3,575 ESTs also by hybridization of a cDNA array and found only 49 genes (1.4%) with altered expression in response to inoculation with Lxx (Ferro et al. 2007). This sharply contrasts with other pathosystems where intense transcriptional reprogramming is commonly reported most likely because our results reflect responses to quantitative changes in population densities of a slow-growing endophytic pathogen rather than to infection by biotrophic and necrotrophic pathogens which result in clearly defined symptoms within a short period. Also, the proportion of genes with altered expression could relate to the bacterial levels in the plant tissue and to the fact that we analyzed leaf whorl and shoot apex rather than xylem tissue where the bacterium grows. The number of differentially expressed genes was 43% higher at 60 DAI than at 30 DAI, suggesting an increased transcriptional response of sugarcane to increased bacterial titers. Moreover, the small overlap between the sets of genes detected at these times suggests that the responses were not only quantitative but also qualitative in nature. Changes at the protein level, however, did not follow the changes in gene expression, since few differentially accumulated proteins were found between treatments at 30 and 60 DAI. This could be due not only to the general poor agreement between transcriptomic and proteomic studies caused by

biological and methodological factors (Maier et al. 2009), but also possibly reflects the slower responses of the proteome to this type of vascular pathogen.

Plants with increased levels of Lxx present down-regulation of genes involved in the cell division cycle

Inoculation with Lxx resulted in the down-regulation of genes involved in the cell division cycle at both DAI. Among these are classical genes regulating the cell cycle progression and cell division, such as cyclin A (CycA), which is considered to be involved in the control of the S-M transition, cyclin B (CycB) and a cyclin dependent protein kinase (CDKB1;1) involved in the G2-M transition (Inzé and De Veylder 2006), and a gene coding for a syntaxin similar to the KNOLLE protein of *Arabidopsis* required for cytokinesis (Lauber et al. 1997). In addition, various genes coding for proteins required for successful chromosome replication and transmission (mini-chromosome maintenance proteins 2-7; origin recognition complex subunit 6; mitotic spindle checkpoint proteins MAD2 and MAD3; chromosomal structural maintenance proteins; microtubule associated proteins, condensins, kinesins, chromatin assembly factor) were down-regulated. These results agree with the observed down-regulation of genes involved in DNA metabolism as the down-regulation of the cell division cycle is expected to be accompanied by a down-regulation in DNA replication and processing as well. Moreover, the repression of all genes of the cytoskeleton functional category at 30 DAI may also be related, since these comprise mostly kinesins, a protein family that includes members essential for cell growth and division by mediating the reorganization of the microtubules in anticipation of the mitotic division (Li et al. 2012) and a gene encoding for a microtubule binding protein similar to TANGLED-1 of maize involved in the orientation of cytokinesis (Smith et al. 2001).

An interesting finding relates to a gene (SCCCLB1003E01.g) similar to one coding for a 14 KDa proline-rich DC 2.15 protein of carrot that at 60 DAI was the most down-regulated gene of this study besides being down-regulated at 30 DAI as well. In carrot as in other plant species, this gene occurs in small gene families

and although not a classical gene of the cell cycle, it was tentatively considered as such because transcripts of similar genes from maize and bean specifically accumulate in the cortical region of the root meristem where cell division occurs (John et al. 1992; Choi et al. 1996). In addition, suppression of its expression in carrot caused retarded growth and development and a reduced ratio of phloem/xylem cells in the vascular bundles, suggesting a role in the differentiation of these cells (Holk et al. 2002). Additional studies should be performed to detail the expression profile of this gene in the intercalary meristem during the interaction of sugarcane with Lxx and its possible contribution to the stunting symptom characteristic of RSD.

Hormone metabolism

Plant hormones play key roles in controlling cell division, plant growth and stress responses, and as such it was expected to find genes involved in their metabolism in this study. Genes related to the metabolism of abscisic acid (ABA), ethylene (ET), gibberellin (GA), auxin (IAA), and JA were all up-regulated in Lxx-inoculated plants, mostly at 60 DAI. By contrast, no genes related to the synthesis of salicylic acid (SA) were detected. Our results are in general agreement with a previous study on hormonal changes in sugarcane conducted in a similar experimental design (Zhang et al. 2016b). The concentrations of ABA, IAA, and GA were evaluated in plants regenerated from Lxx-inoculated setts at 90, 120, 150, and 180 days after shoot emergence. Although the levels of Lxx were not evaluated as in our study, the concentration of ABA increased and of IAA decreased at all evaluation times in inoculated plants compared to non-inoculated, whereas the concentration of GA decreased in the inoculated plants only at 180 days. In our study, the up-regulation of the regulatory gene in the biosynthesis of ABA (9-cis-epoxycarotenoid dioxygenase) at 60 DAI was accompanied by the up-regulation of an ABA 8-hydroxylase-1 gene involved in its degradation at the same time, possibly to cope with the increased levels of this hormone in response to higher bacterial titers. The reported reduced levels of IAA could result from the up-regulation of a gene (indole-3-acetic acid-amido synthetase GH3.8) that prevents

the accumulation of free IAA (Ding et al. 2008). In addition, it is worth mentioning that the over expression of GH3.8 in rice also hinders plant growth and development (Ding et al. 2008). The up-regulation at 30 DAI of a gene coding for an oxidase (cytochrome P450 ent-kaurenoic acid oxidase) involved in the final three steps of the biosynthetic pathway of GA (Helliwell et al. 2001) suggests an expected increase rather than a decrease in the concentration of this hormone, but this could result from the differences in plant genotypes and times of analysis between our study and that of Zhang et al. (2016b). The up-regulation of three genes involved in the last two steps of the synthesis of ET (one ACC synthase and two ACC oxidases) and of two involved in the metabolism of JA (lipoxygenase - LOX and a jasmonate ZIM motif protein) extends the range of hormonal responses of sugarcane to Lxx reported by Zhang et al. (2016b), but additional experiments are needed to confirm their predicted increased levels *in planta* in response to increased Lxx titers.

Our data indicate a more complex hormonal response of sugarcane to Lxx than that previously reported by Zhang et al. (2016b). Given the dynamic nature of plant growth control by hormones in response to various stimuli, the development of RSD symptoms is also expected to respond dynamically to varying environmental conditions. As a fact, in plants with stunting the length of the internodes is not uniformly reduced along the stem compared to healthy plants, with some internodes presenting normal length, possibly corresponding to growth periods under more favorable conditions. The balance between ABA production and degradation in response to Lxx as suggested in this study could represent an underlying mechanism of this growth pattern, since a combination with any other factor that enhances the production of ABA would aggravate the symptoms. The accentuated severity of RSD reported under conditions of moisture stress (Rossler 1974; Ngaruiya et al. 2005) could be explained by this phenomenon given that the production of ABA is well recognized to be drought-responsive. The same applies to ET whose synthesis is reciprocally regulated with that of ABA during stress (Li and Huang 2011) and inhibits the mitotic cell cycle under high osmotic conditions by reducing the activity of CDKA following a suggested pause-and-stop model

(Skirycz et al. 2011) where the cell cycle is rapidly arrested after the sensing of osmotic stress and resumes when conditions improve.

Aside for their role in plant growth, these hormones also play crucial roles in defense, notably ABA, JA and ET. However, the available information on this pathosystem presently does not allow predicting if the hormonal changes induced by Lxx act by increasing or decreasing the susceptibility of sugarcane towards this pathogen given that they exert positive or negative roles in disease promotion depending on the characteristics of the interaction. Moreover, it is difficult to determine changes that are directly related to the pathogen attack from those that are side effects of the extensive crosstalk between these hormones.

Signal transduction and transcription regulation associated to plant growth

The alteration of the cell cycle would be expected to be accompanied by changes in signal transduction and regulation of plant growth processes. Most down-regulated genes at 30 DAI involved in signal transduction comprised kinases and two of these were classified as AURORA kinases whose subtypes play different major roles in the cell division cycle, such as regulation of the cycle progression, chromosomal segregation and cytokinesis. In *Arabidopsis*, the down-regulation of an AURORA kinase by RNAi compromised growth and development by arresting the development of the apical meristem (Petrovská et al. 2012). Two genes coding for Rac GTPase-activating proteins were also down-regulated while a WD40-like beta propeller repeat family protein was up-regulated. Besides the cell cycle, these proteins regulate various other cellular processes. On the other hand, at 60 DAI, genes encoding for receptor Ser/Thr kinases involved both in plant development and defense were up-regulated in LxxI plants perhaps due to the recognition of the pathogen in the mesophyll and bundle sheath cells after a certain level of cell-density has been achieved. The only LRR receptor serine/threonine-protein kinase down-regulated at this time was the ERECTA gene. *Arabidopsis* mutants of this gene showed short lateral organs and internodes, a phenotype attributed to the arrest of the cell cycle (Van Zanten et al. 2009). Pathogen recognition by receptors could then lead to the differential expression of genes

involved in downstream signaling (MAPKKK, calcium dependent protein kinase and calmodulin binding protein) at 60 DAI.

Numerous genes encoding transcription factors (TF) involved in several aspects of plant growth and defense were up or down-regulated at both times, reflecting a complex crosstalk between genes with different developmental roles. At 30 DAI, these include down-regulated genes coding for TFs of the SCARECROW-Like 28 (SCL28) class that controls plant development and of the CXC-domain containing residue involved in cell division (Hauser et al. 2000). At 60 DAI, a different and more diverse set of TFs, mostly involved in growth processes and responses to stress with interplay of phytohormones, notably ABA and ET, were detected. These include genes coding for growth regulating factor (GRF), PLATZ, MYB, and NAC TFs. A member of the WRKY51 class that acts as a negative regulator of gibberellin and a positive of ABA signaling (Xie et al. 2006) was up-regulated, agreeing with the reported levels of these hormones in Lxx-infected plants (Zhang et al. 2016b). Also noteworthy was the up-regulation of a KNOX TF, once members of this protein family are involved in the maintenance of the meristem. On the other hand, two members of the TCP family of TFs (PCF5 e PCF7) were down-regulated. Members of this family may act by stimulating or inhibiting the cell cycle (Müller and Leyser 2011). Six genes coding for AP2/EREBP family members were down or up-regulated at 30 or 60 DAI, two of which were identified as the ethylene-responsive TFs 1 and 4 (ERF1 and ERF4). The latter is a transcriptional repressor of ET and ABA and was up-regulated at 30 DAI and the former is an ET activator whose up-regulation at 60 DAI coincided with the up-regulation of ACC synthase and ACC oxidase. Finally, at both times chromatin remodeling complex ATPase ISW1 genes involved in cell expansion in *Arabidopsis* (Huanca-Mamani et al. 2005) were down-regulated.

Host defense through reactive oxygen species, antimicrobial compounds and hypoxia

Of the few reported defense responses of sugarcane to Lxx, the production of reactive oxygen species (ROS) seems to be a major one as the activity of the

antioxidative stress enzymes superoxide dismutase and peroxidase increased whereas that of catalase decreased in inoculated plants evaluated starting 150 DAI (Zhang et al. 2016a). Also, increased concentrations of O^{2-} were reported in Lxx inoculated plants at 240 DAI. Our data support this previous report and indicated that this response can be detected at earlier times at the gene expression level as indicated by the up-regulation of a NADPH respiratory burst oxidase protein B (Rboh) and of a peroxidase (Pox). Three other peroxidase genes were up-regulated at 60 DAI. These genes are responsible for producing ROS which are secreted to the apoplast as defense signaling or accumulated in the vacuole where they contribute to cell wall reinforcement (Almagro et al. 2009) perhaps contributing to the observed uneven thickness of the cell walls observed in Lxx-infected plants (Zhang et al. 2016b). Also, increased levels of ROS could make the xylem environment more inhospitable to Lxx. The differential expression of a universal stress protein A (USPA) at this time could also be related to ROS production or to a reduction in water availability due to xylem clogging, since its chaperone activity has been related to the control of these oxidative compounds under drought conditions (Isokpehi et al. 2011). In addition to triggering ROS responses, increased bacterial titers at 60 DAI also lead to the up-regulation of genes related to the production of antimicrobial agents, namely phenylalanine ammonia liase (PAL), lipoxygenase (Lox) and cytochrome P450. PAL is the first enzyme in the biosynthesis of phenylpropanoids, from which several antimicrobial compounds are produced (Dixon et al. 2002) whereas Lox, besides participating in the synthesis of JA as mentioned before, generates oxylipins with diverse roles in plant defense against pathogens and insects through the oxidation of fatty acids (Porta and Rocha-Sosa 2002). Cytochrome P450 proteins participate in several cell processes including detoxification of harmful substances and synthesis of defense-related compounds such as phenylpropanoids, alkaloids and terpenoids, as well as the phytohormones GA, auxin and JA (Schuler 1996; Chapple 1998; Schuler and Werck-Reichhart 2003). Metabolomic studies should confirm if these compounds accumulate in sugarcane as a function of Lxx colonization and if they exert any inhibitory effect on its growth.

As an obligate aerobe who colonizes the microaerophilic environment of the xylem vessels, it is expected that any changes in the availability of oxygen would have a substantial impact on the ability of Lxx to grow in the host tissues. The up-regulation of genes commonly expressed under hypoxia both at 30 (pyruvate phosphate dikinase - PPDK; adenylate kinase - AK) and at 60 DAI (pyrophosphate-fructose-6-phosphate 1-phosphotransferase – PFP; pyruvate decarboxylase - PDC; alcohol dehydrogenase – ADH; hypoxia-responsive family protein) in Lxx-inoculated plants indicates a response of sugarcane to higher bacterial titer towards a reduction in the availability of O_2 probably related to hormonal signaling mediated by ET and MYB TFs as suggested by the up-regulation of the ethylene-dependent hypoxia-marker-genes ADH and PDC (Peng et al. 2001).

Pathogen-beneficial transcriptional alterations of the host include suppression of host defenses and up-regulation of genes related to the synthesis of methionine and transport of sugar

Some host physiological modifications induced by pathogens represent remarkable adaptive mechanisms of microorganisms. Viewing Lxx as an obligatory endophyte of sugarcane, modification of the host environment would be crucial for its growth and understanding these mechanisms could provide insights as to means of reducing the impact of RSD on sugarcane production. At 30 DAI, we observed a down-regulation of genes coding for antimicrobial compounds such as phenylpropanoids (4-coumarate CoA ligase) and the pathogenesis-related proteins (PR-proteins) defensin (PR-12) and thaumatin (PR-5) which are markers of systemic acquired resistance. The down-regulation of these genes at 30 DAI may contribute to suppress the plant immune response thus favoring Lxx growth at the early stages of colonization. By contrast, the up-regulation of a gene coding for methionine synthase (MS) at 30 DAI potentially represent another example of pathogen-beneficial modification since Lxx growth *in vitro* is greatly enhanced by the addition of this amino acid because two genes of the methionine biosynthetic pathway are probably non-functional (Monteiro-Vitorello et al. 2004). Thus, an increase in the synthesis of methionine induced by the pathogen at the early

phases of colonization, besides up-regulating the synthesis of ET, could also provide the needed supply of this amino acid for pathogen establishment and posterior growth. On the other hand, methionine synthase is also involved in the synthesis and regeneration of S'adenosyl-L-methionine (SAM), which is a precursor of spermidine, a polyamine that is essential for prokaryotic cell growth and protection from various stresses, including ROS (Shah and Swiatlo 2008). As Lxx does not have the genes necessary for the synthesis of this compound, it would be interesting to determine the role of spermidine on its growth in the host. Another intriguing finding related to the methionine pathway was the up-regulation of a nicotianamine synthase (NS) gene that is known to be induced under conditions of iron deficiency to produce nicotianamine through the trimerization of SAM. As nicotianamine plays an important role in mineral homeostasis, higher bacterial levels would up-regulate NS to cope with mineral sequestering by the pathogen and a candidate mineral would be iron, since hemin is required for its growth *in vitro*. SAM is also important to the biosynthesis of ET indicating a major role of methionine in the interaction of sugarcane with Lxx.

The up-regulation at 60 DAI of a SWEET14 gene in LxxI plants provides another example of Lxx-beneficial alteration of sugarcane as it has been proposed that plant pathogens may divert the carbon flux of their hosts to support their own growth by interfering in the expression of genes of the SWEET class of sugar transporters (Chen et al. 2010). Whether this interference results from the action of an effector as in the case of the *pthXo1* effector of the xylem-dwelling *Xanthomonas oryzae* pv. *oryzae* which induces the expression of OsSWEET11 (Chen et al. 2010) is an interesting line of research on the pathogenicity mechanisms of Lxx. Additionally, since SWEET transporters can also enhance pathogen growth through interaction with copper transporters to remove toxic copper from the xylem (Lapin and Van Den Ackerveken 2013), it is interesting to note that a copper export ATPase, which can be involved in Cu sequestration into the vacuole and/or transport in xylem vessels (Printz et al. 2016) also was up-regulated at 60 DAI, highlighting the activation of a potential toxic copper removal mechanism by Lxx.

Proteomics data

Among the seven differentially accumulated proteins, a calcium-binding chaperone of the endoplasmic reticulum (calreticulin – CRT2) was less abundant in LxxI plants both at 30 and 60 DAI. Recognized primarily as a regulator of calcium homeostasis in both animals and plants, CRT proteins have also been implicated in a variety of plant processes, such as plant growth and responses to stresses, including pathogen attack. CRT2 is a mediator of salicylic acid-dependent immune responses as the overexpression of the CRT2 gene leads to higher levels of SA and of expression of SAR marker-genes, including PR-5 (Qiu et al. 2012). Thus, it is reasonable to assume that the observed reduction in the expression of PR-5 observed in LxxI plants at 30 DAI could be related to the lower abundance of CRT2. At 30 DAI, in contrast with the down-regulation of cyclin transcripts of the A and B classes, higher titers of Lxx lead to increased levels of a H;1 class cyclin, whose role in the control of the cell cycle is not firmly established but in *Arabidopsis* has been associated to drought responses (Zhou et al. 2013). A polyphenol oxidase (PPO) was also more abundant, indicating that the production of quinones with antimicrobial properties may also occur in sugarcane in response to Lxx along with the production of phenylpropanoids and oxylipins as evidenced from the transcriptomic data. At 60 DAI, three spots of gluthatione S-transferase, another key enzyme involved in stress response, differentially accumulated in LxxI plants, evidencing a detoxicating reaction of sugarcane to the accumulation of stressing agents perhaps in response to the generation of reactive electrophile species resultant from the up-regulation of cytochrome P450.

Because of the few proteins identified between treatments when LxxI and MI plants were compared within DAI and since there also was a significant increase in Lxx titers between mock-inoculated plants along the time, we compared the proteomes of the MI and of the LxxI plants between DAI. Since these data are not directly comparable to the results of the microarray analyses because of the variation both in Lxx titers and in time, we highlighted some results that tentatively corroborate the transcriptomic data. For instance, changes in protein abundances

common to both comparisons (69 out of 148) may represent developmental responses of sugarcane or a common response to the growth of the bacterium since bacterial titers increased over time in both treatments. Also a larger set of proteins was uniquely identified in the comparison LxxI60 *vs* LxxI30 where variation in bacterial titer was higher than in the MI60 *vs* MI30 comparison.

In both comparisons, proteins representing the same major functional categories identified in the microarray analysis were detected and, in some cases, there was a direct gene-protein relationship, such as PAL, methionine synthase, ADH, PFP, and UBC9, whereas in other the results complemented the transcriptomic data. For instance, the accumulation of SAM at higher levels in LxxI plants at 30 DAI compared to 60 DAI together with the up-regulation of the methionine synthase, ACC synthase and ACC oxidase genes completes the pathway from the synthesis of methionine to ET and corroborates the involvement of this hormone in the response to Lxx. Also, evidences of responses to hypoxia, besides being supported by the accumulation of ADH and PFP were strengthened by the accumulation of three protein spots identified as sucrose synthases which are also known to accumulate under this condition. Finally, different components of the ubiquitin/proteasome system (UPS) were identified at the transcript (ubiquitin conjugating and ligase enzymes - E2 and E3) and protein levels (five subunits of the proteasome), indicating active protein degradation.

Host growth inhibition and ratoon stunting disease

Despite of its economic importance, there is little information on the effects of RSD on the transcriptome of sugarcane and none so far on its proteome. Once it is still unclear which metabolic pathways are activated as responses of the host to Lxx, our study focused on young susceptible plants at the early times of plant and disease development. By inoculating a set of plants, the experimental conditions simulated an increase in the population densities of Lxx over time and by comparing their responses to the responses of a set of non-inoculated ones, both sets infected with the bacterium, it was possible to detect genes and proteins whose changes in expression or abundances coherently explain the genesis of the

main symptom of RSD. An overview of sugarcane responses at the transcriptome level was elaborated (Figure 5). Our data indicate the negative control of the cell cycle associated with higher bacterial cells as one of the main determinant of stunting. The cumulative negative effects on the division cycle in response to increasing bacterial titers over time modulated by hormones and environmental factors would lead to stunting. Given that the progression of the cell cycle is inhibited by water stress, it is possible that this results from the reported occlusion of the xylem vessels in plants infected with Lxx (Teakle et al. 1973). This conclusion is further supported by changes in other genes and proteins associated to drought and hypoxia here reported. However, since LxxI plants did not display visual symptoms of water stress or growth reduction, this conclusion needs to be confirmed by testing for water restriction or xylem clogging under these experimental conditions. Alternatively, it is possible that a yet uncharacterized compound of bacterial origin, perhaps an analog of ABA, might also negatively affect plant growth (Monteiro-Vitorello et al. 2004) and this could be tested, for example, by assessing gene expression after exposing plants to Lxx-culture filtrates.

Leifsonia xyli subsp. *xyli* behaves similarly to other fastidious and vascular-limited bacteria such as *Herbaspirilum rubrisubalbicans* and *Xanthomonas albilineans* in sugarcane, *Xylella fastidiosa* and *Spiroplasma citri* in citrus and 'Candidatus phytoplasma mali' in apple, which are detected at low levels in asymptomatic plants and at high levels in symptomatic ones (James et al. 1997; Alves et al. 2004; Mello et al. 2010; Baric et al. 2010; Gutierrez et al. 2016). This dual endophytic/parasitic behavior and its modulation by environmental and host factors is an intriguing aspect of the relationships between plants and microorganisms. For instance, drought is a recognized factor that increases losses due to RSD as it compounds with the occlusion of the xylem in aggravating water stress. Other factors such as the population dynamics of Lxx over time and in concert with water stress should be explored. Our study described host responses at the gene and protein level and highlighted metabolic pathways that must be studied in more detail to improve our understanding on the genesis of this disease.

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Figure captions

Figure 1 – Mean bacterial titers ± standard error of mock-inoculated (MI) or inoculated plants with *Leifsonia xyli* subsp. *xyli* (LxxI) at 30 and 60 days after inoculation. Letters indicate different means based on paired Student´s t test ($P < 0.05$).

Figure 2 – Venn diagrams of differentially expressed genes (A) and accumulated proteins (B, C) between treatment contrasts. LxxI –inoculated; MI – mock-inoculated; 30 and 60 refer to days after inoculation. Numbers by up or down arrows indicate the sum of up or down represented genes or proteins in each contrast.

Figure 3 – Numbers of up or down-regulated genes at 30 and 60 days after inoculation with *Leifsonia xyli* subsp. *xyli* based on the SUCEST functional categorization.

Figure 4 – Relative expressions (Log_2 fold-change) of genes differentially expressed at 30 (A) and 60 (B) days after inoculation according to the microarray (stripped bars) and real-time qPCR (solid bars) assays. MCM3, Minichromosome maintenance protein 3; MCM4, Minichromosome maintenance protein 4; MCM6, Minichromosome maintenance protein 6; CycA, Cyclin A; CycB, Cyclin B; CDKB1;1, Cyclin-dependent protein kinase B1;1; SMC4, Structural maintenance of chromosome 4; MAD3, Mitotic spindle checkpoint component MAD3; H4, Histone H4; TOPII, DNA topoisomerase II; CAPH, Non-SMC condensin I complex subunit H; AP2/EREBP-30, AP2/Ethylene Responsive Element Binding Protein; SCL28, Scarecrow-like protein 28-like; Defensin; Lox, Lipoxygenase; MS, Methionine synthase; CS1, Condensin subunit 1; ACCO, ACC oxidase; NCED, 9-cis-epoxycarotenoid dioxygenase; ABAHase, Abscisic acid 8-hydroxylase; WRKY51, WRKY transcription factor 51; AP2/EREBP-60, AP2/Ethylene Responsive Element Binding Protein; bHLH61, Transcription factor bHLH61; CYP450, Cytochrome P450; POX, Peroxidase; ADH, Alcohol dehydrogenase 1; Kinase, Receptor Ser/Thr kinase; USP, Universal stress protein A; and PAL, Phenylalanine ammonia-lyase.

Figure 5 – Schematic representation of the response of sugarcane plants variety SP80-3280 to increased titers of *Leifsonia xyli* subsp. *xyli* at 30 (A) and 60 (B) DAI. Genes are labeled with colors indicating the fold change (Log_2) of their transcript levels based on microarray data (color code is given in the figure). LRR-erecta, LRR receptor-like serine/threonine-protein kinase ERECTA; LRR-Kinase, LRR receptor-like serine/threonine-protein kinase; MAPKKK, Mitogen-activated protein kinase kinase kinase A; Mcm2-7, Minichromosome maintenance protein complex

2-7; DNA topoisomerase; RpA, replication protein A; Cdc7, Cell division control protein 7; Orc6, Origin recognition complex subunit 6; WD40, WD40-like beta propeller repeat family protein; CycA, Cyclin A; CycB, Cyclin B; H4, Histone H4; H2A, Histone H2A; Caf, Chromatin assembled factor; cdkb1;1, Cyclin-dependent protein kinase B1;1; Smc2, Structural maintenance of chromosome 2; Smc4, Structural maintenance of chromosome 4; Tangled1, Microtubule binding protein Tangled1; Mad2, mitotic spindle checkpoint component MAD2; Mad3, mitotic spindle checkpoint component MAD3; Knolle, Syntaxin-related protein KNOLLE; Kinesins; Aur, Aurora kinase A; cs1, condensin subunit 1; Ms, methionine synthase; Ns, Nicotianamine synthase 3; Pox, Peroxidase; Cyp450, Cytochrome P450; Adh, Alcohol dehydrogenase 1; Ppdk, Pyruvate phosphate dikinase; Pdc, Pyruvate decarboxylase; Pfp, pyrophosphate-fructose-6-phosphate 1-phosphotransferase; Ak, Adenylate kinase; Usp, Universal stress protein A; Pal, Phenylalanine ammonia-lyase; 4Cl, 4-Coumarate-CoA ligase; Defensin; Thaumatin; Grf; growth regulation factor; Wrky51, WRKY transcription factor 51; Nac, NAC-domain containing protein; Myb, MYB-like protein; Jaz, Jasmonate ZIM motif family protein; Iswi. ISWI chromatin remodeling complex ATPase ISW1; Cxc, Tesmin/TSO1-like CXC domain; ap2/erebp, AP2/ ethylene responsive element binding protein; Scl28, Scarecrow-like protein 28-like; Pcf7, transcription factor PCF7; ACC oxidase; ACC synthase; Nced, 9-cis-epoxycarotenoid dioxygenase; Abah, Abscisic acid 8-hydroxylase; Lox, Lipoxygenase; GH3.8, Indole-3-acetic acid-amido synthetase GH3.8; Kao, Cytochrome P450 ent-kaurenoic acid oxidase; Rbdoh, NADPH Respiratory burst oxidase homolog protein B; Cdpk, Calcium dependent protein kinase; Sweet14, Bidirectional sugar transporter SWEET14.

Supplementary Figure 1. Representative 2D-DIGE protein patterns of plantlets of the SP80-3280 sugarcane variety (A) mock-inoculated or (B) Lxx- inoculated at 30 DAI and (C) the overlapping image of (A) and (B); (D) mock-inoculated or (E) Lxx-inoculated at 60 DAI and (F) the overlapping image of (D) and (E); (G) Circled spots indicate differentially accumulated proteins.

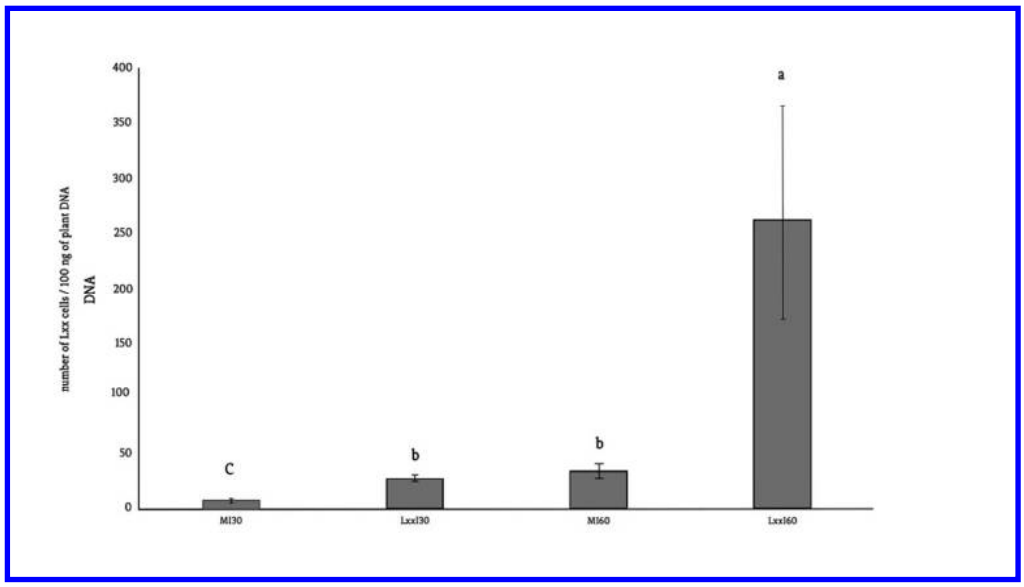

Figure 1 – Mean bacterial titers ± standard error of mock-inoculated (MI) or inoculated plants with *Leifsonia xyli* subsp. *xyli* (LxxI) at 30 and 60 days after inoculation. Letters indicate different means based on paired Student´s t test ($P < 0.05$).

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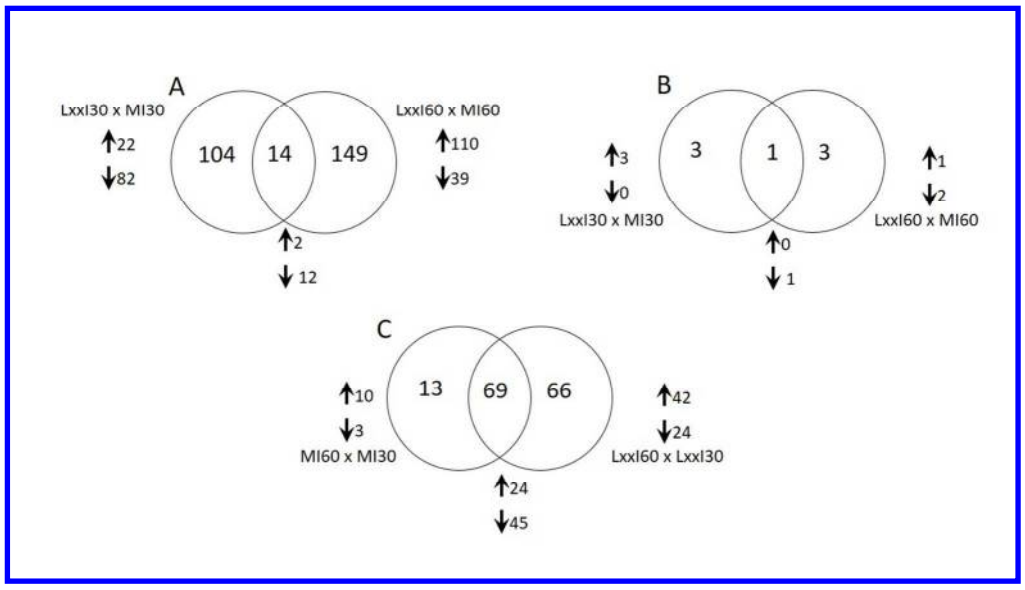


Figure 2 – Venn diagrams of differentially expressed genes (A) and accumulated proteins (B, C) between treatment contrasts. LxxI –inoculated; MI – mock-inoculated; 30 and 60 refer to days after inoculation. Numbers by up or down arrows indicate the sum of up or down represented genes or proteins in each contrast.

338x190mm (96 x 96 DPI)

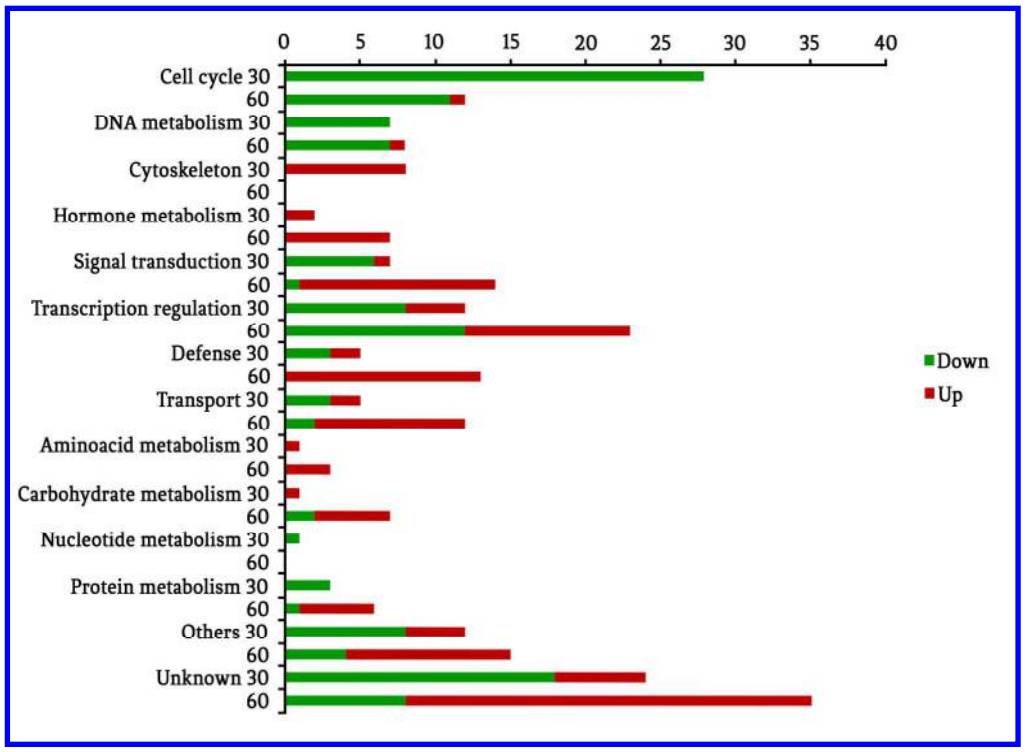


Figure 3 – Numbers of up or down-regulated genes at 30 and 60 days after inoculation with Leifsonia xyli subsp. xyli based on the SUCEST functional categorization.

203x147mm (300 x 300 DPI)

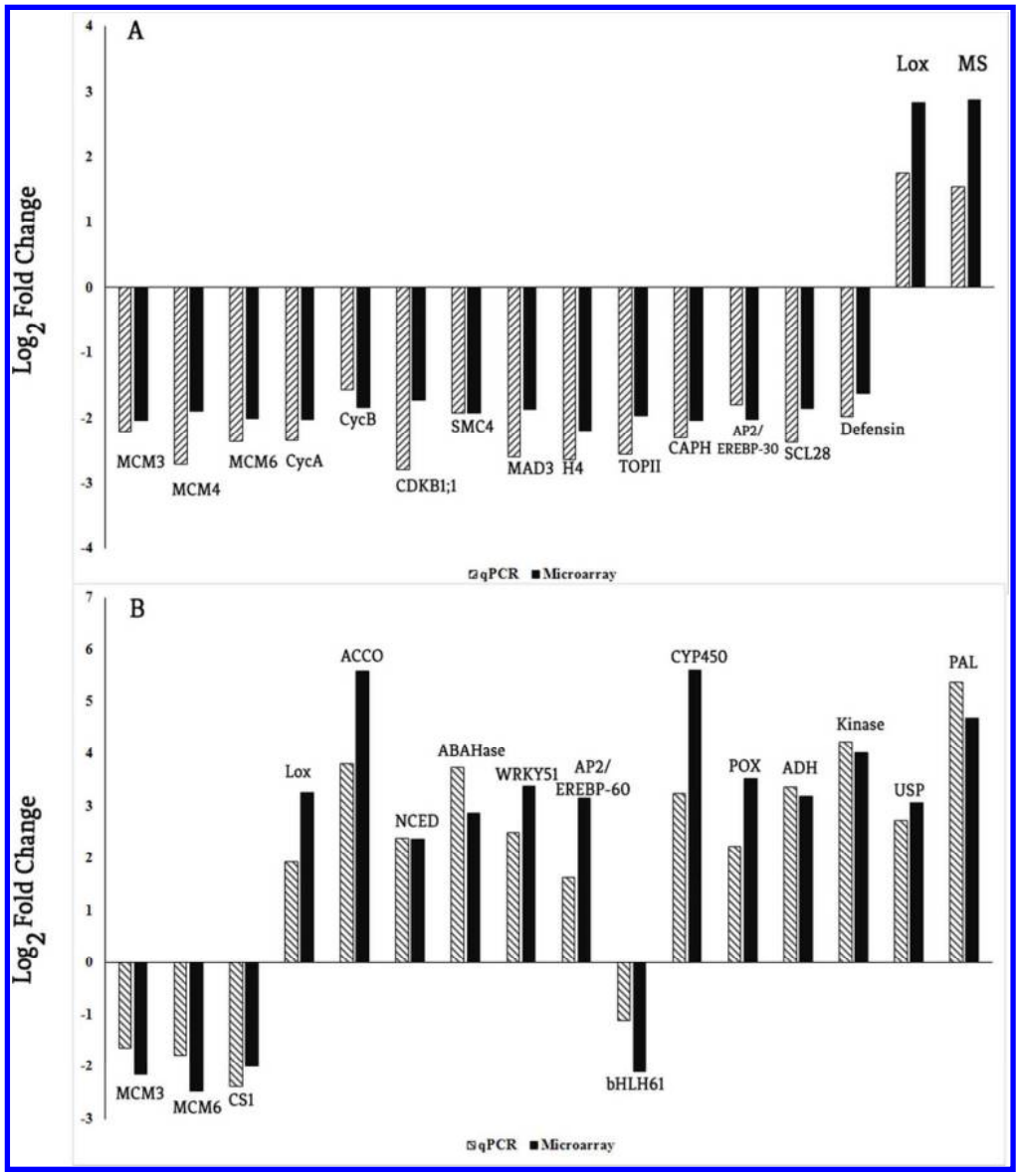


Figure 4- Relative expressions (Log2 fold-change) of genes differentially expressed at 30 (A) and 60 (B) days after inoculation according to the microarray (stripped bars) and real-time qPCR (solid bars) assays. MCM3, Minichromosome maintenance protein 3; MCM4, Minichromosome maintenance protein 4; MCM6, Minichromosome maintenance protein 6; CycA, Cyclin A; CycB, Cyclin B; CDKB1;1, Cyclin-dependent protein kinase B1;1; SMC4, Structural maintenance of chromosome 4; MAD3, Mitotic spindle checkpoint component MAD3; H4, Histone H4; TOPII, DNA topoisomerase II; CAPH, Non-SMC condensin I complex subunit H; AP2/EREBP-30, AP2/Ethylene Responsive Element Binding Protein; SCL28, Scarecrow-like protein 28-like; Defensin; Lox, Lipoxygenase; MS, Methionine synthase; CS1, Condensin subunit 1; ACCO, ACC oxidase; NCED, 9-cis-epoxycarotenoid dioxygenase; ABAHase, Abscisic acid 8-hydroxylase; WRKY51, WRKY transcription factor 51; AP2/EREBP-60, AP2/Ethylene Responsive Element Binding Protein; bHLH61, Transcription factor bHLH61; CYP450, Cytochrome P450; POX, Peroxidase; ADH, Alcohol dehydrogenase 1; Kinase, Receptor Ser/Thr kinase; USP, Universal stress protein A; and PAL, Phenylalanine ammonia-lyase.

88x102mm (300 x 300 DPI)

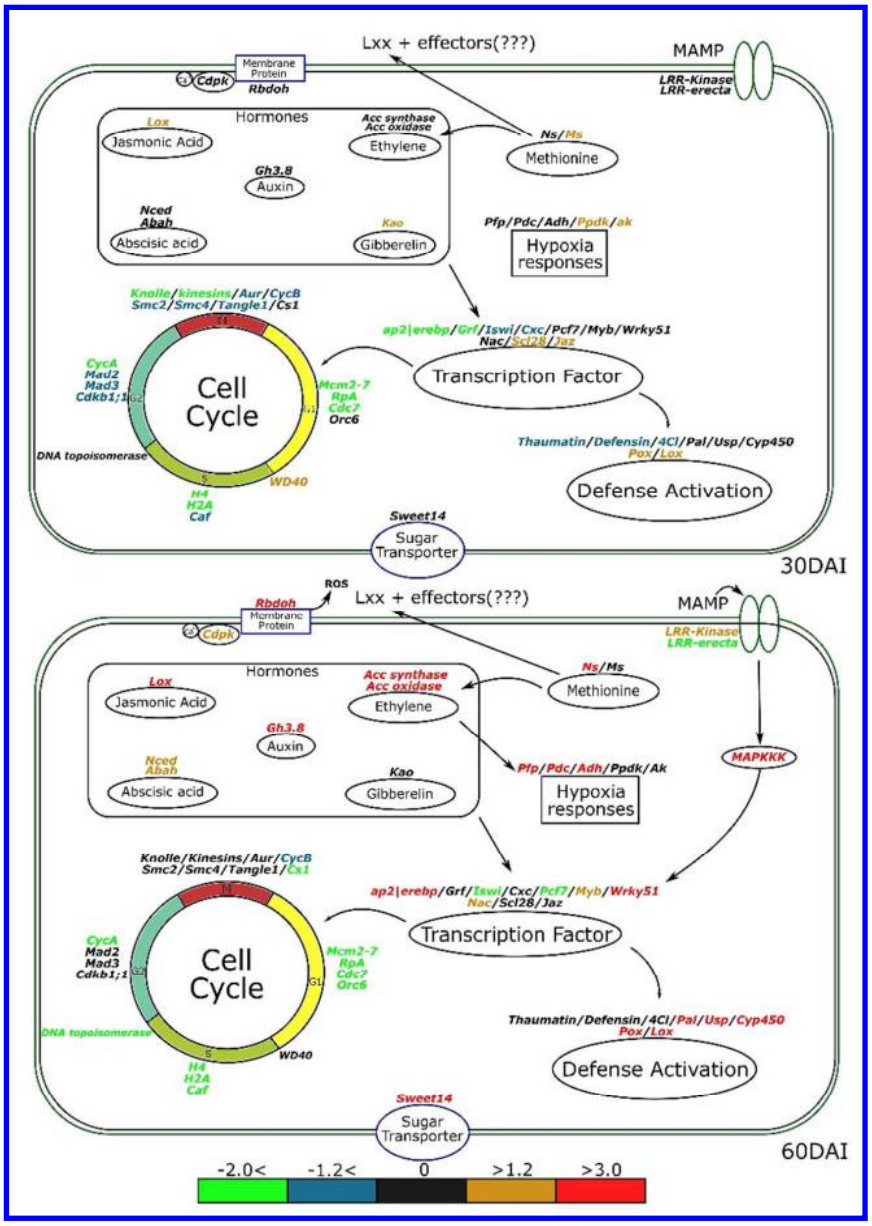


Figure 5 -Schematic representation of the response of sugarcane plants variety SP80-3280 to increased titers of Leifsonia xyli subsp. xyli at 30 (A) and 60 (B) DAI. Genes are labeled with colors indicating the fold change (Log2) of their transcript levels based on microarray data (color code is given in the figure). LRR-erecta, LRR receptor-like serine/threonine-protein kinase ERECTA; LRR-Kinase, LRR receptor-like serine/threonine-protein kinase; MAPKKK, Mitogen-activated protein kinase kinase kinase A; Mcm2-7, Minichromosome maintenance protein complex 2-7; DNA topoisomerase; RpA, replication protein A; Cdc7, Cell division control protein 7; Orc6, Origin recognition complex subunit 6; WD40, WD40-like beta propeller repeat family protein; CycA, Cyclin A; CycB, Cyclin B; H4, Histone H4; H2A, Histone H2A; Caf, Chromatin assembled factor; cdkb1;1, Cyclin-dependent protein kinase B1;1; Smc2, Structural maintenance of chromosome 2; Smc4, Structural maintenance of chromosome 4; Tangled1, Microtubule binding protein Tangled1; Mad2, mitotic spindle checkpoint component MAD2; Mad3, mitotic spindle checkpoint component MAD3; Knolle, Syntaxin-related protein KNOLLE; Kinesins; Aur, Aurora kinase A; cs1, condensin subunit 1; Ms, methionine synthase; Ns, Nicotianamine synthase 3; Pox, Peroxidase; Cyp450, Cytochrome P450; Adh,

Alcohol dehydrogenase 1; Ppdk, Pyruvate phosphate dikinase; Pdc, Pyruvate decarboxylase; Pfp, pyrophosphate-fructose-6-phosphate 1-phosphotransferase; Ak, Adenylate kinase; Usp, Universal stress protein A; Pal, Phenylalanine ammonia-lyase; 4Cl, 4-Coumarate-CoA ligase; Defensin; Thaumatin; Grf; growth regulation factor; Wrky51, WRKY transcription factor 51; Nac, NAC-domain containing protein; Myb, MYB-like protein; Jaz, Jasmonate ZIM motif family protein; Iswi. ISWI chromatin remodeling complex ATPase ISW1; Cxc, Tesmin/TSO1-like CXC domain; ap2/erebp, AP2/ ethylene responsive element binding protein; Scl28, Scarecrow-like protein 28-like; Pcf7, transcription factor PCF7; ACC oxidase; ACC synthase; Nced, 9-cis-epoxycarotenoid dioxygenase; Abah, Abscisic acid 8-hydroxylase; Lox, Lipoxygenase; GH3.8, Indole-3-acetic acid-amido synthetase GH3.8; Kao, Cytochrome P450 ent-kaurenoic acid oxidase; Rbdoh, NADPH Respiratory burst oxidase homolog protein B; Cdpk, Calcium dependent protein kinase; Sweet14, Bidirectional sugar transporter SWEET14.

239x338mm (96 x 96 DPI)

Supplementary Table S1 - Functional categorization, gene annotation, and expression ratios at 30 and 60 days after inoculation (DAI) of differentially expressed Sugarcane Annotated Sequences (SAS) of the variety SP80-3280 inoculated with *Leifsonia xyli* subsp. *xyli*.

SAS	Functional category	Gene	30 DAI	60 DAI
SCEQRT2026F02.g	Cell cycle	Structural maintenance of chromosome 2	-1.68	-
SCCCCL4005H10.g		Structural maintenance of chromosome 4	-1.91	-
SCRFAM1026H03.g		Cyclin1	-1.62	-
SCUTLR1058G02.g		Cyclin III	-2.10	-
SCACAM2042F03.g		Cyclin A	-2.02	-
SCEPCL6029D04.g		cyclin B	-1.83	-
SCJLLR1033H06.g		cyclin B2	-1.97	-
SCACLR1057B10.g		Cyclin-dependent protein kinase CDKB1;1	-1.72	-
SCJFRZ2006F07.g		Mitotic spindle checkpoint protein MAD2	-1.60	-
SCEPAM1023G10.g		Mitotic spindle checkpoint component MAD3	-1.87	-
SCJLLR1103B02.g		Ubiquitin carboxyl-terminal hydrolase superfamily protein	-1.75	-
SCVPLB1016G07.g		Patellin-5	-1.68	-
SCEQLR1029C07.g		Targeting protein for Xklp2	-1.28	-
SCUTHR1065C09.g		Syntaxin-related protein KNOLLE	-2.05	-
SCSGHR1071A09.g		Non-SMC condensin I complex subunit H	-2.02	-
SCACLR1128F01.g		Microtubule-associated protein RP/EB family member 3	-1.77	-
SCQGLR1019E01.g		Microtubule-associated protein RP/EB family	-1.72	-
SCRLAM1013A12.g		65-kDa microtubule-associated protein 5	-1.99	-
SCCCAM2004F12.g		Cell division control protein 7	-1.99	-
SCCCRZ1C01E03.g		Minichromosome maintenance protein 4	-1.89	-
SCCCLB1023D02.g		Minichromosome maintenance protein 5	-1.62	-
SCJLRZ1023B07.g		Minichromosome maintenance protein 2	-1.78	-2.20
SCCCLR1072B11.g		Minichromosome maintenance protein 3	-2.03	-2.17
SCEPCL6029A03.g		Minichromosome maintenance protein 6	-2.00	-2.47
SCCCLR1C05C04.g		Minichromosome maintenance protein 7	-1.91	-2.43

SCEZRZ1012E01.g		Mini-chromosome maintenance complex-binding protein-like	-1.62	-2.20
SCCCLB1003E01.g		14 kDa proline-rich protein DC2.15	-1.96	-4.58
SCMCRT2086D06.g		Chromatin assembly factor 1 subunit B	-1.39	-2.06
SCEPCL6019G04.g		Condensin subunit 1	-	-2.00
SCCCRZ2003E03.g		Centromeric protein E	-	-1.81
SCUTFL3075D03.g		Type A cyclin	-	-2.30
SCJFST1011F10.g		DNA polymerase III	-	-1.72
SCSGLV1008B03.g		Flowering-promoting factor 1-like protein 1	-	3.50
SCCCLB1002E02.g	DNA metabolism	Phosphoribosyltransferases	-1.60	-
SCCCLB1004H09.g		DNA repair protein RAD51 homolog A	-1.84	-
SCBGLR1002A12.g		DNA topoisomerase II	-1.96	-
SCCCLR2C02C12.g		Histone H2A	-2.06	-
SCRFLR2038C05.g		Histone H4	-1.20	-
SCCCLR2002D04.g		Histone H4	-2.19	-
SCVPRZ2036F02.g		Replication protein A 70 kDa DNA-binding subunit	-1.76	-2.17
SCBFST3135H08.g		Histone H2A	-	-1.97
SCRUFL1021B04.g		Histone acetyltransferase type B subunit 2	-	-1.70
SCVPFL1140D05.g		DNA repair helicase RAD5	-	-2.56
SCCCAD1001F12.g		RAD3-related DNA helicase	-	-2.25
SCCCLR1C02F08.g		Replication protein A 32 kDa subunit-like	-	-2.28
SCMCLR1125B04.g		Origin recognition complex subunit 6	-	-2.80
SCQSLR1061C11.g		MATE efflux family protein 1	-	3.80
SCJLFL1052C01.g	Cytoskeleton	125 kDa kinesin-related protein	-1.66	-
SCCCRZ1C01E04.g		125 kDa kinesin-related protein	-1.21	-
SCBFAM2021A04.g		Kinesin-like protein	-2.19	-
SCEPAM1018H01.g		kinesin	-1.60	-
SCACLR1127F03.g		Kinesin	-1.83	-
SCJLRT2050F05.g		Kinesin family member C2/C3	-1.46	-

SCCCCL3001D11.g		Microtubule-associated protein TORTIFOLIA1-like	-1.81	-
SCRLAM1009B07.g		Microtubule binding protein Tangled1	-1.20	-
SCAGRT3046D01.g	Hormone metabolism	Cytochrome P450 ent-kaurenoic acid oxidase	1.22	-
SCCCRT1001E01.g		Lipoxygenase	2.84	3.26
SCCCLR1048H03.g		9-cis-epoxycarotenoid dioxygenase	-	2.38
SCRFRZ3058E03.b		Abscisic acid 8-hydroxylase 1	-	2.86
SCQGAM2028D11.g		ACC synthase	-	4.91
SCCCLR1022A02.g		ACC oxidase	-	5.59
SCSFRT2070A10.g		ACC oxidase	-	1.98
SCCCCL3002B05.b		Indole-3-acetic acid-amido synthetase GH3.8	-	3.02
SCCCFL1097H04.g	Signal transduction	WD40-like beta propeller repeat family protein	2.68	-
SCJLFL4099D04.g		Rac GTPase activating protein	-2.07	-
SCQSLR1061C02.g		Rac GTPase activating protein	-1.73	-
SCBFLR1039G02.g		Serine/threonine-protein kinase	-1.15	-
SCRUFL1112G02.b		Serine/threonine-protein kinase Aurora-1	-1.70	-
SCCCLR2003E06.g		AURORA kinase A	-2.00	-
SCQGFL4078E12.g		Calmodulin binding protein	-1.68	-
SCCCLR1C03F08.g		LRR receptor-like serine/threonine-protein kinase ERECTA	-	-2.31
SCMCRT2108E08.g		Receptor-like protein kinase	-	2.37
SCEQLB2020F07.g		Receptor-like protein kinase	-	3.13
SCBFRT3095E07.g		Receptor Ser/Thr kinase	-	4.01
SCBFAD1045F11.g		Receptor Ser/Thr kinase	-	2.27
SCCCRT1001B08.g		Receptor Ser/Thr kinase	-	2.97
SCBGLR1115B08.g		Receptor Ser/Thr kinase	-	1.99
SCRUHR1074H12.g		L-type lectin-domain containing receptor kinase IX.1	-	2.44
SCJFRZ2015H10.g		L-type lectin-domain containing receptor kinase IX.1-like	-	2.61
SCJLRT1006B11.g		Mitogen-activated protein kinase kinase kinase A	-	3.20
SCCCLR2C02G01.g		Calcium dependent protein kinase	-	2.27

SCVPFL3046E09.b		Zinc(II) purple acid phosphatase	-	2.33
SCCCCL4005C09.g		Light-inducible protein CPRF2	-	2.28
SCCCCL3120A10.b		Auxin response factor 12	-	2.30
SCJFRZ2015E01.g	Transcription regulation	Jasmonate ZIM motif family protein	1.48	-
SCSGLV1009H10.b		Ribonuclease T2	1.79	-
SCQSLR1040B09.g		ISWI chromatin remodeling complex ATPase ISW1	-1.48	-
SCSGFL1079A03.g		Tesmin/TSO1-like CXC domain	-1.36	-
SCBFLR1039G11.g		Tesmin/TSO1-like CXC domain	-1.83	-
SCJFRZ1006B02.g		Transcription factor A mitochondrial	-1.98	-
SCRFAM1028B07.g		Transcription factor jumonji (jmjC) domain-containing protein	-1.55	-
SCJFRZ2031F06.g		Heat stress transcription factor B-4b	-1.28	-
SCEQAM2039A10.g		Scarecrow-like protein 28-like	-1.84	-
SCAGLR1064B08.g		AP2/Ethylene Responsive Element Binding Protein	-2.01	-
SCQSHR1023B08.g		AP2/Ethylene Responsive Element Binding Protein	1.20	-
SCQSLR1090A05.g		Ethylene-responsive transcription factor 4 (ETR4)	1.77	-
SCCCCL4002B07.g		AP2/Ethylene Responsive Element Binding Protein	-	3.15
SCVPRT2073F02.g		Ethylene Responsive Factor like protein	-	3.87
SCCCLR1068A11.g		Ethylene-responsive transcription factor 1A (ETR1)	-	2.15
SCAGLR1021G10.g		Protein Knotted1	-	4.62
SCSGFL5C03H04.g		ISWI chromatin remodeling complex ATPase ISW1	-	-2.50
SCCCFL5096D07.g		Transcription factor PCF5	-	-3.29
SCEPAM1018C05.g		Transcription factor PCF7	-	-2.04
SCJFLR1013D05.g		Homeobox-leucine zipper protein ROC1	-	-1.90
SCAGCL6012F06.g		Homeobox-leucine zipper protein ROC3	-	-1.96
SCCCAD1003G07.g		Homeobox-leucine zipper protein ROC7	-	-1.87
SCJLRZ1020B01.g		Growth-regulating factor 3	-	-2.06
SCCCFL4089D04.g		DNA polymerase alpha subunit B	-	-2.09
SCRFLR1034G09.g		Serine/arginine repetitive matrix protein 1	-	-1.84

SCACLR1130H08.g		Zinc finger protein	-	-3.55
SCJFLR1013F11.g		Histone chaperone ASF1	-	-2.12
SCRURT3061D05.g		Transcription factor bHLH61	-	-2.10
SCCCLR2004B09.g		Transcription factor bHLH96-like	-	2.49
SCCCCL4001A01.g		WRKY transcription factor 51	-	3.38
SCCCRZ1004G12.g		NAC-domain containing protein	-	2.30
SCQGST1032D08.g		MYB-like protein	-	2.44
SCJFRT1060C03.g		Ribonuclease H protein	-	3.44
SCRLAD1097D12.g		Zinc finger protein	-	2.60
SCVPLR2027D09.g		PLATZ transcription factor	-	2.66
SCEZLB1005H06.g	Defense	Defensin (PR-12)	-1.62	-
SCJFRZ2025E05.g		Thaumatin-like protein (PR-5)	-1.24	-
SCVPFL1138B04.g		4-coumarate-CoA ligase	-1.12	-
SCCCLR1066D07.g		Glycine-rich cell wall structural protein	1.69	-
SCCCCL3002F08.b		Peroxidase	1.60	-
SCCCCL2001D02.b		Peroxidase	-	3.53
SCSBSD1033E10.g		Peroxidase	-	2.19
SCCCCL4015G04.g		Peroxidase	-	3.23
SCCCCL5002F03.g		Cytochrome P450	-	5.61
SCAGLR2011D02.g		NADPH Respiratory burst oxidase homolog protein B	-	3.86
SCCCLR1075G12.g		Leucine-rich repeat receptor-like serine/threonine-protein kinase	-	2.92
SCJLRT1006H05.g		Leucine-rich repeats (LRRs)	-	2.89
SCEPRZ3047D01.g		LRR receptor-like serine/threonine-protein kinase	-	2.56
SCSGAM1094D05.g		Phenylalanine ammonia-lyase (PAL)	-	4.69
SCQSRT1035D12.g		Protease inhibitor	-	3.41
SCQSST1037D10.g		Universal stress protein A	-	2.83
SCQSRT1035G12.g		Universal stress protein (USP) family protein	-	3.07
SCEQRT2027C06.g		Wound induced protein	-	2.71

SCQSLR1061H12.g	Transport	Charged multivesicular body protein 2A	-1.66	-
SCRURT2010A08.g		Mitochondrial 2-oxoglutarate/malate carrier protein-like	-1.09	-
SCCCCL4004D08.g		NADH-plastoquinone oxidoreductase subunit k	1.39	-
SCEPSD2005D11.g		NADH dehydrogenase I subunit M	1.29	-
SCJLLR1104H07.g		Early nodulin-like protein 3-like	-2.19	-
SCJFRZ2014D05.g		WAT1-related protein	-	2.06
SCCCCL3004A08.b		Early nodulin 93	-	3.44
SCRFHR1006A11.g		Peptide transporter PTR2	-	-1.82
SCEPAM2013B12.g		Peroxisomal membrane protein	-	-1.68
SCQSHR1020H10.g		Peptide/nitrate transporter	-	1.95
SCSFFL4086H05.g		Potassium transporter 1	-	2.89
SCCCAM1073C01.g		Potassium channel AKT1	-	2.60
SCACLR1057A09.g		Copper-exporting ATPase	-	2.41
SCCCRT2002G08.g		Bidirectional sugar transporter SWEET14	-	3.51
SCSGHR1066C01.g		Sugar transporters	-	2.98
SCEQRT1024C07.g		Pyrophosphate-energized vacuolar membrane proton pump	-	3.45
SCAGLR2018G04.g		RING-H2 finger protein ATL1R	-	2.48
SCCCLR1069H11.g	Aminoacid metabolism	Methionine synthase (metH)	2.88	-
SCMCST1049H08.g		Nicotianamine synthase 3 (NS)	-	3.00
SCJLLR1011D09.g		Arogenate dehydratase / prephenate dehydratase 6	-	3.23
SCEZRZ1012F05.g		Methylenetetrahydrofolate reductase 1	-	2.66
SCJFRT1060F11.g	Carbohydrate metabolism	Pyruvate phosphate dikinase (PPDK)	1.29	-
SCQGLR2025A05.g		Adenylate kinase (AK)	1.37	-
SCCCLB1021E06.g		Granule-bound starch synthase	-	-1.75
SCPIRT3021H05.g		Glucosyltransferase	-	-2.66
SCCCFL4002D04.g		Glucose-1-phosphate adenylyltransferase small subunit	-	3.01
SCCCRZ1003C03.g		Acid phosphatase 1 precursor	-	4.21
SCMCRT2089E02.g		Pyruvate decarboxylase (PDC)	-	4.32

SCCCCL3001B07.b		Pyrophosphate-fructose-6-phosphate 1-phosphotransferase (PFK)	-	2.44
SCEZST3151H02.g		Pyrophosphate-fructose-6-phosphate 1-phosphotransferase (PFK)	-	4.27
SCBFAM2117B08.g		Alcohol dehydrogenase 1 (ADH)	-	3.18
SCRLFL1005F10.g	Lipid metabolism	Anter-specific proline-rich protein APG	-1.58	-
SCUTLR2008F03.g		Glycerol-3-phosphate acyltransferase 1	1.43	-
SCSGHR1068B07.b		GDSL esterase/lipase	-	-2.50
SCSGFL1079D12.g		Cyclopropane fatty acyl phospholipid synthase	-	-3.00
SCVPRZ2038F04.g		Omega-3 fatty acid desaturase	-	-1.66
SCSBAM1085B06.g		Omega-3 fatty acid desaturase	-	2.07
SCQSLR1089H10.g		Lipid binding protein	-	2.66
SCEPAM2054B05.g		Phosphatidylinositol ceramide inositolphosphotransferase	-	2.66
SCJFRT1008C09.g		3-ketoacyl-CoA synthase	-	2.67
SCEZAM2031E09.g	Nucleotide metabolism	Deoxyuridine 5'-triphosphate nucleotidohydrolase	-1.49	-
SCMCRT2086A02.g		Phosphoribosylglycinamide synthetase	-	3.03
SCCCRZ2003H11.g	Protein metabolism	Ubiquitin-conjugating enzyme E2 I	-1.49	-
SCRLAM1006G07.g		E3 ubiquitin ligase SINAT3-like	-1.25	-
SCRLLR1059F03.g		E2 Ubiquitin conjugating enzyme 9 (UBC9)	-	2.29
SCSFAD1107B01.g		DnaJ/Hsp40	-1.79	-1.97
SCAGLB2046F01.g		Trypsin inhibitor	-	2.40
SCJFRZ2009E12.g		Protease	-	4.12
SCRLCL6030H11.g		Pyrrolidone carboxylate peptidase family protein	-	2.87
SCSBRZ2021F12.g		Basic 7S globulin	-	2.94
SCCCLB1026C11.g	Others	Transposon protein	-2.17	-
SCSFSD1066D10.g		Translation initiation factor IF	1.56	-
SCACCL6009A08.g		Cellulose synthase-like protein H1-like	1.27	-
SCQGHR1011A04.g		O-acyltransferase WSD1-like	1.44	-
SCSBAD1052E10.g		ATP binding protein	-1.38	-
SCRLAM1010C03.g		Protein CHUP1 chloroplastic-like	-1.69	-

SCEPCL6023E10.g		EH domain containing protein	-1.56	-
SCEPAM2012H05.g		OB fold nucleic acid binding domain containing protein	-1.45	-
SCCCFL5062D05.g		Tetratricopeptide repeat domain	-2.12	-
SCBGLR1114H02.g		Lamin-like protein	-1.56	-2.21
SCEPAM2015E05.g		Plantacyanin	-1.54	-1.46
SCCCCL3002F05.b		Tetratricopeptide repeat domain	-	3.06
SCQSFL1123E07.g		Aldehyde dehydrogenase family protein	-	-2.19
SCEZRZ1014A06.g		Haloacid dehalogenase-like hydrolase	-	3.41
SCJFRT1010E08.g		3'-N-debenzoyl-2'-deoxytaxol N-benzoyltransferase	-	2.74
SCJFRT1010D11.g		3-hydroxybenzoate 6-hydroxylase 1	-	2.83
SCVPRZ2040D09.g		Pectinesterase	-	2.77
SCMCRZ3065A12.g		Fucosyltransferase	-	2.21
SCAGLR2033H02.g		Oleosin Zm-I 16 kDa	-	-2.25
SCQSRT2035D11.g		Hypoxia-responsive family protein	-	2.61
SCRLLR1110H11.g		2-aminoethanethiol dioxygenase	-	2.18
SCMCLR1010D01.g		Polyprotein	-	3.27
SCCCST2004G03.g		Hydroxyproline-rich glycoprotein 1	-	2.51
SCBGLR1096F11.g	Unknown	Hypothetical protein SORBIDRAFT_02g034460	-1.35	-
SCJLFL1054B04.g		Hypothetical protein SORBIDRAFT_06g023300	-1.28	-
SCCCLR1066B11.g		Hypothetical protein SORBIDRAFT_01g037720	-1.49	-
SCRLFL1004F10.g		Hypothetical protein SORBIDRAFT_01g007940	-1.63	-
SCQGLR2032B01.g		Hypothetical protein SORBIDRAFT_03g042590	-1.79	-
SCSGAM2075E07.b		Hypothetical protein SORBIDRAFT_05g001370	-1.95	-
SCSGFL1083D07.g		hypothetical protein SORBIDRAFT_05g022560	-2.00	-
SCUTRZ2023G10.g		Hypothetical protein SORBIDRAFT_01g027980	-1.74	-
SCBGLR1027F10.g		Hypothetical protein SORBIDRAFT_04g002670	-0.93	-
SCVPLR2012B02.g		Hypothetical protein SORBIDRAFT_03g008530	-1.85	-
SCBFLR1005H01.g		Hypothetical protein SORBIDRAFT_01g006350	-1.98	-

SCCCFL8001C08.g	Hypothetical protein ZEAMMB73_516122	-1.70	-
SCRUFL4020B03.g	Uncharacterized protein DDB_G0287975-like	-1.20	-
SCRLAD1136F04.g	Uncharacterized LOC100831114	-1.75	-
SCBGAM1093E09.g	Uncharacterized LOC100838770	-2.30	-
SCEPAM1021B02.g	Proteophosphoglycan 5	-1.76	-
SCVPRT2075B11.g	Vegetative cell wall protein gp1	1.31	-
SCJFRT1061G09.g	Nucleotide pyrophosphatase/phosphodiesterase	1.44	-
SCJLLR2013D11.g	Hypothetical protein SORBIDRAFT_09g008890	2.04	-
SCBGLR1114D06.g	Hypothetical protein LOC100275145 precursor	1.33	-
SCMCCL6027E12.g	Unknown	-2.33	-
SCUTLR1058H04.g	Unknown	1.74	-
SCBGFL5077B07.g	Unknown	-1.94	-2.46
SCCCCL4001D06.g	phi-1-like phosphate-induced protein	2.30	3.16
SCVPAM1055E12.g	SelT/selW/selH selenoprotein domain containing protein	-	-1.71
SCBFFL1144D08.g	Hypothetical protein SORBIDRAFT_03g040750	-	-2.57
SCQGLB1029D12.g	Hypothetical protein SORBIDRAFT_02g007160	-	-3.24
SCEPAM1017A12.g	Hypothetical protein SORBIDRAFT_02g008000	-	-1.64
SCBFSB1045A05.g	Hypothetical protein ZEAMMB73_084392	-	-2.70
SCSGFL5C03A09.g	Uncharacterized LOC100823323	-	-1.97
SCACRZ3109B12.g	Hypothetical protein SORBIDRAFT_06g018810	-	2.40
SCRLRZ3114C04.g	Hypothetical protein SORBIDRAFT_07g019990	-	2.88
SCRUAD1063A04.g	Hypothetical protein SORBIDRAFT_01g007470	-	2.21
SCEQLR1050B01.g	Hypothetical protein SORBIDRAFT_01g032580	-	2.60
SCQGRZ3014E06.g	Hypothetical protein SORBIDRAFT_08g016800	-	2.98
SCJFRZ3C04A01.g	Hypothetical protein SORBIDRAFT_03g031090	-	3.00
SCAGRT2041E02.g	Hypothetical protein SORBIDRAFT_01g007470	-	2.07
SCUTRZ3070F02.g	Hypothetical protein SORBIDRAFT_02g031530	-	2.84
SCQSLR1090F02.g	Hypothetical protein SORBIDRAFT_01g001260	-	3.24

SCBGRT1047A08.g	Hypothetical protein SORBIDRAFT_03g026010	-	2.41
SCCCLR1078E02.g	Hypothetical protein SORBIDRAFT_04g031500	-	3.09
SCACLR1129A10.g	Hypothetical protein SORBIDRAFT_03g039730	-	3.29
SCRFAD1023D02.g	Hypothetical protein ZEAMMB73_392643	-	2.80
SCEZRZ3050H04.g	Hypothetical protein ZEAMMB73_013833	-	3.10
SCRURT2006H09.g	Uncharacterized LOC100846035	-	2.97
SCCCFL4089H04.g	Unknown	-	-2.76
SCACSD2014F07.g	Unknown	-	2.55
SCSGLV1008C08.g	Unknown	-	2.65
SCMCCL6054C02.g	Unknown	-	2.67
SCQGSD1048C09.g	Unknown	-	3.11
SCRURT2006B11.g	Unknown	-	2.97
SCJLRT1023H11.g	Unknown	-	2.54
SCJFSB1011G12.g	Unknown	-	2.52
SCJFLR1073A01.g	Metal ion binding protein	-	3.08
SCQGLR2017A03.g	36.4 kDa proline-rich protein	-	3.80
SCQGAD1065H07.g	Auxin-inducible protein	-	2.24
SCCCCL3003C03.b	VQ motif family protein	-	2.79

Supplementary Table S2 – Sequences of forward and reverse primers used to amplify differentially expressed Sugarcane Annotated Sequences (SAS) by real time qPCR.

SAS	Gene	Abbreviation	Primer sequence
SCCCLR1072B11.g	Minichromosome maintenance protein 3	MCM3	5′ GATGAAATTGAGCAGGCGGT 3′ 5′ AGGAGATCCACGGGTAAACG 3′
SCCCRZ1C01E03.g	Minichromosome maintenance protein 4	MCM4	5′ GTTTGTTCATCCGCCTTGTC 3′ 5′ TTACAACCCCAGGCTTTCT 3′
SCEPCL6029A03.g	Minichromosome maintenance protein 6	MCM6	5′ GCCAGCCAAACCATCGCCA 3′ 5′ TGACCAAGCAGCGGATAAC 3′
SCACAM2042F03.g	Cyclin A	CycA	5′ATTCAGTAGGGATTTGTGGTG 3′ 5′ GCGACTGTGTAAAGGCATTG 3′
SCEPCL6029D04.g	Cyclin B	CycB	5′ ACAAAGGCACAGCAAATC 3′ 5′ GACAGTCTACATCATCGA 3′
SCAGLR1064B08.g	AP2/Ethylene Responsive Element Binding Protein	AP2/EREBP-30	5′ CGTCCTGCCTACTCTCCTTC 3′ 5′ ATGGTGTCGTCGGTTGCC 3′
SCACLR1057B10.g	Cyclin-dependent protein kinase CDKB1;1	CDKB1;1	5′ CTCTCCCACTCCATCTACG 3′ 5′ TACTTCTTGAGGTCGGTGTCG 3′
SCEZLB1005H06.g	Defensin	Defensin	5′ CGTGTGCCAGACCGAGAACT 3′ 5′ AAACAGCAACGCAGGAGAAC 3′
SCCCCL4005H10.g	Structural maintenance of chromosome 4	SMC4	5′ GGCTCGCTTGTATGGTGA 3′ 5′ AAGTTCCAGTTCGGCATCAC 3′
SCEPAM1023G10.g	Mitotic spindle checkpoint component MAD3	MAD3	5′ TAAATGGCATGGCTCTGTCC 3′ 5′ AAAGTGTGGCTCGAATACGC 3′
SCCCLR2002D04.g	Histone H4	H4	5′ ATGCCCTGGATGTTGTCG 3′ 5′ CGAGCGGAAGAAGCCATG 3′
SCBGLR1002A12.g	DNA topoisomerase II	TOPII	5′ CCTTTGTATTGACTGTCTTCTC 3′ 5′ ATTGGAACTGGATGGAGCAC 3′
SCEQAM2039A10.g	Scarecrow-like protein 28-like	SCL28	5′ CTTTTGGCTGCTGAGAGTC 3′ 5′ CATCCATATACGCTGTGAG 3′
SCSGHR1071A09.g	Non-SMC condensin I complex subunit H	CAPH	5′ TCCAGAAGATTGCCATTACC 3′ 5′ GTCATCATCATTATCCACTG 3′
SCCCRT1001E01.g	Lipoxygenase	Lox	5′ CAGTTGAGGAGGATGGAT 3′ 5′ ATGACGGATGAGGAGTTTGG 3′
SCCCLR1069H11.g	Methionine synthase	MS	5′ TGGAGTGGATGATGTCGTTG 3′ 5′ TGGAGCCTTGTCAGTGTCAG 3′
SCCCLR1022A02.g	ACC oxidase	ACCO	5′ ACAATGGTGGTTCCCGTG 3′ 5′ TCACGAGCTGGAAGAATC 3′
SCCCLR1048H03.g	9-cis-epoxycarotenoid dioxygenase	NCED	5′ TAGCAAGAGGCACCACCAGC 3′ 5′ GTAGGAGAGAGAAGAAAGGCG 3′
SCRFRZ3058E03.b	Abscisic acid 8-hydroxylase	ABAHase	5′ CGTGGTTTCCTTTTGACTCC 3′ 5′ CCTGATTGGGATTGTATGTAAGC 3′
SCCCCL4001A01.g	WRKY transcription factor 51	WRKY51	5′ GCCATCGGGATTAGTTGAGG 3′ 5′ AGCGGGACAAGGACGACC 3′

SCCCCL4002B07.g	AP2/Ethylene Responsive Element Binding Protein	AP2/EREBP-60	5′ CATTTGATACATCGGCACTG 3′ 5′ GTTTTGGGTGCCTGGAGTTC 3′
SCCCCL5002F03.g	Cytochrome P450	CYP450	5′ CGACGACAAGTACCTCCG 3′ 5′ GGGTTCTTGGACAGGAGC 3′
SCCCCL2001D02.b	Peroxidase	POX	5′ AGGGCTTGTTCATCTCCGAC 3′ 5′ TAGTGGTTGACGACGAAGC 3′
SCBFAM2117B08.g	Alcohol dehydrogenase 1	ADH	5′ GAGGCTGGAGGGTACATTATC 3′ 5′ CCAGAGTCCACGCAACATAAC 3′
SCBFRT3095E07.g	Receptor Ser/Thr kinase	Kinase	5′ CCTCCACAGACCTTGCTCATC 3′ 5′ CTGGACCCCGACATGAAG 3′
SCQSRT1035G12.g	Universal stress protein A	USP	5′ CACGACAACGAGCATGACC 3′ 5′ GGCAGCAGGAAAATAAGGAC 3′
SCSGAM1094D05.g	Phenylalanine ammonia-lyase	PAL	5′ TGGAATACAGTCAGCCTTTGC 3′ 5′ TAGGGAGAAAGAATGGAGTG 3′
SCRURT3061D05.g	Transcription factor bHLH61	bHLH61	5′CGCATCGTCGTACATCAAAGA 3′ 5′GCCTCAAGAATGGAAGCAAGT 3′
SCEPCL6019G04.g	Condensin sub. 1	CSI	5′ TCAGAAGCGTAATATCAAAGTGC 3′ 5′ GCCAGAAATTCATCAAGCGAGC 3′

Supplementary Table S3 – Functional category, accession number and volume fold-change of proteins differentially accumulated (P < 0.05) according to contrasts between plants inoculated with *Leifsonia xyli* subsp. *xyli* or mock-inoculated (LxxI and MI, respectively) at 30 or 60 days after inoculation (DAI).

Functional category	Protein	Accession	Protein fold change in contrasts			
			LxxI30 vs MI30	LxxI60 vs MI60	MI60 Vs MI30	LxxI60 vs LxxI30
Cell cycle	cyclin H;1	CBB36490	1,33	-	-	-1,39
	translationally-controlled tumor protein	ACG47605	-	-	-	1,42
	proliferation-associated protein 2G4	NP_001149216	-	-	-	1,56
Signal transduction	non-TIR-NBS-LRR type resistance protein	ABM30222	-	-	1,76	-
	small Ras-related GTP-binding protein	AAL30396	-	-	-	2,17
	14-3-3-like protein	AAP48904	-	-	-	2,56
	small Ras-related GTP-binding protein	AAL30396	-	-	-2,76	-3,24
	putative protein phosphatase 2C	BAD43676	-	-	-1,46	-1,60
	auxin-binding protein ABP20 precursor	ACG30276	-	-	-6,22	-9,72
	nucleoside diphosphate kinase	AAF91407	-	-	-1,90	-1,52
Transcription regulation	lysyl-tRNA synthetase	NP_001146902	-	-	-	1,6
	pre-mRNA-splicing factor 19	NP_001150622	-	-	-	1,38
	RNA recognition domain containing protein expressed	CBH32582	-	-	-	1,91
	NHP2-like protein 1	ACG28000	-	-	-	1,37
	glycine-rich RNA-binding protein	AAG23220	-	-	-	1,9
	DEAD-box ATP-dependent RNA helicase 56	Q0JM17	-	-	-	1,63
	transcription factor BTF3-like isoform 1	XP_003574078	-	-	-2,83	-3,17
	Tat binding protein	BAA04615	-	-	-1,56	-1,93

	heterogeneous nuclear ribonucleoprotein A3	XP_003563737	-	-	1,71	2,18
	DNA-binding protein	CAA46876	-	-	-2,53	-2,20
Defense	calreticulin2	Q9SP22	-1,6	-1,8	-	-
	polyphenol oxidase	AAB94293	1,3	-	1,81	-
	glutathione S-transferase C-terminal domain	ACG41887	-	-1,43	1,74	-
	glutathione S-transferase12	NP_001104981	-	-	1,83	-
	glutathione S-transferase12	NP_001104981	-	-	2,02	-
	glutathione S-Transferase-I	1AXD_A	-	-	1,3	-
	caffeic acid 3-O-methyltransferase	AAQ67347	-	-	-	-1,77
	isoflavone reductase homolog IRL	NP_001105699	-	-	-	-1,58
	putative respiratory burst oxidase homolog protein G	Q9SW17	-	-	-	-1,5
	phenylalanine ammonia-lyase	ABM63378	-	-	-	1,31
	polyphenol oxidase	AAB94293	-	-	1,48	1,54
	polyphenol oxidase	AAB94293	-	-	1,77	2,00
	polyphenol oxidase	AAB94293	-	-	1,62	1,48
	caffeic acid 3-O-methyltransferase	AAQ67347	-	-	-1,54	-1,81
	caffeic acid 3-O-methyltransferase	AAQ67347	-	-	-1,43	-1,65
	APx1 - Cytosolic Ascorbate Peroxidase	NP_001152249	-	-	1,51	1,71
	formate dehydrogenase	AAP80655	-	-	1,46	1,50
	isoflavone reductase homolog IRL	NP_001105699	-	-	-1,49	-1,69
	osmotin-like protein precursor	NP_001147098	-	-	1,50	1,74
Aminoacid metabolism	methionine synthase	AAL73979	-	-	-	-1,99
	methylenetetrahydrofolate reductase 1	NP_001104947	-	-	-	-1,99
	S-adenosylmethionine synthetase	CAJ45548	-	-	-	-1,59
	S-adenosylmethionine synthetase	CAJ45549	-	-	-	-1,53

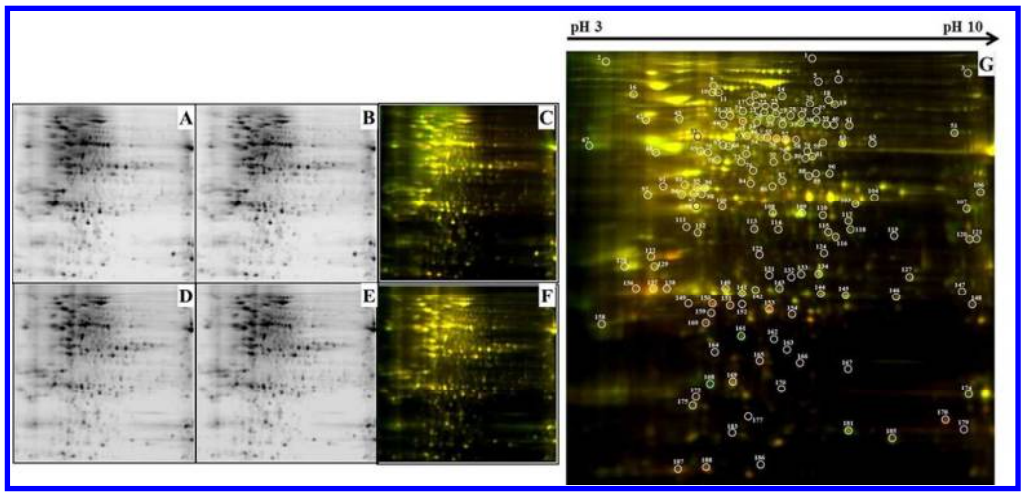
	glutamine synthetase	AAW21273	-	-	-	-1,44
	asparaginyl-tRNA synthetase, cytoplasmic	ACG28959	-	-	1,42	1,39
Carbohydrate metabolism	isocitrate dehydrogenase2	NP_001140324	-	-	-1,3	-
	chlorophyll a-b binding protein. chloroplastic	P06671	-	-	-2,45	-
	transketolase, chloroplastic	Q7SIC9	-	-	-	-1,74
	transketolase, chloroplastic	Q7SIC9	-	-	-	-1,73
	exoglucanase1 precursor	NP_001130296	-	-	-	-1,49
	pyrophosphate-fructose-6-phosphate1-phosphotransferase	ABL10095	-	-	-	-1,57
	Succinate dehydrogenase [ubiquinone] flavoprotein subunit	NP_001058845	-	-	-	-1,82
	ATP synthase CF1 alpha subunit	YP_024377	-	-	-	-1,45
	UDP-glucose 6-dehydrogenase	B6TBY8	-	-	-	-1,4
	ribulose-1.5-bisphosphate carboxylase/oxygenase large subunit	Q6ENV5	-	-	-	-2,69
	NADH-ubiquinone oxidoreductase 51 kDa	B6T6U3	-	-	-	-1,38
	cytoplasmic aldolase	BAA02729	-	-	-	-1,84
	protochlorophyllide reductase B	NP_001167680	-	-	-	-2
	glycosyltransferase	CAI93183	-	-	-	1,66
	sucrose synthase:ISOTYPE=2	2008300A	-	-	-	1,74
	sus1 (sucrose synthase)	AAA68209	-	-	-	2,13
	sucrose-phosphate synthase	BAA19241	-	-	-	1,56
	pyrophosphate-fructose-6-phosphate1-phosphotransferase	ABL10095	-	-	-	1,69
	endo-1.4-b-glucanase BAA94257	CBB36505	-	-	-	1,87
	trehalose-6-phosphate synthase	ADA70118	-	-	-	1,5
	putative trehalose-phosphatase	CAZ96191	-	-	-	1,51
	alcohol dehydrogenase class-3 (ADH)	NP_001105485	-	-	-	1,53
	glyceraldehyde-3-phosphate dehydrogenase	AEA30218	-	-	-	1,85
	fructokinase-2	ACG34322	-	-	-	1,33

mitochondrial ATP synthase gamma chain	ACF06556	-	-	-	1,68
chain A. Crystal Structure of Dhurrinase 1	Q41290	-	-	-	1,44
guanine nucleotide-binding protein beta subunit	ABR25943	-	-	-	1,7
putative r40c1 protein - rice	AAN64997	-	-	-	1,44
ATP synthase CF1 alpha subunit	YP_024377	-	-	-2,26	-2,02
ATP synthase CF1 alpha subunit	YP_024377	-	-	-2,91	-2,86
ATP synthase CF1 beta subunit	YP_024386	-	-	-2,01	-2,05
pyrophosphate--fructose 6-phosphate 1-phosphotransferase	XP_003564006	-	-	1,59	1,63
ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	YP_024387	-	-	-2,56	-2,33
ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	YP_024388	-	-	-3,33	-2,86
ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	YP_024389	-	-	-2,40	-2,41
ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	YP_024390	-	-	-1,41	-1,46
Rubisco large subunit-binding protein subunit alpha, chloroplastic	XP_003558045	-	-	-2,06	-2,01
RuBisCo subunit binding-protein beta subunit	AAT90346	-	-	-2,55	-2,31
chloroplast ribulose-1,5-bisphosphate carboxylase/oxygenase	ABY66908	-	-	-3,04	-2,21
glyceraldehyde-3-phosphate dehydrogenase, cytosolic 3	NP_001105385	-	-	1,57	1,60
glyceraldehyde 3-phosphate dehydrogenase, putative	XP_002513328	-	-	1,39	1,30
photosystem I reaction center subunit IV A	ACG30530	-	-	-4,72	-4,44
fructose-bisphosphate aldolase	ACG36798	-	-	-2,65	-2,74
plastid high chlorophyll fluorescence 136 precursor	ABQ53629	-	-	-1,72	-2,09
ferredoxin	NP_001105568	-	-	-1,89	-2,13
oxygen-evolving enhancer protein 1	ACG31595	-	-	-2,76	-2,75
Chlorophyll a-b binding protein, chloroplastic	P06671	-	-	-1,45	-2,41
chloroplast-localized Ptr ToxA-binding protein1	ACG46365	-	-	-2,66	-2,85
Chlorophyll a-b binding protein, chloroplastic	P06671	-	-	-3,26	-3,48
chlorophyll a-b binding protein 1, chloroplastic precursor	NP_001147639	-	-	-2,68	-2,86
chlorophyll a-b binding protein 8	ACG28457	-	-	-3,94	-3,91
chlorophyll a/b binding protein precursor	ACD13267	-	-	-4,43	-4,14

	23 kDa polypeptide of photosystem II	BAA08564	-	-	-3,18	-3,25
	23 kDa polypeptide of photosystem II	BAA08564	-	-	-3,07	-2,95
	cytochrome c oxidase subunit II PS17	P84733	-	-	-2,39	-2,38
	quinone oxidoreductase	CAD31838	-	-	-1,46	-1,63
Protein metabolism	chaperonin 60	AAA33450	1,32	-	-	-
	eukaryotic translation initiation factor 3 subunit G-like isoform 1	XP_003570267	-	-	1,53	-
	eukaryotic translation initiation factor 5A	NP_001105606	-	-4,38	-	-2,69
	eukaryotic translation initiation factor 5A	NP_001105607	-	-	-	1,4
	eIF-4A	ACF98296	-	-	-	-1,54
	40S ribosomal protein SA	NP_001149295	-	-	-	-1,37
	poly(A)-binding protein	AAB38974	-	-	-	1,66
	TCP-1/cpn60 chaperonin family protein	NP_198008.1	-	-	-	1,67
	TCP-1/cpn60 chaperonin family protein	AAT77033	-	-	-	1,55
	26S protease regulatory subunit S10B	NP_001148485	-	-	-	1,54
	20S proteasome alpha subunit	NP_001105138	-	-	-	1,52
	proteasome subunit alpha type-4-1-like	XP_003564259	-	-	-	1,43
	protein-O-fucosyltransferase 1	CAH40838	-	-	-	1,66
	Arabidopsis protein targeted to mitochondria	CBB36496	-	-	-	1,3
	SUMO-conjugating enzyme UBC9	NP_567791	-	-	-	1,95
	26S protease regulatory subunit 6B	B6TDT1	-	-	-	1,85
	cyclophilin	ACT53879	-	-	-	1,38
	chloroplast hsp 70	ABP65327	-	-	-2,01	-1,96
	TCP-1/cpn60 chaperonin family protein	NP_186902	-	-	1,73	2,36
	putative t-complex protein 1 theta chain	BAD45605	-	-	1,62	1,91
	T-complex protein 1 subunit delta-like	XP_003573152	-	-	1,52	1,65
	TCP-1/cpn60 chaperonin family protein	AAT77033	-	-	1,41	1,55
	eukaryotic initiation factor 4A	NP_001105372	-	-	1,58	1,61

	translation initiation factor 1	YP_024411	-	-	-2,75	-2,17
	dnaJ subfamily B member 5	NP_001149016	-	-	1,32	1,37
	26S proteasome non-ATPase regulatory subunit 14	ACG37608	-	-	-1,90	-2,42
	proteasome component2	NP_001148490	-	-	-2,72	-2,79
	40S ribosomal protein S12	ACG24471	-	-	1,49	1,93
	EF1a-like protein	AEI29162	-	-	1,38	1,50
	acidic ribosomal protein P40	NP_001141003	-	-	-1,91	-1,89
	nascent polypeptide-associated complex alpha subunit-like protein	NP_001148944	-	-	1,62	1,59
	peptidyl-prolyl cis-trans isomerase	XP_002514874	-	-	-3,23	-3,24
	peptidyl-prolyl cis-trans isomerase	XP_002514874	-	-	-1,48	-1,54
Hormone metabolism	1-aminocyclopropane-1-carboxylate synthase	AAV63950	-	-	-1,52	-1,95
Transport	annexin p33	NP_001105728	-	-	2,02	-
	annexin p33	NP_001105728	-	-	-	1,39
Uknown	uncharacterized protein	XP_002466586	-	1,65	-4,88	-
	rice homologue of Tat binding protein	BAA04614	-	-	1,31	-
	uncharacterized protein [Saccharum hybrid cultivar R570]	CAZ96142	-	-	1,48	-
	uncharacterized protein SORBIDRAFT_09g021240	XP_002448064	-	-	-	-1,52
	uncharacterized protein SORBIDRAFT_09g021240	CAZ96108	-	-	-	1,69
	uncharacterized protein SORBIDRAFT_09g021240	XP_002452667	-	-	-	1,43
	uncharacterized protein	CAZ96176	-	-	-	1,74
	Uncharacterized Protein	XP_002461241	-	-	1,39	1,90
	hypothetical protein SORBIDRAFT_05g005150	XP_002450420	-	-	-3,28	-3,48
	uncharacterized protein At2g34160-like	XP_003569410	-	-	1,41	1,76
	uncharacterized protein LOC100276911	NP_001144080	-	-	3,47	4,73

	Putative r40c1 protein - rice	AAN64997	-	-	1,69	1,89
	Uncharacterized Protein	XP_002461230	-	-	-1,89	-1,94



Supplementary Figure S1 - Representative 2D-DIGE protein patterns of plantlets of the SP80-3280 sugarcane variety (A) mock-inoculated or (B) Lxx- inoculated at 30 DAI and (C) the overlapping image of (A) and (B); (D) mock-inoculated or (E) Lxx-inoculated at 60 DAI and (F) the overlapping image of (D) and (E); (G) Circled spots indicate differentially accumulated proteins.

177x83mm (150 x 150 DPI)