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Comparative Transcriptome Profiling in Winter Wheat Grown under Different Agricultural Practices

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Supporting Information

ABSTRACT: Wheat (*Triticum aestivum* L.), one of the three most important cereal crops worldwide, has a dominant position in Europe due to its adaptability and consumer acceptance particularly as an organic food commodity. Organic agriculture is developing rapidly, and its authenticity is presently a subject of great concern to food authorities, as incorrect labeling can represent commercial fraud. A comparative transcriptome profiling was conducted on winter wheat flag leaves of several cultivars growing in open fields under different agricultural production systems. Performing a microarray study, 10 transcripts differentially expressed in organic and conventional growing conditions were identified in Tommi and Centenaire cultivars. Transcript abundance profiles of selected probe sets were independently confirmed by quantitative reverse-transcription PCR analysis, tested on Tommi, Centenaire, and Cubus cultivars from different growing-year and geographical sites. Univariate and multivariate statistical analysis showed that the global wheat transcriptome is influenced by the agricultural system indicating a promising approach for analytical verification of the production system of wheat at the farm level.

KEYWORDS: *transcriptome profiling, gene expression, winter wheat flag leaves, Triticum aestivum, organic agriculture, multivariate statistics*

INTRODUCTION

In the past decade, the new techniques of functional genomics such as microarray technology has been applied to plant species to analyze the responses to several stress conditions[1,2] or various treatments,[3,4] and only recently proposed as a new approach for identification of changes in transcript accumulation as an effect of agricultural practice.[5,6] The wheat (*Triticum aestivum* L.) transcriptomics approach has been used with a high potential to reveal the expression dynamics of genes that control many important traits for agriculture.[7−9] The broad range of gene expression data sets provides future opportunities for integrating these data in a systematic approach that may reveal gene coexpression networks that underline important traits and represents valuable resources, which can be exploited in the developmental context and environmental conditions such as drought, high-salinity, or cold.[10]

Organic agriculture is developing rapidly over the world, and food authenticity is presently a subject of great concern to food authorities, as the incorrect labeling of foodstuffs can represent commercial fraud.[11] Organic agricultural methods are believed to be more environmentally friendly than conventional agriculture, which depends on the routine use of herbicides, pesticides, and inorganic nutrient applications in the production of crops and animals. It has also been suggested that organic practices can enhance the biodiversity in agricultural landscapes.[12] Most used fertilizers in agricultural practices are nitrogen based. Nitrate is a natural constituent in plants and is present in all vegetables. Several factors influence the accumulation of nitrate in plants including lack of sunlight or water, variety, maturity, and high levels of fertilization.[13] For example, numerous studies comparing the nitrate contents of organically and nonorganically grown fruits and vegetables revealed a trend toward significantly lower nitrate contents in organically grown crops; however, another study found inconsistent or not significant differences.[14] This is likely to be due to the use of lower amounts and less available sources of nitrogen in an organic farming system (e.g., composts). The nitrogen of the organic fertilizers is not soluble and has to be mineralized before its assimilation by the plants, while the nitrogen from mineral fertilizers is directly assimilated. Other studies have shown that in the case of cereal an increase of the nitrogen rate could imply an increase of proteins but a decrease in their baking quality.[15] However, nitrate fertilization leads among other things to higher levels of amino acids and protein and rapid changes in the levels of a wide range of transcripts encoding enzymes in nitrogen and carbon metabolism to facilitate the assimilation of nitrate and its incorporation into amino acids.[16,17]

Transcription is a biological process known to be influenced not only by abiotic stress conditions but also biotic ones[18] and thus is expected to be also modulated by the differences in the amount and type of plant protection products applied during the developmental stages of the plant.

Agricultural research related to organic farming has been mainly focused on nutritional benefits and food safety aspects (e.g., pesticides, antibiotics, mycotoxins, and nitrites) of organic husbandry in order to appreciate the specificity and eventually superiority of organic food.

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Recently, a number of papers reported the use of modern molecular techniques for the identification of diagnostic factors such as transcripts, proteins, and metabolites for food authentication.[5,19,20] For example, transcriptome analysis was performed in two cultivars of potato tubers grown in two controlled agricultural systems including differences in fertilizer (manure or compost).[6] The most significant differences were observed between compared cultivars (genetic background) instead of between growing conditions.[6] In wheat, Lu et al.[21] analyzed the gene expression changes using either organic (farmyard manure) or inorganic nitrogen. Several genes were differentially expressed in grain endosperm when nitrogen was supplied in either organic or inorganic form. Nonetheless, this unique study on wheat was carried out mainly on one variety grown under controlled experimental conditions.

Four main approaches (i.e., retail purchase comparison, fertilizer treatment comparisons, whole farm comparison, or animal feeding and human health) are used when comparing the two agricultural systems with their advantages and disadvantages and complement each other in terms of the statement they can make.[22] Farm studies are conducted on products from selected farms with different forms of cultivation for which the production conditions are recorded.[23] A mean number of samples reflecting as much as possible the reality can be examined. Environmental factors such as climate and soil conditions can be made suitable for comparison by selecting neighboring farms. The disadvantage of this method is that accuracy of the information given cannot be easily verified. Another problem is that it is very difficult to select the farms and fields in such a manner that they truly represent the cultivation forms which are to be compared regarding the fact that various organic and conventional production systems exist. Considering the intrinsic objective of this study which is to evaluate potential analytical methods that could be used by inspectors to support their work within the certification system of the organic agriculture, the whole farm comparison is the most appropriate experimental design to provide samples that best represent the ones used by the inspectors for taking their decision.

Despite intensive research on the genetic improvement of wheat, the knowledge on how agricultural growing conditions affect the transcription is still weak and under investigation. In addition, up to now there is no validated analytical method for the authentication of organic food products.[24] Availability of wheat microarray platforms may enable the identification of gene expression profiles associated with particular growing conditions and allow the development of diagnostic markers to verify the type of growing conditions. For addressing this issue, the Affymetrix GeneChip wheat genome array was applied to the flag leaves of two winter wheat cultivars grown under three different agricultural production systems in order to screen for differentially expressed transcripts. The flag leaves were chosen based on its importance in nitrogen accumulation leading to the increase of grain yield and on the fact that synthetic liquid fertilizer can still be applied and leading further to changes in the transcriptome. We report here a comprehensive analysis of transcriptome profiles in winter wheat flag leaves, and the results represent a promising approach for the evaluation of the origin of a production system in relation to food authentication.

MATERIALS AND METHODS

Plant Material and Study Design. For any of the winter wheat (*Triticum aestivum* L., *Poaceae*) samples collected, one flag leaf (the last leaf before the ear) per plant was sampled at the milk stage of kernel development. The flag leaves harvested from the fields were further sliced longitudinally, submersed in RNA*later* buffer (Ambion, Gent, Belgium), and stored on ice upon arrival in the lab. The tissue was ground to powder in liquid nitrogen with a MM301 mixer mill (Retsch, Aartselaar, Belgium) and the powder stored at −80 °C until processing. A geographical localization of all the fields sampled in this study is provided in Figure S1 (Supporting Information).

For the microarray analysis, two winter wheat cultivars (i.e., Tommi and Centenaire) were collected in 2008 from the surroundings of Ciney in the Walloon region (Belgium) on plants grown under three agricultural systems: no nitrogen fertilization, certified organic agriculture, and conventional agriculture. In this environment, the organic fields and the ones under no nitrogen fertilization of both wheat cultivars were placed side by side. A total set of 30 samples was used (i.e., 5 flag leaves per field × 3 fields per cultivar × 2 cultivars).

For the quantitative reverse-transcription PCR (RT-qPCR) confirmatory analysis, a total of 60 samples of winter wheat cultivar Cubus (i.e., 4 fields × 15 samples per field) were collected in 2006 from Marche (2 fields), Fronville (1 field), and Dinant (1 field).

Fertilization applied by the farmers during the full study was recorded (Table S1, Supporting Information). In order to check the homogeneity of the soils in a specific geographical site, samples were collected from the four fields of cv. Cubus grown in 2006 (Table S2, Supporting Information). The fields were characterized by a loamy soil (particle size <50 μm) percentage higher than 45% and a clay (particle size <2 μm) percentage lower than 30%. The combination of fertility and moisture holding capacity with good drainage makes loamy soil systems an excellent medium for growing wheat. The pH (KCl) which is a fundamental property controlling biological and chemical processes in the soil was generally within the target zone for such soil types (target zone: pH 6.2−7.3).[25] Cation exchange capacity (CEC) is an important property of soil and is directly related to soil texture. Soil particles are negatively charged, which allows the soil to prevent cations from being leached away. A CEC greater than 10 cmol kg^{-1} was observed across all sites indicating good base cation holding capabilities. For the fields located in the zone of Ciney, the soils were also loamy ones according to the digital soil map of the Walloon Region (http://cartopro3.wallonie.be/CIGALE/).

RNA Extraction and Microarray Analysis. Total RNA was isolated from wheat flag leaves (100 mg powder) using the Qiagen RNA isolation kit (Qiagen, Carlsberg, CA, USA) following the manufacturer's instructions. The RNA quality was assessed with the 2100 Bioanalyzer (RNA Nano chip 6000, Agilent Technologies, Diegem, Belgium) and their quantity estimated with the NanoDrop ND3300 fluorospectrophotometer (Thermo Scientific, Wilmington, DE, USA). The samples with a RNA integrity number (RIN) higher than 5.5 were selected for further experiments. The selected samples (n = 30) used for microarray analysis fitted the requirements for gene expressions screening (i.e., the ribosomal peaks were present and not degraded). The genome-wide expression profile was performed using the GeneChip Wheat Genome Array (http://www.affymetrix.com/products/arrays/specific/wheat.affx), and the expression profiling experiments were conducted on the Affymetrix GeneChip microarray platform (Affymetrix Inc., Santa Clara, CA, USA). All RNA samples were processed following the Affymetrix GeneChip Expression Analysis Technical Manual.

Microarray Data Preprocessing. GeneChip hybridization quality was ensured using the standard Affymetrix controls. Four data processing methods have been used: (a) Probe Log_2 Intensity Error (PLIER) (www.affymetrix.com); options were chosen such that only perfect match (PM) intensities were taken into account and quantile normalization was applied to the probes. The analysis was performed within Bioconductor (http://www.bioconductor.org/); (b) dChip as described by Li and Wong,[26] performed with the dChip software (http://biosun1.harvard.edu/complab/dchip/); (c) Robust Multiarray Average (RMA) as described in Irizarry et al.[27] This method makes use only of the PM intensities, consists of a background correction, and applies a quantile normalization and median polish to summarize the intensities in an expression value, performed within the bioconductor;

(d) positional-dependent-nearest neighbor model (PDNN) as described by Zhang et al.[28] The binding energy parameter file and the expression values were computed with the Perfect Match software (http://bioinformatics.mdanderson.org/software.html).

The normalized expression values (of each of the four data processing methods) of the different conditions were compared with the Linear Models for Microarray Data, Limma Package of Bioconductor.[29,30] For each probe set, the number of MAS 5.0 (Affymetrix Microarray Suite 5) detection calls that were present was counted over all the hybridizations and ranged between 0 (none are present) and 30 (all are present). The probes that were absent for all samples (i.e., # present calls = 0) were omitted. This subset consists of 20,157 probe sets. Also, the control probes were omitted for analysis; hence, the analysis was performed for 41,048 probes. The average expression value for all conditions was estimated using the Limma package, and finally, a contrast matrix (contrast of estimates) was estimated. For each of the contrasts, a moderated t-statistic implemented in Limma was used to test whether it deviated significantly from zero. The resulting p-values were corrected for multiple testing with Benjamini-Hochberg[31] to control for the false discovery rate. For the selection of differentially expressed genes, a method with a more stringent cutoff of the uncorrected p-values (e.g., $p < 0.001$) was applied. Probe sets were allocated to tissue and biological process categories according to the sequence similarity of the Affymetrix target known sequences (Table S3, Supporting Information). Gene annotation was derived from the Affymetrix Web site, National Center for Biotechnology (NCBI), TIGR Wheat genome, and PLEXdb (http://www.plexdb.org). The microarray data were submitted to ArrayExpress (Accession # E-MTAB-903).

Quantitative TaqMan Real-Time PCR Analysis. The quantitative reverse-transcription PCR (RT-qPCR) analysis was performed for selected transcripts identified as differentially expressed in microarray analysis (Tables S3 and S4, Supporting Information). Total RNA isolated from flag leaves was treated with DNaseI (Turbo DNaseI, Ambion) to remove contaminating DNA before being used as a template for complementary DNA (cDNA) synthesis. First strand cDNA was synthesized using the high-capacity cDNA reverse transcription kit (M-MLV reverse transcriptase) according to the manufacturer's instruction (Applied Biosystems, Life Technologies Corp., Carlsbad, CA). Applied Biosystems (assay-by-design service) was used for designing primers and minor-groove-binding non-fluorescent sequence (MGB-NFQ) TaqMan probes (Table S5, Supporting Information). The total PCR reaction volume was 20 μL, comprising 10 μL of TaqMan buffer (2×), 1 μL of 20× TaqMan assay, and 9 μL of cDNA (1:20 dilution) using an ABI standard protocol. The reactions were carried out in duplicates in a 384 well plate. The C_q value (threshold method) was determined automatically using the SDS 2.2.2 software (Applied Biosystems). The PCR amplification efficiency ranged from 93% to 107% (Table S5, Supporting Information) and was determined by the slope of a standard curve obtained by plotting the fluorescence versus concentrations of a mixture of all sample cDNAs (ranging from 1:1 to 1:10000 dilution of the cDNA mixture sample) tested for each primer combination and was determined using the equation: $E = 10^{(-1/\text{slope})} - 1$.[32] No signal was detected in no-template and no-RT (no reverse-transcription) controls. C_q values extracted from the qPCR instrument, and the relative quantities were determined according to Biogazelle qBase^{PLUS} software[33] considering the PCR efficiency calculated for each primer/probe combination. The normalization of RT-qPCR reaction was performed using Ferredoxin-NADP (H) oxidoreductase (AJ457980) and Actin II (TC234027) selected as the most stable expressed genes in the winter wheat flag leaves grown under three different agricultural production systems.[34]

Statistical Analyses. Systat 13 (Erkrath, Germany) was used in order to perform descriptive statistics and the principal component analysis (PCA) for wheat cv. Tommi and Centenaire gene expression analyzed by microarray. For the PCA, the normalized relative quantities of the 10 selected candidate genes measured by RT-qPCR were considered.[35]

SIMCA P+ v12.0 (Umetrics, Umeå, Sweden) was used to build a predictive model based on the orthogonal projections to latent structures − discriminant analyses (OPLS-DA) method considering the three wheat cultivars and the log transformed normalized relative quantities of the selected 10 candidate genes measured by RT-qPCR. From the total of 113 samples analyzed (51 conventional, 48 organic, and 14 no nitrogen fertilization), a calibration set and a validation set were selected randomly for the development and validation of the model, respectively. Seven randomly selected segments were used for the cross-validation. In addition, a prediction set (20% of the total analyzed samples) was selected for the estimation of the prediction rates. The development, validation, and prediction were performed on five random samplings from the sample set. For each OPLS-DA model, some performance characteristics were evaluated: (i) the total explained variance due to the agricultural system (R^2_{ycum}); (ii) the total amount predicted (Q^2_{cum}); (iii) the root mean square error of estimation (RMSEE); and (iv) the root mean square error of prediction (RMSEP). Each OPLS-DA model was applied on "unknown" samples (i.e., prediction set) and the prediction rates (%) for the three classes estimated.

Outliers (0.5% of the total data set) were removed before the PCA and PLS modeling.

RESULTS

Global Transcriptome Changes in Winter Wheat Flag Leaves Revealed by Microarray. Comparative transcriptome profiling was carried out on flag leaves collected from two winter wheat cultivars, Tommi and Centenaire, grown under organic and conventional agricultural production systems in similar pedoclimatic conditions in order to identify subsets of genes differentially expressed as a response to crop husbandries. Depending on the stringency of the data extraction method used (PLIER, PDNN, RMA, and dChip), different numbers of differentially expressed probe sets were obtained by applying a cutoff on the uncorrected p-values at $p < 0.001$ and a fold-change of two (Table 1). In addition, we performed comparisons of organic or conventional versus samples grown

Table 1. Comparisons of Differentially Expressed Probe Sets Depending on the Applied Data Extraction Methods and Treatments to Two Winter Wheat Cultivars (Tommi vs Centenaire)

		uncorrected p-values <0.001		
data extraction[a]	treatment[b]	$\log_2$-ratio < −1 (down-regulated)	$\log_2$-ratio >1 (up-regulated)	total regulated probe sets
PLIER	O vs C-Tommi	238	120	358
	O vs C-Centenaire	884	523	1407
dChip	O vsC-Tommi	162	76	238
	O vsC-Centenaire	686	276	962
RMA	O vs C-Tommi	200	88	288
	O vs C-Centenaire	722	333	1055
PDNN	O vs C-Tommi	38	12	50
	O vs C-Centenaire	196	50	246

[a]PLIER, probe log 2 intensity error; RMA, robust multiarray average; PDNN, positional-dependent-nearest neighbor model. [b]Wheat cultivars grown under an organic production system (O) and under a conventional production system (C).

in no-nitrogen supply in both winter wheat cultivars (Table S6, Supporting Information). Notably, not many transcripts considering the 41048 probes sets analyzed were found as differentially expressed when organic vs no-nitrogen samples were compared, while many transcripts were different when conventional were compared with no-nitrogen grown samples. Table 1 shows clearly (i) that independent of the data extraction method, the number of regulated probe sets was significantly higher in cultivar Centenaire than in the cultivar Tommi and (ii) the stringency of the selected data extraction methods in the identification of differentially expressed genes with PLIER being the least and PDNN the most stringent method. Since a higher amount of false positive results can be expected when using the PLIER method, this method was discarded. Using the PDNN method to select differentially expressed transcripts in both wheat cultivars and the two agricultural conditions (organic/conventional), and using a cutoff higher than 2-fold-change, five probe sets were found as down-regulated in both organically grown cultivars, while no probe sets were up-regulated in both organically grown cultivars. On the basis of RMA and dChip methods with an intermediate stringency, several probe sets were identified as significantly down-regulated in the flag leaves of both organically grown winter wheat cultivars, while one probe set was up-regulated in both organically grown cultivars. To compensate for lowered stringency, a cutoff value of 3-fold change was used for the RMA and dChip methods. The choice of threshold values is somehow arbitrary since every change in gene expression can be biologically important, especially for regulatory genes. However, in the first instance, by increasing the stringency we expected to select the genes significantly differentially expressed in both cultivars since we were interested in selecting transcripts with altered expression related to the agricultural production system and common to all cultivars.

At the selected cutoff, 10 transcripts exhibited cultivar-independent differences between organic and conventional growing conditions (Tables S3 and S4, Supporting Information). The most common functional group among the down-regulated genes in organically grown Tommi and Centenaire samples was represented by transcripts related to light regulated protein precursors (LTP family). Only probe set Ta.28063.1.S1_x_at had higher expression in both organic cultivars samples than the conventional ones. PCA of the 10 selected probe sets produced a clear separation between conventionally and organically or wheat grown without nitrogen for the Tommi and Centenaire wheat cultivars (Figure S2, Supporting Information). Taken together, these findings suggest that microarray analysis is a promising approach, which can be used to screen for differentially expressed transcripts in wheat plants grown under different agricultural conditions.

Validation of Specific Gene Expression Profiles by Quantitative Real-Time PCR Analysis. The relative expression levels determined by RT-qPCR showed that selected transcripts were in agreement with the microarray data. Differences could be observed between organic and conventional samples, and to some extent, a difference in expression levels were also seen (data not shown), which may be explained by the fact that the techniques used (microarray and RT-qPCR) present different normalization procedures. Figure 1 depicted the normalized relative expression of the probe set Ta.30807.3.S1_x_at in conventional and organic samples. This transcript had moderate similarity to an

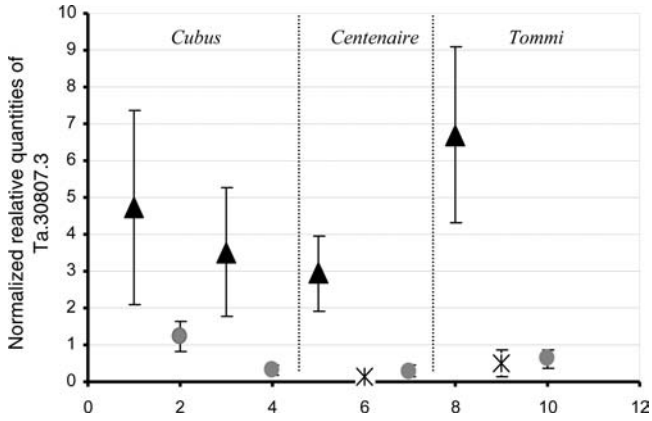

Figure 1. Normalized relative quantities ($\bar{X}$ ± SD) of the gene Ta.30807.3.S1_at estimated in winter wheat flag leaves of three cultivars grown under different agricultural production system. *X*-axis: lanes 1−4, cv. Cubus; lanes 5−7, cv. Centenaire; lanes 8−10, cv. Tommi; triangle, conventional agriculture; circle, organic agriculture; ×, no nitrogen applied.

Arabidopsis protein involved in the metabolism of the glutamate group (Tables S3 and S7, Supporting Information).[36] A blast analysis against the rice genome showed a sequence similarity to the light-induced protein 1-like, which is a light and circadian regulated protein.[37] There is evidence that the transcriptome is strongly influenced by the circadian clock.[38] Another study in wheat grains have shown that this transcript which is involved in aldehyde detoxification is differentially expressed in grains grown under drought conditions.[1] However, the environmental input has an effect on the whole plant transcriptome, and the response level is highly dependent on the time at which the stress is applied.[39] Only one probe set, Ta.28063.1.S1_x_at, was identified as being up-regulated in organic Tommi and Centenaire samples (Figure 2). This transcript had some

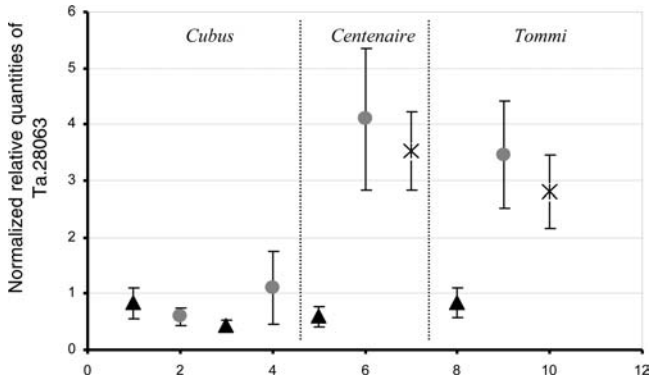

Figure 2. Normalized relative quantities ($\bar{X}$ ± SD) of the gene Ta.28063.1.S1_x_at estimated in winter wheat flag leaves in three cultivars grown under different agricultural production systems. *X*-axis: lanes 1−4, cv. Cubus; lanes 5−7, cv. Centenaire; lanes 8−10, cv. Tommi; triangle, conventional agriculture; circle, organic agriculture; ×, no nitrogen applied.

similarity with the gene *DOT1*, defectively organized tributaries 1 of *Arabidopsis*, which encodes a glycine-rich protein involved in leaf vascular patterning (Table S3, Supporting Information). The function of this gene is not known yet, and further studies will be important for the identification of the biological function of this gene. Despite the fact that a constant trend was observed between both agricultural production systems, we noted in

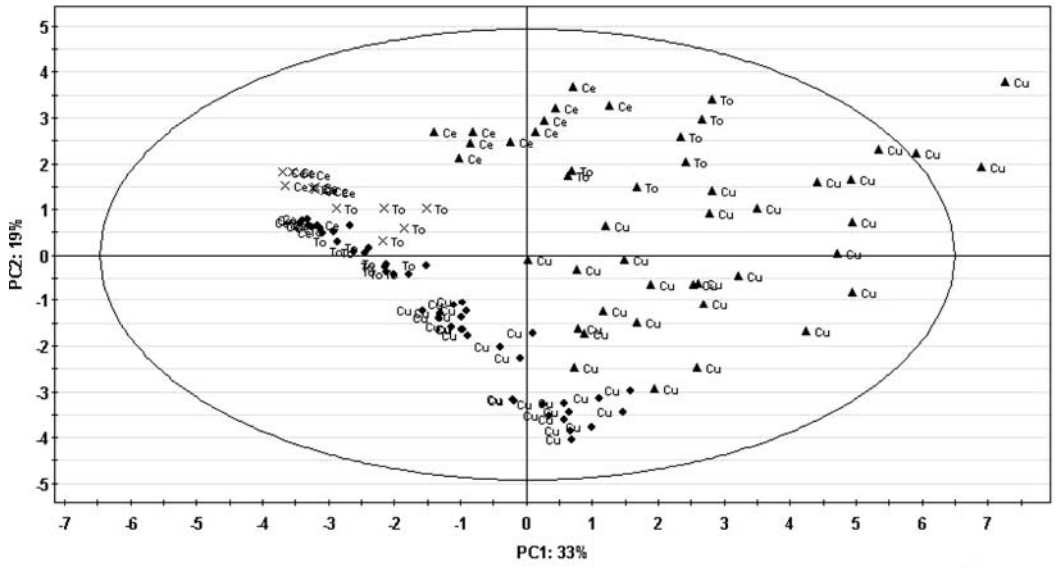

Figure 3. Principal component analysis of genes expressed in flag leaves of wheat grown under three agricultural production systems. Triangle, conventional agriculture; ○, organic agriculture; ×, no nitrogen applied; Cu, cv. Cubus; Ce, cv. Centenaire; To, cv. Tommi.

some cases, such as for probe sets Ta.28394.3.S1_x_at and TaAffx.131611.1.S1_at, a variation in fold change of gene expression (data not shown). This variation may be attributed to the wheat genotype or to environmental conditions that can affect the expression of such transcripts in these plants.

Furthermore, we investigated the expression pattern of the 10 transcripts in cv. Cubus samples collected in 2006 from three different geographical sites and two agricultural conditions (organic vs. conventional) to see whether the observed changes in gene expression is consistent and independent of cultivar, growing year, and geography.

The relative expression of some of the selected probe sets showed a slight modification of the transcriptome in the case of cv. Cubus. Not all transcripts determined as being down-regulated in organic wheat in cv. Tommi and Centenaire were found to be down-regulated in cv. Cubus. The trend of a decreasing gene expression in organic samples observed for the probe set Ta.30807.3.S1_at (Figure 1) was also noted for Ta.30807.2.S1_s_at and Ta.30807.1.S1_at (probe sets from the same transcript). The range of expression levels differed between the geographical sites but was still consistent in terms of a lower expression in organic samples. In some cases, the expression pattern was probably influenced by the environments. For example, the probe sets Ta.8495.1.a1_at and Ta.28233.1.S1_at showed a difference between organic and conventional Cubus samples grown in Fronville and Dinant (geographical distance = ±35 km), respectively, but not in the case of Cubus samples collected in the surrounding of Marche (distance between fields = 5 km). While in other cases, genotypes influencing the expression pattern as in the case of the probe set Ta.28063.1.S1_x_at did not show any significant difference in gene expression between organically and conventionally grown samples of cv. Cubus (Figure 2). These results illustrate the importance in analyzing several cultivars that were grown in different environments and crop seasons.

Prediction Model by OPLS-DA. PCA using the normalized relative quantities of the 10 selected genes of wheat samples showed a clear separation of samples collected in organic growing fields (or fields without nitrogen fertilization) and samples collected from conventional growing fields (Figure 3). The first principal component explained 33% of the total variance due to the agricultural production systems, while the second principal component explained 19%. Four principal components were significant, which together explained 73% of the total variance. In the case of cv. Cubus, the differentiation between both agricultural production systems is weaker than for cv. Tommi and Centenaire due to the fact that in the former not all the selected genes (e.g., Ta.28063.S1_x_at) were differentially expressed between the organically grown samples and the conventionally grown ones (Figure 2). A higher variation in gene expression could be also observed in conventionally grown samples.

The orthogonal projections to latent structures−discriminant analysis (OPLS-DA) was performed in order to sharpen the separation between groups of observations (differences between samples grown under organic/conventional/no nitrogen) by rotating PCA components such that a maximum separation among classes is obtained, and by separating the systematic variation in the predictor variables (i.e., normalized relative quantities) into two parts, one that is linearly related to the response variable and one that is unrelated (orthogonal) to this variable. The advantage of OPLS-DA is its ability to separate predictive from nonpredictive variation. All of the five OPLS-DA models comprised two predictive components and one orthogonal component. On average, the total explained variance due to the agricultural system (R^2_{ycum}) and the estimate of the predictive ability of the model (Q^2) are above 0.50 (Table 2). Therefore, the models developed are valid as far as the biological material is used.[40] Good models were obtained showing a fair correlation between the quantified (observed) gene expressions and the predicted gene expression. Indeed, on average RMSEE equals RMSEP. The mean prediction rates estimated from the five independent prediction sets equal 88%, 96%, and 43% for the conventional, organic, and no-nitrogen wheat samples, respectively. The low prediction rate of wheat samples grown without nitrogen fertilizer is explained by the limited number of samples in this category (n = 14). Figure S3 (Supporting Information) shows the individual cumulative R^2 and Q^2 for every response variable (treatment) and illustrates

Table 2. Validity and Prediction Rates of Five Independent OPLS-DA Models Based on Normalized Gene Expression of Organically and Conventionally Grown Winter Wheat

					prediction rate (%)[f]		
prediction set[a]	R^2_{Ycum}[b]	Q^2_{cum}[c]	RMSEE[d]	RMSEP[e]	C	O	ON
1	0.54	0.51	0.26	0.28	91	89	0
2	0.55	0.52	0.26	0.28	90	100	50
3	0.52	0.50	0.27	0.22	90	100	67
4	0.52	0.50	0.27	0.23	100	89	67
5	0.56	0.53	0.26	0.30	70	100	33
$\bar{X}$	0.54	0.51	0.26	0.26	88	96	43

[a]Each independent prediction is made by selecting 20% of the total sample set (N = 113). [b]Cumulative Y-variation modeled. [c]Estimate of the predictive ability of the model. [d]Root mean square error of estimation (RMSEE). [e]Root mean square error of prediction (RMSEP). [f]Prediction rates of unknown to three classes of treatment (C, conventional; O, organic; ON, no nitrogen).

the low cumulative contribution of these samples to the predictive model.

In total, 14% of the unknown samples used for prediction were misclassified, and half of this percentage was in fact a misclassification of wheat samples grown without nitrogen as organically grown wheat. This similarity is in favor of field experiments simulating organic agriculture by testing crops under no nitrogen treatment. Considering the organic samples, only 5% were false positives, and 1% was false negative. All those samples were in fact from cv. Cubus, which was not involved in the microarray study and consequently not contributing to the importance of the preselected predictor variables. As mentioned above, the profile of expression of several genes in cv. Cubus is different from the one observed for cv. Tommi and Centenaire. Estimating the variable importance in projection for the five models highlighted, the variables with the lowest correlation to all the responses were Ta.28233.1, TaAffx.131611.1, and Ta.28394.3. Nonetheless, removing them had no significant effect on the prediction rates (data not shown).

DISCUSSION

Nitrogen availability under field conditions can vary from one site to another one as a result of the fertilizers applied but also due to environmental factors, and the inappropriate use of fertilizers can lead to the changes in physiological state of the plant triggered by internal or external factors under natural field conditions and be further reflected in the changes of the global transcriptome. A number of projects worldwide are therefore focusing on understanding the processes that determine the efficiency of nitrogen uptake, assimilation, and utilization of nitrogen in order to improve the efficiency of nitrogen recovery in the grain.[42,43] While the physical processes of nitrogen and sulfur remobilization have been studied in detail, the genetic control of these processes and their contribution to agronomic productivity are less well understood.[41] The particular complex structure of the flag leaf allows for an efficient translocation of assimilates until the very late stages of leaf senescence,[42] and the relative contribution of the flag leaves to the final grain nitrogen level is essential.[43] Nitrogen fertilization during the developmental growth of plants influences gene expression.[21,44,45] A more recent gene expression study in maize[46] identified several nitrogen biomarkers in response to different sources of nitrogen fertilizer applied in different agronomic practices. They have shown that while a single gene alone was often able to assess nitrogen status in one experiment, the composite score approach using multiple genes was able to quantitatively assess nitrogen status across all experiments.

In relation to the agricultural systems for organic food production, an inspector will need an analytical method that can be applied on plant material samples grown in field conditions and coming from various environments. As already reported,[47] a risk for plants grown in organically managed farms is low nitrogen availability, which lends to negative effects on yield and fruit quality. To avoid this problem, some unscrupulous farmers could apply synthetic nitrogen by direct fertilization of the crop (e.g., nitrogen fertilizer in solution applied on the flag leaves), and in this case, the fraudulent product is difficult to detect.

When comparing two agricultural systems (organic vs conventional), farm studies are conducted on products from selected farms with different forms of cultivation for which the production conditions are recorded.[23] Environmental factors such as climate and soil can be made comparable by selecting neighboring farms as was done in our study.

In the present study, we have selected those transcripts whose expression differed between samples grown in organic and conventional agricultural production systems, independently of the effect of cultivars (genetic background), crop husbandry, and crop seasons. Similarly, the gene profiling analysis done by the team of Lu et al.[21] showed differences in gene expression between wheat grains fertilized with organic (farmyard manure) and inorganic nitrogen. Using a cutoff of more than 2-fold change, we have identified 10 probe sets which showed differences between organic and conventional samples in winter wheat cultivars (i.e., Tommi and Centenaire). These probes selected by microarray analysis in winter wheat flag leaves belong putatively to defense metabolism, flavonol metabolism, light regulated proteins, and storage proteins. Moreover, we observed that the number of differentially expressed transcripts is lower when organic versus no-nitrogen-added-to-the-soil-growing samples were compared and were higher in the case of the conventional one. Nonetheless, considering the distance between conventional fields and organic ones, an effect of the microenvironment could not be completely excluded. The accuracy of microarray results was confirmed by real-time quantitative PCR not only by repeating the analyses on Tommi and Centenaire cultivars but also by testing a third cultivar, i.e., Cubus. Among down-regulated probe sets in organically grown samples or those grown under no-nitrogen fertilization, the most significant differences have been observed for probe sets Ta.30807 and Ta.23297.1. We have shown that several genes had a similar range of expression applying microarray or quantitative PCR to both Tommi and Centenaire cultivars grown under three different conditions. *In silico* analysis using publicly available data in Genevestigator (https://www.genevestigator.com/gv/plant.jsp) revealed that those transcripts are widely expressed although their expression level varied at different stages of development in flag leaves and other organs (Figure S4, Supporting Information). In general, the heat-map expression pattern showed that those transcripts are up-regulated by different stress and treatments conditions such as nitrogen treatment (e.g., fertilizer), drought stress, etc. (Figure S5, Supporting Information). The expression of transcripts Ta.30807 and Ta.23297, is up-regulated under nitrogen conditions, while transcript Ta.28063.1.S1_x_at,

which was the only up-regulated transcript in organic and no-nitrogen samples, is shown here as down-regulated in wheat samples grown under nitrogen conditions. Consistent with the outcomes of the in silico analysis, the latter transcript is also up-regulated under the low nitrogen condition observed in Ciney and to a lesser extent in Fronville (Figure 2 and Table S1, Supporting Information). However, this transcriptome study highlights that there is a molecular response to different growing conditions, which is most likely linked to the type of treatment and type of chosen germplasm used in a given experiment. For example, the stress responses of reproductive tissues have more relevance to agriculture than juvenile or most vegetative tissues.[51] Therefore, relevant changes in gene expression in specific cells may be diluted by transcripts from surrounding cells and may not be determined.[48] Nevertheless, the spatial resolution of tissue sampling analyzed by a microarray has an impact on all transcriptome profiles. Most performed microarray analyses are conducted on samples grown under laboratory conditions, which allow for a better control of treatment conditions and the germplasm used.[2] Thus, the kinetics of stress treatments is very important and has an impact on the interpretation of results. With regards to the field condition, a recent review highlighted the weakness of the majority of transcription profiles due to the fact that early response to several stress conditions is more related to treatment conditions and does not reflect the growing conditions in fields.[49] Global transcriptome profiling of samples grown under natural field conditions might be different from the case of samples collected from controlled growth conditions (e.g., hydroponic conditions). In natural growing conditions, it is much more difficult to monitor all the parameters that may have an impact on the whole plant transcriptome.

Despite the large amount of analyzed transcripts provided by the Affymetrix chip, a small number was differentially expressed in winter wheat flag leaves grown under different agricultural conditions at the selected cutoff. Although many of these transcripts have not been fully characterized in wheat, the transcriptome profiling performed in several crops grown in different conditions and experimental setups indicates that expression of several gene/proteins responds to the nitrogen supply. Overall, the variation of gene expression may be explained by the cultivar itself, crop seasons, geographical sites, plant-to-plant variation, etc. The differential gene expression could be caused by different physiological stages of the flag leaves in organic versus conventional fields. Nonetheless, several target genes such as RuBisCO small and large subunits and ferredoxin-NADP(H) oxidoreductase, which are abundant in leaves and regulated by nitrogen,[3] were stably expressed in the samples collected from the three cultivars and different agricultural growing conditions. This may argue for comparable physiological stages of the flag leaves in both agricultural production systems knowing that RuBisCO is degraded during senescence.[50] In a transcriptome analysis of potato tubers, van Dijk et al.[6] have shown that there were more pronounced differences among plants grown under a conventional agricultural regime than among the different types of organic fertilizer and plant protection regimes applied. The gene expression profile observed in the studied wheat cultivars seems to confirm this statement (Figures 3 and S2 (Supporting Information)). A hypothesis could be that conventional wheat samples might have higher variability in their physiological stage than organic samples due to the different amounts of nitrogen fertilizer applied in both agricultural systems (Table S1, Supporting Information). Under field growing conditions, nitrogen is one of the major factors that influence plant growth.[16] Taken together, the transcriptome profile analysis in wheat flag leaves collected from plants grown under different agricultural conditions reflected a specific gene expression signature under field conditions. The selected differentially expressed genes are involved in amino-acid metabolism, assimilation of ammonia, metabolism of the glutamate group, or C-carbohydrate group (sugar metabolism) in cellular transport (Table S7, Supporting Information).[51] Previous investigations in *Arabidopsis* showed that many genes involved in carbon metabolism, such as sugar transporter genes, and amino acid transporter genes were up-regulated when the long-term availability of nitrogen increased.[48] Additionally, the light-induced synthesis of carbohydrates also appears to affect the expression of genes involved in nitrogen assimilation.

Similarities of gene expression profiles and the concomitant misclassification rate higher than 5% between organically grown wheat and wheat grown under no-nitrogen fertilization imply the identification of additional biomarkers and also predict the challenges in comparing organic agriculture and integrated agriculture or sustainable agriculture. However, overall these results showed that the agricultural production system had a significant effect on the global transcriptome of winter wheat. The transcriptome profiling represents a valuable source exploited for functional genomics in wheat and offers a new approach in understanding the alteration of gene expression as results of changes in environmental conditions for identification of the origin of crop production systems.

■ ASSOCIATED CONTENT

Supporting Information

Geographical localization of wheat fields, PCA plots, overview plots, microarray data, and additional data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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