A Comprehensive Transcriptomic Analysis of the Effect of Silicon on Wheat Plants Under Control and Pathogen Stress Conditions

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The supply of soluble silicon (Si) to plants has been associated with many benefits that remain poorly explained and often contested. In this work, the effect of Si was studied on wheat plants under both control and pathogen stress (*Blumeria graminis* f. sp. *tritici*) conditions by conducting a large transcriptomic analysis (55,000 unigenes) aimed at comparing the differential response of plants under four treatments. The response to the supply of Si on control (uninfected) plants was limited to 47 genes of diverse functions providing little evidence of regulation of a specific metabolic process. Plants reacted to inoculation with *B. graminis* f. sp. *tritici* by an upregulation of many genes linked to stress and metabolic processes and a downregulation of genes linked to photosynthesis. Supplying Si to inoculated plants largely prevented disease development, a phenotypic response that translated into a nearly perfect reversal of genes regulated by the effect of *B. graminis* f. sp. *tritici* alone. These results suggest that Si plays a limited role on a plant's transcriptome in the absence of stress, even in the case of a high-Si-accumulating monocot such as wheat. On the other hand, the benefits of Si in the form of biotic stress alleviation were remarkably aligned with a counter-response to transcriptomic changes induced by the pathogen *B. graminis* f. sp. *tritici*.

Soluble silicon (Si) is not considered to be essential for plant growth even though it can be absorbed by plants at levels exceeding most macronutrients (Epstein 1999). In general, monocots are able to take up more Si than dicots, with rice being the crop accumulating one of the highest levels (approximately 10% dry matter) (Epstein 1994; Hodson et al. 2005). This proficiency would be attributable to the presence of specific Si transporters recently discovered in rice roots and shoots (Ma et al. 2006; Yamaji et al. 2008). There is some indication that Si transporters are present in other species and, thus, the absorption capacity of plants would depend on the density or efficiency of these transporters (Rains et al. 2006). Despite these recent breakthroughs linking Si with plant metabolism, the physiological role of Si remains undefined and controversial.

Essential or not, the role of Si in plants has been gaining in interest in recent years because of many reports describing its

benefits. It is now established that Si amendments can improve crop yields through alleviation of abiotic and biotic stresses in plants known to absorb and accumulate Si. In fact, the prophylactic effect of Si toward fungal pathogenic agents causing rice blast and powdery mildews is arguably the best described effect of Si on plants (Epstein 1999). However, the exact mechanisms by which Si exerts its beneficial effects are still misunderstood, a situation that has hindered a wide-scale application of Si for agricultural purposes.

Fauteux and associates (2006) conducted a transcriptomic analysis of the effect of Si feeding in the *Arabidopsis*–powdery mildew pathosystem. Interestingly, the effect of Si feeding appeared to be negligible on control plants but manifest as a stress alleviator in infected plants. In a similar smaller-scale study on rice, other authors could not see any Si effect on a number of selected genes and suggested that applying a pathogen stress or looking at a greater number of genes might have yielded more insightful results (Watanabe et al. 2004).

Ideally, one would want to perform a complete transcriptomic analysis on a crop known to accumulate Si to high levels and that has been shown unequivocally to be protected against a pathogen stress as a result of Si supply. The wheat–*Blumeria graminis* DC. f. sp. *tritici* Ém. Marchal interaction meets these conditions because i) a large part of the wheat genome has been sequenced and gene annotation is progressing at a fast rate, ii) wheat microarrays are commercially available, iii) wheat plants accumulate large amounts of Si, and iv) the response of wheat plants to *B. graminis* f. sp. *tritici* following Si supply has been studied extensively through microscopic and chemical analyses (Bélanger et al. 2003; Guével et al. 2007; Rémus-Borel et al. 2005). In this work, we performed a comprehensive analysis of the influence of Si on the wheat transcriptome under both control and pathogen stress conditions. Genes that were differentially expressed as a result of Si feeding were categorized. These results highlight both the benefits of Si amendments and the complex nature of its interaction with plants in general and wheat in particular.

RESULTS

In this work, wheat plants were subjected to four different treatments: control (Si–B–), addition of soluble Si to the nutrient solution (Si+B–), inoculation with the powdery mildew pathogen *B. graminis* f. sp. *tritici* (Si–B+), and addition of soluble Si to the nutrient solution and inoculation with *B. graminis* f. sp. *tritici* (Si+B+).

X-ray microanalysis.

X-ray microanalyses were performed on wheat leaves (three leaves per treatment) to follow Si deposition in plants in relation to the four treatments. Based on the X-ray analysis of Si deposition, Si was efficiently absorbed and readily deposited in silicaphile cells of wheat plants regardless of the plants being infected or not when it was supplied at a concentration of 1.7 mM (Fig. 1C, D, G, and H). In contrast, in Si⁻ plants, only a faint presence of Si was detected, primarily at the base of trichomes (Fig. 1A, B, E, and F) and in a few silicaphile cells (Fig. 1B).

Fig. 1. Scanning electron microscopy (SEM) and X-ray microanalysis of silicon (Si) accumulation in wheat leaves across the different treatments. SEM (left) and corresponding X-ray mapping (right) for the different treatments: **A** and **B**, without Si amendment and without inoculation with *Blumeria graminis* f. sp. *tritici*; **C** and **D**, with Si amendment and without subsequent inoculation; **E** and **F**, without Si amendment and with subsequent inoculation; and **G** and **H**, with Si amendment and with subsequent inoculation. Colored scales in panels b, d, f, and h represent Si concentration, where red is the highest concentration and black is an absence of detectable silicon. All pictures were taken in the same conditions (voltage, 15 kV; current, 20 nA; acquisition time, 1 h).

Disease evaluation.

Disease was visually scored on wheat plants to monitor its development by evaluating the leaf area covered by *B. graminis* f. sp. *tritici* on whole plants in order to assess the prophylactic effect of Si supply. Wheat plants inoculated with *B. graminis* f. sp. *tritici* started showing the first macroscopical signs of disease 7 days after being inoculated and infections were more manifest in Si⁻B⁺ plants. This trend progressed over the course of the observation period and, by the end of the experiment, Si⁻B⁺ plants were significantly more infected than Si⁺B⁺ plants that were nearly free of macroscopically visible disease symptoms (Fig. 2). As expected, no signs of disease were observed on noninoculated plants whether or not they were supplied with Si.

Microarray data analysis.

In all, 12 microarrays (three per treatment) were used in this study to assess the impact of the four conditions (Si⁻B⁻, Si⁺B⁻, Si⁻B⁺, and Si⁺B⁺) on gene expression. On a given microarray, hybridized RNA corresponded to the leaves of one plantlet. Thus, each treatment was analyzed through three biological replicates. Data quality assessment performed on the microarray data indicated a high consistency among replicates. Overall, our study identified 880 regulated genes in the four comparisons studied, some of them being present in more than one comparison. Patterns of gene regulation in four comparisons are shown in Figure 3.

On a comparative basis, the supply of Si to control (non-inoculated) plants did not influence the expression of many genes because only 47 of 55,052 were significantly altered in their expression by the treatment (Fig. 3; Si⁺B⁻ versus Si⁻B⁻). Among these, 27 were upregulated and 20 were downregulated. On the other hand, in the absence of Si supply, inoculation of plants with *B. graminis* f. sp. *tritici* affected the expression of 518 genes (Fig. 3; Si⁻B⁺ versus Si⁻B⁻). Most of them (459) were upregulated as a result of the infection caused by the fungus. In contrast, the effect of Si on inoculated plants (Si⁺B⁺ versus Si⁻B⁺) yielded some surprising results (Fig. 3). The expression profiles appeared to be nearly the mirror image of the previous comparison, whereby genes that were upregulated were now downregulated and vice versa, with 418 genes in common. Specifically, in total, 699 genes were regulated, with the vast majority of them being downregulated (685). In the final comparison (Fig. 3; Si⁺B⁺ versus Si⁺B⁻), it appeared that treatment with Si alleviated the negative impact of the inoculation with *B. graminis* f. sp. *tritici* because few genes ($n =$

Fig. 2. Disease assessment. Percentage of leaf area covered by *Blumeria graminis* f. sp. *tritici* for the four treatment combinations 3 weeks after inoculation. Si⁺ or Si⁻ stand for the presence or absence, respectively, of Si in the fertilizing solution; B⁺ or B⁻ indicate whether an inoculation with *B. graminis* f. sp. *tritici* took place or not.

81; 25 up and 56 down) were regulated when plants were first supplied with Si. This is in line with results in Figure 2, showing that inoculated Si+ plants had a very low level of infection and were phenotypically similar to noninoculated plants. The alleviation of stress caused by *B. graminis* f. sp. *tritici*, as a result of Si supply, is also corroborated by comparing lanes 2 and 4 (Fig. 3); in the absence of Si, large changes in gene expression are seen whereas, in its presence, the transcriptomic response is much attenuated.

Annotations.

Categorization of the regulated genes proved to be challenging. Of the 880 genes identified as significantly regulated in at least one of the comparisons, only 10.8% ($n = 95$) came with an identified function provided with Affymetrix's description

of the microarray. Our interrogation of additional databases increased this number to 349 (39.6%). The proportion of genes of "unknown function" ranged from 36% in the comparison Si+B- versus Si-B- to 70% in the comparison Si-B+ versus Si-B-.

Results of the functional classification of genes are presented in Figure 4. Among the few genes that appeared to be regulated by Si in noninoculated plants (Si+B- versus Si-B-), most of the successfully annotated genes (20 of 30) were nonetheless identified as genes involved in the response to stress or plant defense. Expectedly, infection following inoculation by *B. graminis* f. sp. *tritici* (Si-B+ versus Si-B-) induced gene changes involved in translation ($n = 37$ of 153), primary metabolic processes ($n = 30$), transport ($n = 19$) response to stress ($n = 18$), and photosynthesis ($n = 4$). Conversely, Si

Fig. 3. Visual representation of differential gene expression in wheat leaves for plants having received or not received silicon (Si) amendment or *Blumeria graminis* f. sp. *tritici* inoculation. The columns represent contrasts between the treatments: control (Si-B-), Si alone (Si+B-), inoculation alone (Si-B+), or a combination of both (Si+B+). In total, 880 genes have been identified as differentially expressed ($P < 0.01$, ≥ 1.5 -fold change) in at least one contrast. Each gene corresponds to a colored line indicating the mean ($n = 3$) relative transcript level: green corresponds to a $\log_2$ ratio of -2 (downregulation) and red corresponds to a $\log_2$ ratio of 2 (upregulation).

Fig. 4. Functional categorization of differentially expressed genes in wheat plants. The columns represent contrasts between the treatments: control (Si-B-), Si alone (Si+B-), inoculation with *Blumeria graminis* f. sp. *tritici* alone (Si-B+), or a combination of both (Si+B+). For each gene category and each contrast, colored boxes indicate the direction of regulation (upregulation = red boxes or downregulation = green boxes). Figures indicate the number of regulated genes ($P < 0.01$, ≥ 1.5 -fold change).

treatment of inoculated plants (Si+B+ versus Si-B+) revealed regulation in translation ($n = 96$ of 247), primary metabolic processes ($n = 41$), transport ($n = 17$), and response to stress ($n = 25$). Finally, in the comparison of Si+ plants inoculated or not with *B. graminis* f. sp. *tritici* (Si+B+ versus Si+B-), regulation concerned mainly genes involved in the response to stress ($n = 30$ of 47).

Validation of microarray results

by quantitative real-time polymerase chain reaction.

Quantitative real-time polymerase chain reaction (PCR) was performed on 10 selected genes that were regulated by the different treatments under our experimental conditions. These genes were randomly selected among genes that were i) strongly regulated and ii) suitable to design quantitative PCR primers. All treatments were included in this validation. Quantitative PCR and microarray results were linearly correlated ($R^2 = 0.93$) (Fig. 5).

DISCUSSION

After 150 years of research, the role of Si in plant physiology remains ambiguous because the element is still considered nonessential to plant growth while benefits associated with its absorption by plants continue to accumulate (Fauteux et al. 2006). In this study, we compared the differential expression of more than 55,000 tentative transcripts in wheat plants supplied or not with Si and infected or not with *B. graminis* f. sp. *tritici* in an attempt to shed some light on the elusive biological properties of Si in plants. To our knowledge, this is the most comprehensive transcriptomic analysis aimed at explaining the role of Si in an active accumulator monocot plant.

As a prerequisite, we first had to validate that our biological material did respond to Si in a manner consistent with the expected outcome. This was done by comparing the amount of Si deposition and disease protection against *B. graminis* f. sp. *tritici* between wheat plants supplied or not with Si. This step had important implications because it assured that the transcriptomic response reflected the phenotypic variations observed. As reported previously, our X-ray microanalysis confirmed the typical pattern of Si deposition in wheat plants (Côté-Beaulieu et al. 2009). The faint presence of Si in control plants is indicative of the ubiquity of the element in the environment. This may explain the experimental difficulties in establishing the essentiality of Si in plants but, nevertheless, this low level of background contamination was clearly insufficient to have the prophylactic effect provided by an amendment of 1.7 mM, as demonstrated here and in numerous other reports (Guével et al. 2007; Rémus-Borel et al. 2005). Incidentally, the protection against *B. graminis* f. sp. *tritici* conferred by Si on wheat in this work was nearly complete in contrast with the *Arabidopsis*-powdery mildew model, where disease reduction was clearly less manifest (Fauteux et al. 2006).

Our comparative transcriptome analysis of four different treatments provided novel insights regarding the responses of wheat to Si and powdery mildew. First, the effect of Si alone resulted in the altered regulation of a relatively small number of genes. However, the relevance or importance of these genes lies more in the analysis of their identity than in their number. Indeed, there is accumulating evidence that supports the concept that the benefits of Si feeding are more evident under conditions of stress, and our analysis corroborates this concept. As a first observation, it is interesting to note that two-thirds of the annotated genes (20 of 30) were related to a stress or plant defense response in spite of the controlled conditions that prevailed. The remaining genes did not seem to have a common link that would associate Si supply with a specific role or function.

Our results are in agreement with the report by Fauteux and associates (2006), who found that only two genes had been altered in Si-fed *Arabidopsis* plants. As a premise, one may have expected a different response in wheat, and a possible effect on the plant's primary metabolism genes, considering that the species is recognized as an active Si accumulator. It has been sometimes argued that Si can directly influence photosynthetic activity (Ma et al. 2004). However, similar results were obtained with rice where the authors, looking strictly at the effect of Si supply, reported no influence of Si on the expression of a number of selected genes (Watanabe et al. 2004). The authors concluded that imposing a stress on the plants would have probably generated more significant results. Therefore, it does appear that the effect of Si alone on plants in general is minimal, at best, when plants are not subjected to a stress, regardless of their ability to absorb Si.

Inoculation of wheat plants with *B. graminis* f. sp. *tritici* led to the regulation of several hundred genes, most of which were upregulated. Among them, the expression of several genes known to be involved in the wheat defense reaction against *B. graminis* were significantly stimulated, including β -1,3-endoglucanase; pathogenesis-related proteins; and germin-like, superoxide dismutase, and peroxidase genes (Bruggmann et al. 2005). It is also interesting to note that several genes involved in energy-demanding primary metabolic processes were upregulated. It is well known that a biotroph such as *B. graminis* f. sp. *tritici* will interfere with the plant's metabolic processes by deriving resources for its own development, thus requiring a greater effort on the plant's part to meet its own demands. At the same time, it was also relevant to observe the downregulation of genes involved in photosynthesis, one of the most common symptoms linked with powdery mildew attacks.

Treatment of wheat plants with a 1.7 mM soluble Si solution provided a nearly complete protection against *B. graminis* f. sp. *tritici*, a result that translated into an almost perfect mirror image of the previous transcriptomic profile comparison looking into the effect of powdery mildew on wheat plants. In other words, most of the genes that had been upregulated in response to *B. graminis* f. sp. *tritici* attack were downregulated in relative terms if plants were treated with Si prior to inoculation, and the opposite occurred for downregulated genes. This suggests that the stress imposed by the pathogen, which translated into nearly 900 genes being regulated, has been basically eradicated by Si. This conclusion is reinforced by the comparison Si+B+ versus Si+B-, where it is quite clear that very few genes are regulated in response to the pathogen when Si is

Fig. 5. Correlation between microarray and real-time polymerase chain reaction (PCR) data. Log₂ of fold change determined by microarray (x axis) and real-time PCR (y axis) are plotted for a subset of 10 differentially expressed genes in wheat leaves. A linear regression was performed and the line equation is given with its squared correlation coefficient.

supplied. These results contrast with the *Arabidopsis*–powdery mildew study by Fauteux and associates (2006), where supply of Si followed by powdery mildew inoculation resulted in the regulation of a very large number of genes. This disparity is actually very informative in terms of how monocots and dicots differ in their response to Si. As useful as *Arabidopsis* can be as a model plant, it remains a passive (low) accumulator of Si, like most dicots, compared with active (high) accumulators such as rice, sugarcane, wheat, and most monocots that will contain more than 1% Si in their tissues (Hodson et al. 2005). Accordingly, the beneficial effects of Si have always been more manifest and documented in monocots (Ma et al. 2004). Our results certainly support this model, given that wheat plants supplemented with Si were nearly devoid of powdery mildew colonies in this study whereas Si+ *Arabidopsis* plants from the study of Fauteux and associates (2006) were still largely infected albeit, to a lesser extent than Si– plants. This would confirm that the ability of a plant to accumulate high levels of Si (presumably through active transport) is correlated with the benefits it obtains.

In order to assess the level of common genes regulated by Si and powdery mildew between wheat and *Arabidopsis*, we performed a comparison of our results with those previously published by Fauteux and associates (2006). In their work, only two *Arabidopsis* genes were identified as regulated by Si supply alone. These genes, At5g22460.1 and At1g21860.1, code for an esterase lipase thioesterase family protein and a multicopper oxidase type I family protein, respectively. No correspondence was found when we looked for equivalent functions in the list of genes regulated in the wheat assay. To determine if this was attributable to a lack of annotation in wheat, we performed a blast using the sequences of At5g22460.1 and At1g21860.1 against the National Center for Biotechnology Information database and found no match with known wheat sequences. We further used the sequences of the 880 primers of the genes that were regulated in the wheat assay to find possible homologs in the regulated *Arabidopsis* genes reported in the study of Fauteux and associates (tblastx, e-value threshold = $1e^{-10}$). Of these 880 sequences, 139 (15.8%) yielded results. Several of them (21 of 139) were assigned to *Arabidopsis* sequences without known functions. Other identified homologs were mainly classified into response to stress (20 of 139), primary metabolic process (19 of 139), secondary metabolic process (18 of 139), and defense response (12 of 139) categories. Although this indicates that there is a certain level of overlap in genes regulated by stress such as powdery mildew infection, it does appear that wheat and *Arabidopsis* do not react to Si supply and powdery mildew infection in a similar manner. This result is consistent with the better prophylactic role played by Si on wheat compared with *Arabidopsis*.

As conclusive as was the prophylactic role of Si in this study, our transcriptomic analysis could not delineate whether it was the result of a mechanical barrier or induced resistance, a commonly debated point in the literature (Fauteux et al. 2005). On the one hand, the results would intuitively support the hypothesis of a mechanical barrier, because a minimal transcriptomic response is coherent with a protection afforded by a physical sheath. On the other hand, the very low disease pressure on Si+B+ plants would indicate that only a limited proportion of cells had to react to *B. graminis* f. sp. *tritici* attacks, a response far too diluted among the other cells to register significantly under our stringent analytical conditions. Localized sampling at sites of infection might generate more insightful data as to what specific defense reactions are involved in wheat supplied with Si under pathogen stress conditions.

This comprehensive transcriptomic analysis of wheat plants reveals that supplemental Si alone has very little incidence on the plant's transcriptome, a result explaining the difficulties that plant physiologists have had over the last 150 years in establishing the role and essentiality of Si in plant nutrition. Although scientists have argued that a difference may exist between active Si accumulators such as monocots and passive ones such as dicots, our results tend to confirm that the benefits conferred by Si are qualitatively the same for both types of plants, albeit at different levels. Indeed, under stress imposed by the powdery mildew pathogen *B. graminis* f. sp. *tritici*, wheat plants supplied with Si had both a phenotypic and a transcriptomic response similar to that of control plants, thus confirming the intrinsic property of Si to alleviate stress in plants.

MATERIALS AND METHODS

Wheat culture in hydroponic system.

Hydroponic systems were used to precisely control Si feeding of plants and to reduce external Si contamination. Seed of wheat cv. AC Drummond, chosen for its known high susceptibility to *B. graminis* f. sp. *tritici* (CRAAQ and CEROM 2005), were sown in 9-cm pots in a nylon bed consisting of nylon stockings cut into small ribbons. Hydroponic systems were set up to immerse roots for 15 min every 30 min. The plants were grown in a greenhouse (16 h of light at 22°C and 8 h of darkness at 18°C, 80% humidity). Plants were immersed in distilled water only during the first week following sowing.

Si amendment and *B. graminis* f. sp. *tritici* inoculation.

Wheat plants were grown in the presence (Si+) or absence (Si–) of Si and inoculated (B+) or not (B–) with *B. graminis* f. sp. *tritici* for a total of four treatments: Si–B–, Si+B–, Si–B+, and Si+B+. These treatments were applied in the following manner. One week after sowing, distilled water was replaced by a Hoagland solution amended or not with potassium silicate (Kasil 6; PQ Corp., South Gate, CA, U.S.A.) at a concentration of 1.7 mM (Si+ Hoagland). This concentration was chosen as the standard, because it is the highest one under which Si will remain in soluble form and available to the plant at a pH below 7 (Knight and Kinrade 2001). Twice a week, Si was added to the Si+ nutrient solution in order to maintain Si concentration at 1.7 mM. Every other week, both Si– and Si+ Hoagland solutions were renewed and the pH adjusted to 5.8 in all systems at each Si addition or nutrient solution renewal.

At 4 weeks after sowing (i.e., 3 weeks after Si amendment started), half the plants were inoculated with *B. graminis* f. sp. *tritici* as described previously. Briefly, 1 day prior to inoculation, reservoir wheat plants heavily infected with *B. graminis* f. sp. *tritici* were gently shaken to remove old spores and to stimulate the production of fresh ones. The inoculation was performed by shaking these infected wheat plants over the experimental plants (Guével et al. 2007; Rémus-Borel et al. 2005).

X-Ray microanalysis.

X-ray microanalysis mapping was used to confirm the presence of Si in wheat leaves as previously described (Côté-Beaulieu et al. 2009; Guével et al. 2007). The third leaf of plants from each treatment was harvested 3 weeks after inoculation. Collected leaves were frozen, lyophilized, and then coated with gold and palladium. Samples (at least three per treatment) were analyzed using a CAMECA SX-100 Universal EPMA microscope (Cameca Instruments, Inc., Trumbull, CT, U.S.A.) operating at a voltage of 15 kV under a current of 20 nA. The acquisition time for each picture was 1 h.

Disease evaluation, harvest of leaf material, and preparation for microarray experiment.

Following inoculation, the percentage of leaf area covered by *B. graminis* f. sp. *tritici* was determined by examining the plants daily over 3 weeks in each treatment. At the end of this period, total RNA was extracted from leaves of three plants per treatment (three biological replicates) with the RNeasy plant kit (Qiagen, Hilden, Germany). Concentration and quality of RNA were assessed on a Nanodrop spectrophotometer (Nanodrop, Wilmington, DE, U.S.A.) and 2100 Bioanalyzer (Agilent, Palo Alto, CA, U.S.A.). Biotin-labeled cRNAs were synthesized using a MessageAmp II-biotin enhanced kit (Ambion, Austin, TX, U.S.A.) following the manufacturer's instructions, using 1 µg of total RNA. Labeled cRNA was then fragmented using 5× Array Fragmentation Buffer supplied with the MessageAmp II-biotin enhanced kit.

Microarray hybridization.

The labeled samples were added to an Affymetrix GeneChip wheat genome array (Affymetrix, Santa Clara, CA, U.S.A.), according to the manufacturer's instructions. As stated by the manufacturer, the wheat microarray contains probe sets representing 55,052 transcripts for all 42 chromosomes in the wheat genome and corresponding to 20,312 unigenes. The microarrays were hybridized for 16 h at 45°C in a rotisserie oven at 60 rpm. Following hybridization, the arrays were washed and stained in an Affymetrix Fluidics Station 450, according to the standard protocol from Affymetrix, and scanned using an Affymetrix Scanner 3000. Twelve microarrays were prepared and corresponded to three biological replications of each of the four treatments (Si-B-, Si+B-, Si-B+, and Si+B+).

Microarray data analysis.

The R 2.6 software (R Development Core Team 2007) and Bioconductor (Gentleman et al. 2004) were used to perform data analysis using dedicated packages Limma (Smyth 2004), Affy (Gautier et al. 2004), and AffyPLM available on the Bioconductor website. Data quality assessment was performed as described by Bolstad and associates (2005). These packages were also used to correct for background (robust multiarray averaging method), normalize the microarray data (quantile method), and generate lists of differentially expressed genes according to the fold change and *t* test *P* values. Gene lists were created by initial filtering on confidence at *P* = 0.01 using the *t* test *P* value as the measure of confidence and the Benjamini and Yekutieli false discovery rate (2001) as multiple testing correction, followed by filtering on expression level (1.5-fold change). The complete data set is available through the Gene Expression Omnibus (accession number GSE12936). Selected gene lists (log ratio data) were loaded into Java Tree View 1.1.1 to generate data display (Saldanha 2004).

Annotations.

Preliminary annotations for differentially expressed genes were obtained from the Affymetrix website. These annotations were later updated using information available in the following databases: Wheat plex, The Gene Index project, InterPro, and UniProt. Annotated genes were classified using GO terms. All generated annotations were manually curated.

Validation of microarray results by quantitative real-time PCR.

Following analysis of microarray results, 10 genes were randomly selected among those that showed a significant up- or downregulation in response to the treatments. The differential expression of the selected genes was verified by quantitative real-time PCR with a Mastercycler RealPlex 2S (Eppendorf, Germany). cDNA was made from 2 µg of total RNA, previously digested by RQ1 RNase-free DNase (Promega Corp., Madison, WI, U.S.A.), for each biological replicate using Oligo(dT)₁₈ and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, Ca, U.S.A.). A pair of primers for each of the selected genes (Table 1) was designed with Primer 3 software (Rozen and Skaletsky 2000) to amplify approximately 100-bp fragments with similar GC content and Tm. Target sequences were obtained from the Affymetrix website. PCR amplifications were performed in 20 µl with the QuantiTect SYBR Green PCR kit (Qiagen) using 1 µl of sample cDNA. Each sample of each biological replicate was analyzed twice. The specificity of primer pairs was verified by both gel electrophoresis and melting curve analysis. β-Tubulin was identified as a constitutively expressed gene in a previous study in wheat inoculated with *Fusarium graminearum* (Bernardo et al. 2006) and, therefore, was used as an internal control for normalization. Quantification of the relative changes in gene expression was performed by using the 2^{-ΔΔCT} method (Livak and Schmittgen 2001).

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Table 1. Primers used in the quantitative polymerase chain reaction validation of microarray data^a

Gene identifier	Forward primer (5' to 3')	Reverse primer (5' to 3')	Comparison
Ta.6123.1.A1_s_at	CCTGGGGTACTTCAAGATCAC	ATCCTCACCTCCATGAACG	Si+B- vs. Si-B-
Ta.7022.1.S1_s_at	CCATGAACCAGGGTAAGCAC	TCTGAACCAAGGCATCTTCTC	Si+B- vs. Si-B-
Ta.12076.1.S1_at	GGCGAGTCTGGTAGATTGG	CACAACCTAGCACCTAATGTCC	Si+B- vs. Si-B+
Ta.22668.2.S1_x_at	GCATCATCACAGCTATCTCTCC	AACCGATGAAACAATGTGGATT	Si+B+ vs. Si+B-
Ta.27763.1.S1_at	GCGGGCTTCTATAAATGTGG	GTATTCTGGTACGGAGGAGTAGATC	Si+B+ vs. Si-B+
TaAffx.54100.1.S1_at	CTGATATGGCTAAAAGCATGTGTC	CTCAAAATGGCACTTATTTCTCT	Si-B+ vs. Si-B-
Ta.261.1.S1_at	TGATCCCTTTCTGACTGTAGTTCC	ATTCCGAACCTACCTAAATCAGCAC	Si-B+ vs. Si-B-
Ta.9140.2.S1_at	ATGGGTGAGTGAGTTCTAGTGGTT	ATCTCATATCATACATCGCCAGAA	Si+B- vs. Si-B-
Ta.21379.1.S1_s_at	CTCAAAGAACTGGGGAAGATC	GGATTGTGCCTGAGTAAGGTC	Si-B+ vs. Si-B-
Ta.25927.1.A1_at	CCCTTGTTGATTCTCTTTCAG	CATCGACACACTTTTGTGCTG	Si-B+ vs. Si-B-
β-Tubulin	ATGTTCCGTGGCAAGATGAGCA	CACACGCTTGACTTGACGTTGT	All

^a The corresponding comparison used to validate the gene is also indicated.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

AffyPLM: bmbolstad.com
 Bioconductor: www.bioconductor.org
 The Gene Index project: compbio.dfci.harvard.edu/tgi
 InterPro: www.ebi.ac.uk/interpro
 UniProt: www.uniprot.org
 Wheatplex database: www.plexdb.org