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Growth stage-based metabolite profiling of drought-tolerant transgenic rice under well-watered and deficit conditions

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Abstract

Metabolite profiling of transgenic crops is useful when evaluating the intended and unintended effects of genetic modifications. Our study objective was to investigate variations in metabolites from drought-tolerant transgenic rice (*Oryza sativa* L.) that over-expresses *AtCYP78A7*, a gene encoding cytochrome P450 protein. Two transgenic Lines, '10B-5' and '18A-4', plus the wild-type 'Hwayoung', were cultivated under either well-watered or water-deficit conditions in a rainout shelter. Their shoots were collected at the tillering, heading, and ripening stages and polar extracts were subsequently analyzed by ^{1}H–NMR and GC–MS. Principal component analysis revealed that the metabolite profiles could be clearly distinguished during those stages. Soil-water conditions also contributed to the variation in profiles. However, a marked discrimination of metabolites between transgenic and non-transgenic rice was apparent only under water-deficit conditions at the heading stage. This was mainly a result of differences in the sugar-related NMR profiles among genotypes. Our data suggested that the genetic contribution to metabolite profiles is constrained by growth stage and water status. In addition, sugar content is of great importance when separating metabolite profiles in shoots from rice plants that over-express *AtCYP78A7*.

Keywords: Cytochrome P450, Drought, GC–MS, Growth stage, Metabolite profiling, NMR.
Abbreviations: CE–MS_capillary electrophoresis–mass spectrometry; CE–TOF–MS_capillary electrophoresis–time of flight–mass spectrometry; FT–IR_Fourier transform–infrared spectroscopy; GC–FID_gas chromatography–flame ionization detection; GC–MS_gas chromatography–mass spectrometry; GM_genetically modified; LC–MS_liquid chromatography–mass spectrometry; NMR_nuclear magnetic resonance; PCA_principal component analysis; WT_wild-type.

Introduction

Worldwide, genetically modified (GM) crops continue to be developed with improved agronomic traits, higher nutritive quality, and tolerance to biotic or abiotic stresses. However, it is desirable that such plant systems be comprehensively investigated not only for the targeted expression of the introduced gene under standard culturing practices but also so that one can identify any unintended alterations caused by secondary or pleiotropic effects of those modifications. The adoption of "-omics" techniques, such as transcriptomics, proteomics, and metabolomics, is a good approach when considering the experimental data as a whole that are obtained (Cellini et al., 2004; Metzdorff et al., 2006; Ricroch et al., 2011). For example, metabolomics relies upon nuclear magnetic resonance (NMR), gas chromatography–mass spectrometry (GC–MS), liquid chromatography–mass spectrometry (LC–MS), capillary electrophoresis–mass spectrometry (CE–MS), and Fourier transform–infrared spectroscopy (FT–IR), all of which are widely applied for analyzing metabolic changes in various GM crops (Charlton et al., 2004; Levandi et al., 2008; Alcantara et al., 2010; García-Cañas et al., 2011). Manetti et al. (2006) have used NMR to demonstrate that the expression of *Cry1Ab* leads to metabolic alterations in the primary nitrogen pathway of transgenic maize (*Zea mays*) grains. A metabolomics study of glyphosate-tolerant transgenic soybean (*Glycine max*), using capillary electrophoresis–time of flight–mass spectrometry (CE–TOF–MS), has revealed significant differences in several metabolites, especially 4-hydroxy-L-threonine, when compared with its non-transgenic parental line (García-Villalba et al., 2008). Based on investigations by gas chromatography–flame ionization detection (GC–FID) and GC–MS, the levels of sucrose, mannitol, and glutamic acid in transgenic rice (*Oryza sativa*) grains are considerably enhanced by the introduction of *cry1Ac* and *sck* (Zhou et al., 2009). Despite these applications described above, only a few reports are available on the use of metabolomics to evaluate the components of abiotic stress-tolerant transgenic crops growing under adverse conditions. It is impractical to conduct a targeted analysis to investigate the influence of stress because unexpected pleiotropic effects may arise in tolerant transgenic crops (Kasuga et al., 1999; Miki et al., 2009). Moreover, the complex response mechanism of plants against abiotic stresses can make it difficult to predict possible changes in composition. In such situations, metabolomics, which examines the integrated metabolic pathways from upstream to downstream, can be a powerful tool for assessing the utility of abiotic stress-tolerant transgenic crops. Recently, drought-tolerant transgenic rice was developed by inserting *AtCYP78A7* to encode a cytochrome P450 protein

(Kim and Choi, 2012). Such proteins are ubiquitous in plants and play crucial roles in various biochemical pathways, where they catalyze oxygenation/hydroxylation reactions in primary and secondary metabolism (Feldmann, 2001; Schuler and Werck-Reichhart, 2003; Mizutani and Ohta, 2010). Therefore, in terms of the potential of GM crops, it is important to trace the metabolic changes in this transgenic rice that arise upon overexpression of *AtCYP78A7*. Previously, we reported results obtained from our targeted analysis of major and minor nutrients in grains of drought-tolerant transgenic rice that contains *AtCYP78A7*. There, we found substantial equivalence between transgenic and non-transgenic crops in a well-irrigated rice paddy (Nam et al., 2013). We also tested the influence of limited soil moisture on the chemical composition of those same transgenic grains. To do so, we established artificial drought conditions in a rainout shelter and confirmed that the levels of some nutritional components in both transgenic and non-transgenic rice were altered by water-stress treatment (Nam et al., 2014). In the present study, we examined how well-watered versus water-deficit conditions might affect the metabolite composition of shoots from drought-tolerant transgenic rice when compared with its non-transgenic parental line. We also analyzed whether growth stage affects their profiles. Our experimental data were obtained by ^{1}H–NMR and GC–MS.

Results and Discussion

Principal component analysis of metabolite profiles from ^{1}H–NMR and GC–MS

We compared the metabolite profiles of two transgenic rice lines, '10B-5' and '18A-4', with that of wild-type (WT) 'Hwayoung'. Transgenic and non-transgenic rice were grown under either well-watered or water-deficit conditions and their shoots were collected at the tillering, heading, and ripening stages. Principal Component Analysis (PCA) was conducted on the metabolite data obtained via ^{1}H–NMR and GC–MS. The score plot presented three clusters that corresponded to those individual developmental stages (Figs. 1, 2A). In particular, the tillering stage was largely separated from the other two, indicating that metabolic variations between the vegetative and reproductive periods were more significant than those of shoots sampled only within the reproductive period. The PCA loading plot generated from GC–MS data suggested that soluble sugars, including fructose, glucose, and sucrose, were the main contributors to this discrimination among stages (Fig. 2B). Higher levels of fructose and glucose were accumulated at the tillering stage, whereas sucrose was more abundant at the heading and ripening stages. In addition, levels of γ-aminobutryric acid, shikimic acid, phosphate, and quinic acid were highest at the tillering stage. The pattern of clustering found here, which indicated compositional differences among growth stages, was not surprising because the kinds and amounts of metabolites are known to change according to the phase of plant development (Brown et al., 2003; Argyropoulou et al., 2007). Significant stage-dependent variations in metabolite profiles have also been described for *Artemisia annua* L. and *Withania somnifera* (Ma et al., 2008; Sidhu et al., 2011). In our current study, three factors – growth stage, soil water conditions, and genotype – influenced the metabolite profiles in rice shoots. Among these, growth stage appeared to play a more dominant role in the steering metabolism.

Comparison of metabolic profiles at each growth stage

To investigate further the effects of soil water conditions and genetic modification, we conducted PCA on the dataset from each growth stage (Figs. 3, 4). The PCA score plot derived from ^{1}H–NMR data showed two distinct clusters of well-watered and water-deficit conditions in transgenic Line '18A-4' and in the WT 'Hwayoung' at their tillering stages (Figure 3A). However, a clear separation among genotypes was not found under either water status. During heading, good separation between watering conditions was observed only for the two transgenic lines (Fig. 3B). Under drought stress, a clear discrimination appeared for transgenic versus non-transgenic rice, but their results overlapped under well-watered conditions (Fig. 3B). Only a negligible difference in metabolites was detected between watering conditions and genotype at the ripening stage (Fig. 3C). Results from our GC–MS analysis allowed us to divide all samples into two groups of well-watered and deficit conditions at the tillering stage, but no clear grouping among genotypes (Fig. 4A). The loading plot for that stage revealed that the levels of sucrose, fructose, shikimic acid, inositol, quinic acid, and raffinose were significantly greater under the water deficit while those of γ-aminobutryric acid, maltose, glycerol, and phosphate were higher in well-watered plants (Fig. 4B). During heading, a clear separation between watering conditions was observed for the transgenic lines (Fig. 4C). Levels of most compounds, except citric acid, stigmasterol, malic acid, and hexadecanoic acid, were enhanced by the drought treatment (Fig. 4D). By contrast, the datasets associated with ripening could not be separated by PCA (Figs. 4E, 4F). Differences in metabolite levels between soil water conditions by relative quantification are presented in Supplementary Table 1. Drought stress induces numerous metabolic responses in plants (Hare et al., 1998; Seki et al., 2007; Urano et al., 2010; Obata and Fernie, 2012). Endogenous levels of abscisic acid as well as several amino acids, sugars, sugar alcohols, and amines are significantly increased under such conditions. Metabolomics studies have revealed that the expression and accumulation of drought-inducible metabolites are altered during different time intervals of dehydration stress in the leaves of *Arabidopsis thaliana* and wheat (*Triticum aestivum*) (Urano et al., 2009; Bowne et al., 2012). Here, we confirmed such metabolite alterations between watering conditions at the tillering and heading stages. However, as development progressed from tillering to heading to ripening, the earlier boundary between water-deficit and well-watered groups became indistinguishable. These results suggested that drought stress differentially affects metabolite profiles in rice shoots as a function of growth stage.

Metabolite variations between transgenic and non-transgenic rice

Our data showed that genotype had a less significant impact on metabolic changes than did either growth stage or watering conditions. In fact, a specific dissimilarity in metabolites between transgenic and non-transgenic rice was observed only under drought stress at the heading stage (Fig. 3B). To ascertain which metabolite(s) had the greatest influence, we individually compared the datasets of transgenic lines with their non-transgenic counterpart (Fig. 5).

Table 1. Chromatographic and spectrometric data for 49 metabolites from rice shoots analyzed by GC–MS.

Metabolite	RT[a]	QI[b]	Metabolite	RT[a]	QI[b]
Lactic acid*	11.81	117	Phenylalanine*	26.88	218
Glycolic acid	12.22	147	Asparagine*	27.90	116
Alanine*	13.05	147	Ribitol*	29.39	217
Oxalic acid*	13.90	147	Glutamine*	30.10	156
N-carboxy-glycine	14.17	147	Shikimic acid*	30.93	204
Leucine*	14.51	86	Citric acid*	31.25	183
Malonic acid*	15.98	147	Isocitric acid*	31.25	245
Valine*	16.36	144	Quinic acid*	32.24	345
Serine*	17.48	116	Fructose*	32.53	217
Aminoethanol	17.79	174	Glucose*	33.07	319
Phosphate	18.02	299	Lysine*	33.16	174
Glycerol	18.06	147	Mannitol*	33.70	319
Isoleucine*	18.54	158	Hexadecanoic acid*	35.22	313
Glycine	18.89	174	Inositol	36.71	217
Succinic acid*	18.97	147	Octadecanoic acid*	38.73	341
Glyceric acid	19.61	147	Uridine	42.47	217
Fumaric acid*	19.83	245	Adenosine	45.38	236
Malic acid*	23.65	233	Sucrose*	45.93	437
Salicylic acid	24.13	267	Lactose*	46.90	204
Pyroglutamic acid	24.40	156	Trehalose*	47.42	361
Aspartic acid*	24.42	232	Maltose*	52.49	204
γ-Aminobutyric acid	24.59	174	Campesterol	53.78	73
Norvaline	25.09	142	Stigmasterol	54.27	73
Threonic acid	25.55	147	β-Sitosterol	55.22	73
Glutamic acid*	26.72	128	Raffinose*	57.25	361

[a] Retention time. [b] Quantification ion. * Metabolites were identified using purified standards.

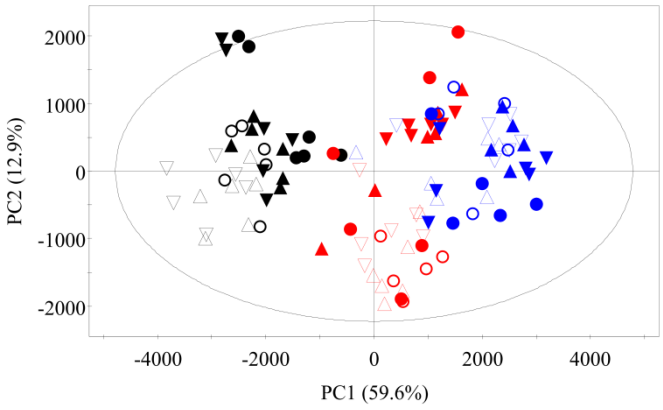

Fig 1. PCA score plot derived from ^{1}H-NMR data for transgenic rice Lines '18A-4' (▼, ▽) and '10B-5' (▲, △), and wild-type (●, ○) 'Hwayoung' grown under well-watered (open symbols) or water-deficit (filled symbols) conditions, as examined at tillering (black), heading (red), and ripening (blue) stages. Ellipse represents Hotelling T2 with 95% confidence in score plots.

Exploiting the PCA score plots, metabolites were obviously distinguished between transgenic and non-transgenic lines (Figs. 5A, 5C, 5E). Furthermore, tracing through those loading plots enabled us to conclude that this variance originated in the sugar region (δ 3.0~5.5) (Figs. 5B, 5D, 5F).

Ma et al. (2008) have reported that differences in metabolites between an *Artemisia annua* L. control and its transgenic counterpart are most prominent at the pre-flower budding, flower budding, and full-flowering stages, but that no separation is displayed at the tender- or adult-seedling stages. Together with that result, our determination that drought-stressed transgenic and non-transgenic rice show specific separations in metabolites during heading strongly indicates that growth stage plays a crucial role in regulating metabolic variations in GM crops. We also were able to assign the metabolites from our tolerant transgenic rice to two groups of well-watered and deficit conditions at the heading stage. By contrast, metabolites from the non-transgenic plants were not affected by drought treatment. For rice, plant growth and yield are extremely sensitive to drought stress, with the effects varying in timing and intensity. Drought that occurs during the vegetative stage can lead to reduced plant height, tiller number,

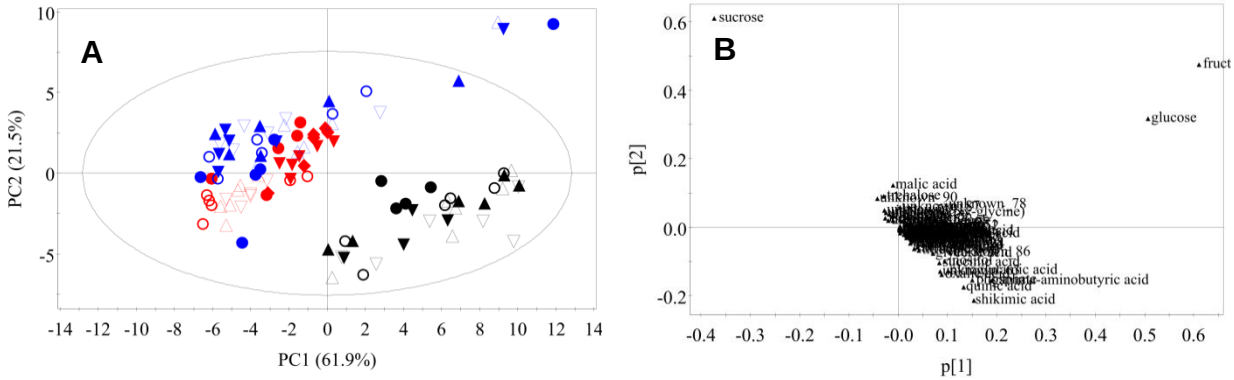

Fig 2. PCA score (A) and loading (B) plot derived from GC–MS data for transgenic rice Lines '18A-4' (▼, ▽) and '10B-5' (▲, △), and wild-type (●, ○) 'Hwayoung' grown under well-watered (open symbols) or water-deficit (filled symbols) conditions, as examined at tillering (black), heading (red), and ripening (blue) stages. Ellipse represents Hotelling T2 with 95% confidence in score plots.

and leaf area (Yoshida, 1981) whereas drought at the reproductive stage greatly influences grain number and size (Dolferus et al., 2011). In particular, plants exposed to drought stress at the heading stage will show large losses in both grain quantity and quality. In rice, heading is a critical trait with complex inheritance, and is regulated by numerous endogenous genes. We presumed in this study that the role of *AtCYP78A7* is important especially during the heading period.

During the heading stage, metabolite levels did not change in an apparent way between transgenic and non-transgenic plants under well-watered conditions. However, individual metabolites were altered under deficit conditions because of differences in sugar content. Sugars play critical roles in plant growth and development processes as signaling molecules, energy sources, and regulators of gene expression (Rolland et al., 2002). In particular, soluble sugars are closely involved in the mechanism for tolerance against drought stress. A metabolic analysis of Italian rice lines with different tolerances to osmotic stress has demonstrated that drought-tolerant and -sensitive cultivars can be clearly distinguished according to their accumulations of some primary metabolites, predominantly sugars (Baldoni et al., 2013). In response to drought, barley (*Hordeum vulgare*) plants increase their levels of glucose and fructose, stabilize their amounts of sucrose, but reduce their starch contents (Wingler et al., 1999). Exogenous applications of glucose and fructose to wheat seedlings boost their drought tolerance whereas treatments with sucrose and mannose are linked with reduced survival rates for drought-stressed plants (Bogdan and Zagdańska, 2006). Furthermore, transgenic overexpression of trehalose-biosynthetic genes, which help confer enhanced drought tolerance in transgenic rice, leads to a rise in soluble sugar contents and photosynthetic capacity (Garg et al., 2002). Accordingly, our observed changes in sugar contents that occurred only under drought conditions implied that overexpression of *AtCYP78A7* in our tolerant transgenic rice improves stress tolerance by regulating and balancing sugar metabolism in the shoots during droughty periods.

Materials and Methods

Plant materials

Two transgenic rice Lines, '10B-5' and '18A-4', and their WT counterpart were used. The transgenics were produced by inserting *AtCYP78A7*, which encodes cytochrome P450 protein, via *Agrobacterium*-mediated transformation (Kim and Choi, 2012). Briefly, the PCR product containing *AtCYP78A7* was cloned between the *CaMV* 35S promoter and ocs3' of pART7, and the transgenic cassette was sub-cloned into the pCAMBIA1301 binary vector. The binary vector construct was then transformed via *Agrobacterium tumefaciens* strain AGL1 into *Oryza sativa japonica* cv. 'Hwayoung'. The insertion of a single-copy gene into the transgenic lines was verified by Southern blotting and inverse polymerase chain reactions.

Water-deficit stress treatments

The entire experimental period ran from June to November 2012. Seedlings were initially grown in a greenhouse controlled to day/night temperatures of 28±3°C/22 ± 3°C and long-day conditions (16 h light/8 h dark). At five weeks of age (10 July), they were transplanted into field soil within a rainout shelter where both well-watered and water-deficit systems were maintained. Before transplanting, fertilizer was applied with 4.6, 5.4, and 5.2 kg per 10 a of nitrogen (N), phosphorus (P), and potassium (K), respectively, as a basal dressing. Soil pH values for the well-watered and water-deficit systems were 5.3 and 5.5, respectively. Soil organic matter and total N for the well-watered system were 4.30% and 0.03%, respectively, while those for the deficit system were 4.00% and 0.03%, respectively. Available P and K in soils for well-watered system were 77.2 mg/kg and 50.0 mg/kg, respectively, versus 68.7 mg/kg and 49.1 mg/kg, respectively, for the deficit system. The shelter was located at the Korea Research Institute of Bioscience and Biotechnology (KRIBB), Cheongwon-gun, Republic of Korea (36°43' N, 127°26' E; elevation 35 m). A complete randomized block design with three replicated plots was applied. Rainfall was automatically intercepted by a moisture sensor installed in the shelter, and water was supplied through sub-surface drip irrigation.

Moisture stress was induced by withholding water beginning on 27 July (17 d after transplanting), when tillers first appeared. For the water-deficit system, irrigation was interrupted for two weeks, then watering resumed for another one week before being halted again until harvesting occurred. For the well-watered system, seedlings were irrigated daily for 30 min. This practice continued for 13 weeks after transplanting. Soil water content at 10, 20, 30, 40, and 50 cm depths was measured using capacitance probe (EasyAG, Sentek, Australia). Throughout the water-holding periods, the moisture content in the well-watered system and water-deficit system remained 9.6% ~ 16.1% and 7.0% ~ 11.4%, respectively. Two seedlings were collected from each plot at the stages of tillering (9 August), heading (25 September), and ripening (31 October). Their shoots were

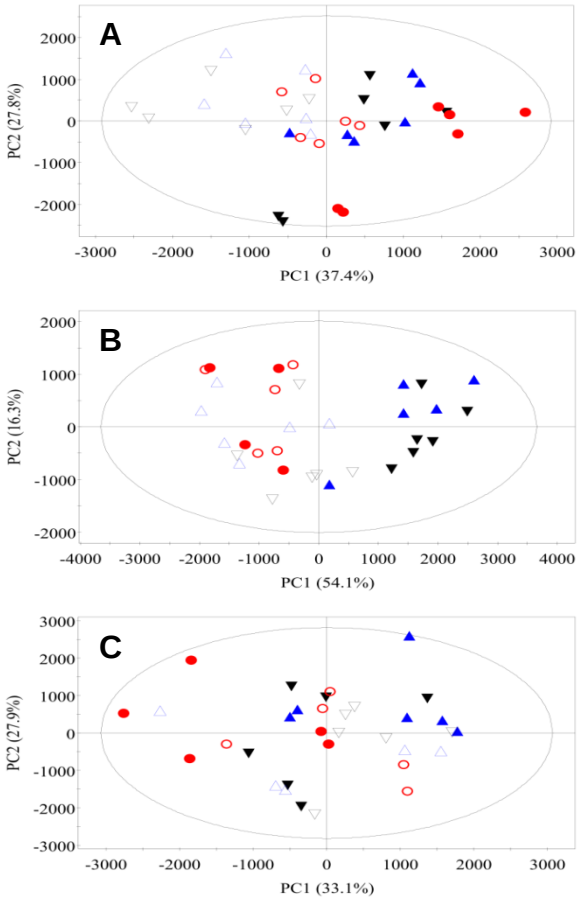

Fig 3. PCA analysis of ^{1}H-NMR data for transgenic rice Lines '18A-4' (▼, ▽) and '10B-5' (▲, △), and wild-type (●, ○) 'Hwayoung' grown under well-watered (open symbols) or water-deficit (filled symbols) conditions over time. Score plot at tillering (A), heading (B), and ripening (C) stages. Ellipse represents Hotelling T2 with 95% confidence in score plots.

freeze-dried (FreeZone 2.5 Freeze Dry System; Labconco, Kansas City, MO, USA), then ground, and stored at –80°C. Six biological replicates were randomly analyzed for each genotype cultivated under well-watered or deficit conditions during three different growth stages.

Chemicals

For NMR analysis, methanol-d_4 (99.8%), deuterium oxide (99.9%), and 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TSP, 98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). For GC–MS analysis, *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA), methoxyamine hydrochloride, anhydrous pyridine, and ribitol were also obtained from Sigma-Aldrich. Other sources included HPLC-grade methanol and water from Burdick & Jackson (Ulsan, Korea), and chloroform and ethanol from Merck (Darmstadt, Germany). Standards of amino acids, sugars, and fatty acids for identifications were acquired from Sigma-Aldrich, while those of organic acids, sugar alcohols, and oligosaccharides were obtained from Supelco (Bellefonte, PA, USA).

^{1}H–NMR analysis

Shoot samples (100-mg dry weight) were extracted as described by Kim et al. (2010), with slight modifications. They were dissolved in 750 μL of methanol-d_4 and 750 μL of deuterium oxide containing 0.03% TSP. This was followed by sonication for 20 min and centrifugation at 17,000 *g* for 10 min at room temperature. The resulting upper layer was transferred into a 5-mm NMR tube.

All ^{1}H–NMR spectra were acquired at 298 K on a Bruker Advance III 400 MHz NMR spectrometer (Bruker Biospin, Karlsruhe, Germany) operating at 400.13 MHz with a gradient two-channel 5 mm broad band BBFO probe. A NOESYGPPR1D pulse sequence was applied to suppress the residual water peak during the relaxation delay of 4.0 s and a mixing time of 100 ms. In all, 32 scans were collected into 64k data points over a spectral width of 4801.54 Hz, pulse width of 9.46 μs, and acquisition time of 6.82 s. The spectra were subsequently Fourier-transformed with 0.3 Hz line-broadening, phased, and baseline-corrected.

The ^{1}H–NMR spectra were processed using AMIX software (version 3.8.6; Bruker Biospin, Germany). The regions between δ 3.31 and δ 3.34, corresponding to the solvent, were removed and the remaining spectral regions were divided into 0.01-ppm bins. Prior to statistical analysis, the spectra segments were integrated by sums of intensities and normalized to total spectral intensity between samples.

GC–MS analysis

For GC–MS analysis, metabolites were extracted from freeze-dried shoot samples (100 mg), based on a modified version of a method by Lisec et al. (2006). The samples were extracted with 1.4 mL of methanol and 60 μL of a ribitol internal standard (0.4 mg mL^{-1}) for 10 min at 70°C. The extracts were then centrifuged at 11,000 *g* for 10 min. All of the supernatant was separated into a new tube, to which 750 μL of chloroform and 1.4 mL of water were added. After this mixture was centrifuged at 2,200 *g* for 15 min, 150 μL of the upper aqueous layer was evaporated in a centrifugal vacuum concentrator (Genevac, Ipswich, UK). The dried polar extract was derivatized for 2 h at 37°C with 40 μL of methoxyamine hydrochloride in anhydrous pyridine (20 mg mL^{-1}). Subsequently, the samples were allowed to react with 70 μL of MSTFA at 30°C for 30 min, after which the glass inserts were transferred to a GC vial and sealed with moisture-proof Parafilm.

The GC–MS analysis was performed on a Clarus 680/600T (Perkin-Elmer, Waltham, MA, USA). A 1-μL sample was injected in the split mode at a ratio of 10:1, with GC separation conducted on a DB-5 MS capillary column (30 m × 0.25 mm, 0.5 μm; J&W Scientific, Folsom, CA, USA). The initial oven temperature of 60°C for 2 min was ramped, at 5°C min^{-1}, to 320°C, and held for 11 min. Helium was used as a carrier gas at a constant flow rate of 1.2 mL min^{-1}. The mass spectrometer was operated with an electron ionization of 70 eV, scanning the mass range of 44 to 620 *m/z* at 0.15 scans s^{-1}. The ion source and interface temperatures were set at 250°C and 280°C, respectively.

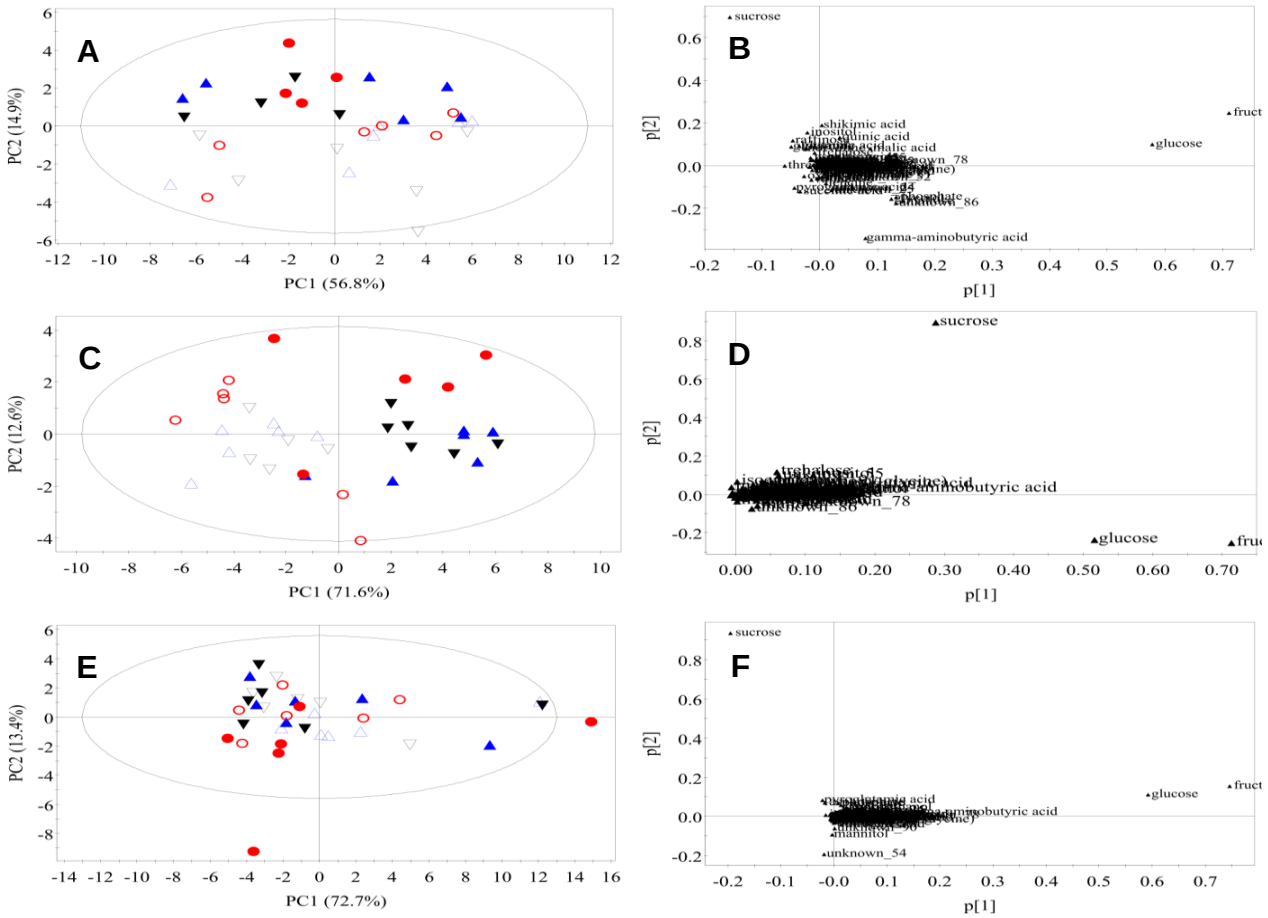

Fig 4. PCA analysis of GC–MS data for transgenic rice Lines '18A-4' (▼, ▽) and '10B-5' (▲, △), and wild-type (●, ○) 'Hwayoung' grown under well-watered (open symbols) or water-deficit (filled symbols) conditions over time. Scores and loading plots at tillering (A, B), heading (C, D), and ripening (E, F) stages. Ellipse represents Hotelling T2 with 95% confidence in score plots.

All GC–MS data were processed with TurboMass software (version 5.4.2.1617; Perkin-Elmer). The entire ion chromatogram was obtained from the EI-scan by time. Peaks with a signal to noise ratio (S/N) > 200 were extracted by the software. Mass spectral deconvolution of each peak was conducted to clean up the mass spectra of adjacent peaks and noise by employing the named 'combine' function in TurboMass software before the library search. Peaks for metabolites were identified by comparing a mass spectrum of peaks with reference spectra from the NIST (National Institute of Standards and Technology, USA) mass spectral library. The combined mass spectrum was identified as a metabolite compound by reviewing the highest-scoring one among several candidates that had a match factor of more than 750. For most cases, it was confirmed through comparisons with the spectrum and retention time of an authentic compound. In all, 49 metabolites, including amino acids, organic acids, sugars, sugar alcohols, steroids, and fatty acids, were identified from the GC–MS spectra of the rice shoots (Table 1). All peak areas were further normalized to the internal standard.

Data analysis

Chemometric analysis was performed with SIMCA-P+ software (version 12.0; Umetrics AB, Kinnelon, NJ, USA). All data obtained from the ^{1}H–NMR and GC–MS analyses were Pareto-scaled prior to the multivariate analysis. Principal Component Analysis was applied as an unsupervised method to visualize variance and clustering in the datasets. We first examined three principal components (PC1, PC2, and PC3) before choosing two that could clearly visualize the differences between samples.

Conclusion

We examined the metabolite changes that occur in drought-tolerant transgenic rice over-expressing *AtCYP78A7* and its non-transgenic parental line during three growth stages while exposed to either well-watered or water-deficit conditions. Our PCA results, based on ^{1}H–NMR and GC–MS data, suggested that growth stage (tillering, heading, or ripening), soil water conditions (well-watered vs. water-deficit), and genotype (WT or two transgenic lines) influence the discrimination of metabolite profiles in shoot samples. In particular, stage of development has the most prominent role in driving metabolism. Variations in metabolites between soil water conditions or genotypes are growth stage-specific. A distinct separation in metabolites between transgenic and non-transgenic rice appears only under drought conditions at the heading stage. We attribute this difference to changes in sugar contents. These results suggest that developmental and environmental factors strongly affect the metabolic responses of transgenic crops. Moreover, a large change in sugar metabolism occurs during the heading stage for drought-stressed transgenic rice that over-expresses *AtCYP78A7*. Thus, the findings from our metabolomics study will be an important tool in furthering our understanding of the relationships between environmental constraints and transgene expression.

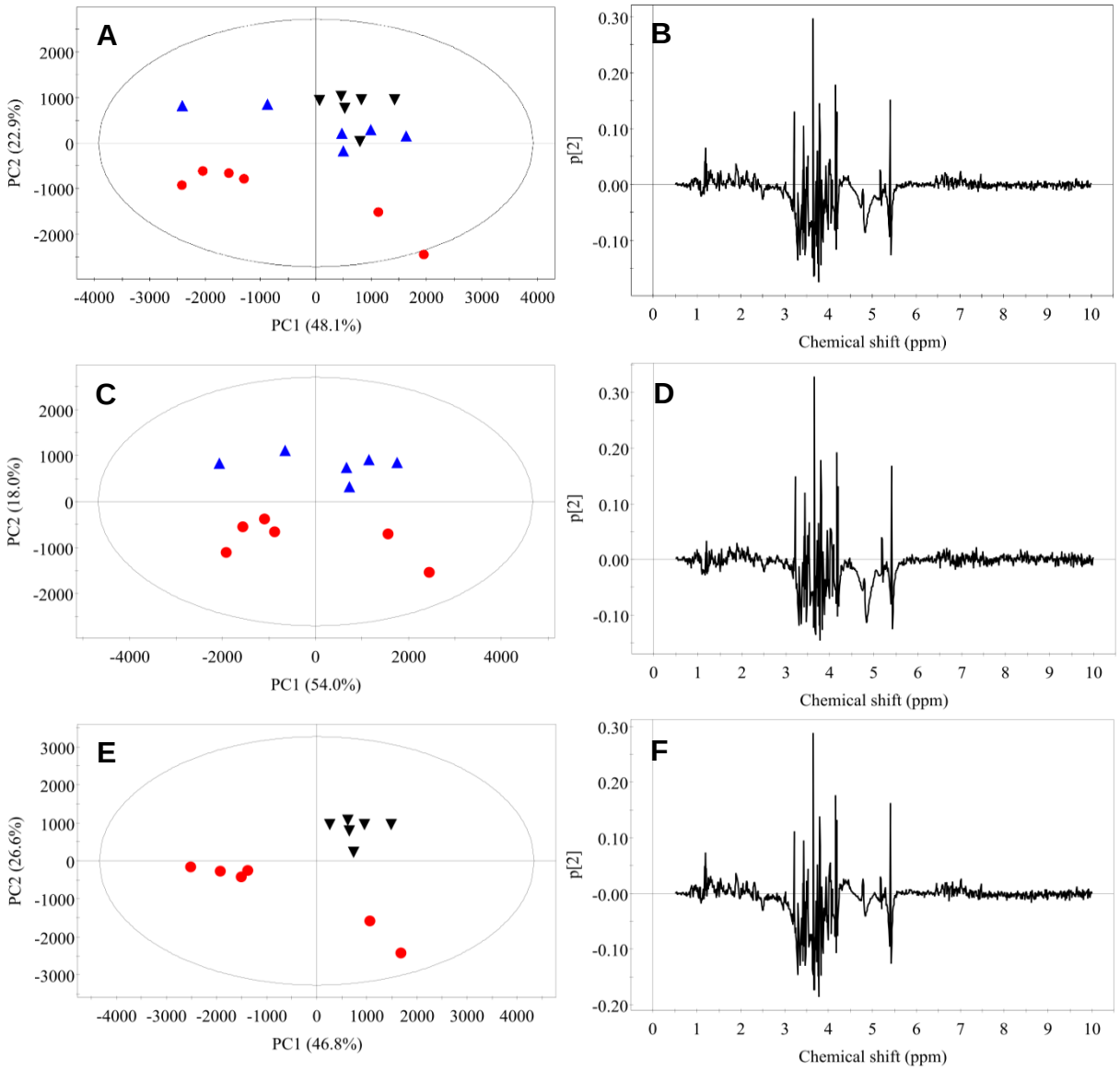

Fig 5. PCA analysis of ^{1}H-NMR data for transgenic rice Lines '18A-4' (▼) and '10B-5' (▲), and wild-type 'Hwayoung' (●) grown under water-deficit conditions, as examined at heading stage. Score plot of transgenic Lines vs. wild-type (A) and loading plot of PC2 (B); Score plot of '10B-5' vs. wild type (C) and loading plot of PC2 (D); Score plot of '18A-4' vs. wild type (E) and loading plot of PC2 (F). Ellipse represents Hotelling T2 with 95% confidence in score plots.

Acknowledgments

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