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Overexpression of Maize (*Zea mays* L.) Malate Dehydrogenase (*ZmMDH*) in IR64 Rice (*Oryza sativa* L.) Leads to Altered Carbohydrate Metabolism as Revealed by Transcriptomics and Metabolite Analysis

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Malate dehydrogenase (MDH) (EC 1.1.1.40) is among the major genes identified by the C_4 Rice Consortium to play an important part in inducing C_4 photosynthesis in a C_3 rice system. Despite the vast information on the biochemical characteristics of the enzyme, the effects of engineering maize (*Zea mays* L.) MDH on IR64 rice (*Oryza sativa* L.) for the induction of the C_4 pathway was never thoroughly studied in the light of transcriptomics. Two transformed IR64 rice events conferring overexpression of maize MDH (*ZmMDH*), namely MDH22 and MDH48, were analyzed using transcriptome analyses to assess differential expression of endogenous genes due to MDH overexpression. Sugar metabolite quantification was also conducted to identify changes in the concentration of simple and complex carbohydrates against the wild type (untransformed) IR64 rice. Transcriptome analyses revealed 301 differentially expressed genes in transformed rice with significant enriched effects in the downregulation of simple carbohydrate degradation. This result implies lower degradation of sucrose, fructose and glucose content as eight strongly associated genes for this function were downregulated. Carbohydrate characterization revealed significant differences in terms of simple and complex sugar content in MDH 22 and MDH 48, respectively, which aligned with the results of the transcriptome analyses. The resulting phenotype of the MDH-transformed lines indicate an increase in plant height and biomass which can be attributed to the association of growth due to the rapid conversion of simple sugars to starch and traced further to the implied change in the rate of simple sugar degradation. The study successfully established the effect of maize MDH in rice based on its transcriptome, sugar content, and phenotype.

Key Words: carbohydrate metabolism, C_4 photosynthesis, MDH, metabolite analysis, transcriptomics

Abbreviations: CFT – confined field testing, FDR – False Discovery Rate, GO – gene ontology, IRRI – International Rice Research Institute, MDH - malate dehydrogenase, SEA – Singular Enrichment Analysis, xg – times gravity, *ZmMDH* – *Zea mays* L.-derived MDH

INTRODUCTION

Malate dehydrogenase (MDH) (EC 1.1.1.40) plays diverse roles in plant survival. Among C_4 plants, MDH catalyzes the oxidation of oxaloacetate to malate (Vance 1997). Malate is a major metabolite in plants with related contributions to other processes, namely: nutrient uptake, respiration, fatty acid oxidation, nitrogen assimilation, and pathogen response, among others (Vance and Heichel 1991; Vance 1997). Induced overexpression experiments of MDH on crops have also been conducted to improve other agronomic traits such as aluminum resistance in alfalfa (*Medicago sativa* L.) (Tesfaye et al. 2001) and tobacco (*Solanum tabaccum* L.) (Wang et al. 2010). At present, the C_4 Rice Consortium identified MDH as one of the key enzymes necessary to induce the C_4 photosynthetic pathway in rice (Karki et al. 2013). The conversion of the photosynthetic pathway of rice from C_3 to C_4 is expected to make rice more resilient to the increasing atmospheric temperatures in the advent of global warming and more efficient in utilizing the declining availability of resources such as water

(Langdale and Nelson 1991). This study is the first to analyze the effects on the transcriptome in overexpressing maize (*Zea mays* L.)-derived MDH (*ZmMDH*) in IR64 rice (*Oryza sativa* L.).

Maize MDH is one of the many candidate genes for the induction of C_4 photosynthesis in rice. Hence, characterizing the effect of maize MDH in the transcriptome, sugar metabolism and phenotype in rice is of paramount importance. Existing lines which are homozygous for the gene were analyzed at the level of the transcriptome to characterize the resulting differential gene expression on the endogenous genes in rice. Carbohydrate analyses were also conducted to evaluate the effect of the overexpression of MDH on the concentration of glucose, fructose, sucrose, and starch compared to the wildtype. Lastly, the phenotypes of *ZmMDH*-transformed rice lines were compared against the wild type IR64 control to evaluate the changes in terms of important agronomic traits, namely: plant height, tiller number, number of leaves, biomass, grain yield and leaf greenness.

MATERIALS AND METHODS

Differential Gene Expression

Read count data derived from RNA Sequencing of MDH-transformed events (MDH 22 and MDH 48) and two IR64 wildtype controls were retrieved from the International Rice Research Institute (IRRI). Differential gene expression analyses were done using the DESeq2 package (Love et al. 2014) on the R platform, at a threshold value of α=0.05 and False Discovery Rate (FDR) of 0.05. The script was coded according to the specifications of the package's authors with minor adjustments to the inputted script to fit the experimental design. The created script still followed the standard pipeline of the program which includes prefiltering and calculation of Wald's statistics and negative binomial distribution. The results of the analyses yielded a list of genes which are differentially expressed along with the average base mean value and amount of change as reflected in the log2-fold values, α values, and FDR. Genes which were differentially expressed were selected based on the threshold criteria (α=0.05; FDR=0.05). These were annotated using the batch annotation tool in the Rice Genome Annotation Project (Kawahara et al. 2013) online database.

Singular Enrichment Analysis and Pathway Visualization

Singular Enrichment Analysis (SEA) was conducted using the AgriGO V.2.0 online tool (Tian et al. 2017) to determine significant concerted effects. All differentially expressed genes were grouped into three partitions independently composed of: (1) all differentially expressed genes, (2) downregulated genes only, and (3) upregulated genes only. The three established partitions were individually inputted into the search tool to be analyzed for significant concerted effects based on their ontologies. Significant results were identified to be less than the threshold value of FDR=0.05.

All differentially expressed genes in the transformed lines were also loaded to MapMan (Thimm et al. 2004) to visualize specific pathways and functions associated with each gene along with their Log-2 fold values. Every gene associated with a specific pathway was plotted by the program as colored data points: blue indicating upregulation and red if otherwise.

Soluble Sugars and Starch Quantification

The youngest fully expanded leaf from the 6-wk-old transformed lines and the control were sampled at full sunlight, between 1000H–1100H. Three biological replicates were obtained for each plant sample. The soluble sugars (glucose, fructose, and sucrose) and starch were simultaneously isolated using the modified perchloric acid-based extraction by Smith and Zeeman (2006) and Delatte et al. (2005). Leaf tissues were ground in liquid nitrogen using a mortar and pestle then immediately treated with 0.7 M perchloric acid. The solution was centrifuged at 14300 rpm in refrigerated conditions for 10 min. The supernatant and pellet were separated afterwards. The pH of the supernatant was adjusted to 6.0–7.0 using a neutralization buffer (2 M potassium hydroxide, 0.4 M 2-(N-morpholino) ethanesulfonic acid, and 0.4 M potassium chloride) until the white pellets of starch appeared. The supernatant which contained the soluble sugars was separated from the pellet. The latter was washed thrice with 80% ethanol then suspended in distilled water and stored for further starch content quantification.

Sugar assay cocktail (25 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)) at pH 7.5, 1 mM magnesium chloride ($MgCl_2$), 1 mM adenine triphosphate (ATP), 1 mM nicotinamide adeninedinucleotide (NAD), and 1.4 U hexokinase) was prepared. Fifty (50) μL cocktail was added to 20 μL soluble sugar along with 128 μL nanopure water in the clear bottom 96-well plate. Four technical replicates were prepared for each sample. The absorbance was initially read at 340 nm until the reading has stabilized. To measure glucose content, 2 μL of glucose-6-phosphate dehydrogenase (Roche) was added, then 1 μL of phosphoglucoisomerase (Roche) was introduced to measure fructose content. Lastly, 1 μL of invertase (Sigma-Aldrich®) was added to measure sucrose content. The differences in the absorbances before

and after addition of the enzymes were analyzed to determine the concentration of soluble sugars.

The starch pellets were gelatinized at 95°C for 4 h. Starch digestion setup was prepared by mixing 100 µL of the gelatinized starch and 100 µL of the starch digestion master mix (200 mM sodium acetate (pH 4.8), amyloglucosidase and α-amylase). Also, a control setup of undigested starch was prepared by mixing 100 µL of the gelatinized sample and 100 µL of 200 mM sodium acetate (pH 4.8). The setup was incubated overnight at 37°C and centrifuged the following day at 16000 xg for 10 min. The assay setup was prepared by mixing 50 µL assay cocktail (25 mM HEPES pH 7.5, 1 mM MgCl2, 1 mM ATP, 1 mM NAD and 1.4 U hexokinase), 20 µL digested and undigested sample and 128 µL nanopure water. The initial absorbance was read at 340 nm and then 2 µL of glucose-6-phosphate dehydrogenase was added. Reading of the final absorbance followed afterwards. The readings were compared and treated statistically using Student's T-Test using STAR.

Phenotype

Three 2 x 2-meter plots were assigned respectively for MDH 22, MDH 48, and IR64 wildtype. These were situated randomly across IRRI's Confined Field Testing (CFT) Facility. A total of 100 individuals were planted for each plot on a 10 x 10 scheme and were apart by 20 cm.

The plants were allowed to mature with constant weekly monitoring, weed removal and pesticide application following weekly inspections.

Data for plant height, leaf tiller, estimated chlorophyll content, and tiller count were obtained during their middle tillering and late flowering stage. Data on biomass and total grain yield (for both filled and unfilled grains) were also collected. Ten biological replicates for each sample were randomly selected from the plots to measure the six agronomic traits mentioned. Plant height measurements were collected in centimeters using a meter stick from the surface of the soil to the furthest extent of the longest leaf while the estimated chlorophyll content was identified using a Konica Minolta hand-held SPAD meter.

Postharvest agronomic traits of MDH 22 and MDH 48 were collected once fully matured and ready to harvest. Data on shoot biomass was determined per plant by measuring the fresh weight of the shoot system of each plant and its average value was calculated.

Grain yield was calculated per plot by:

$$[(\text{total fresh weight of panicle+grain})/(\text{planting distance} = (m^2 \times 10))] \times 10 \text{ kg/ha}$$

This was derived for all the three plot replicates and was compared with the grain yield per hectare of the IR64 wildtype control using Student's T-Test using STAR.

RESULTS AND DISCUSSION

Transcriptomics

Several genes in transformed rice lines had altered gene expression levels as revealed by DESeq2 analysis. A total of 301 differentially expressed genes were identified where 161 genes were significantly downregulated and 140 were significantly upregulated in both MDH 22 and MDH 48 events compared to the transcriptomes of two wild type IR64 rice controls.

MapMan pathway visualization revealed that out of the total number of differentially expressed genes, 38 were directly related to the regulation of transcription or gene expression specifically for pre-transcriptional control functions. This procedure validated the effect of maize MDH on the mechanisms that control the expression of other genes which explains the differences in the transcriptomes of the MDH-transformed events and the wild type control (Fig. 1a). Pathway visualization also revealed differentially expressed genes as a direct response to the presence of maize MDH.

Thioredoxin-coding gene (loc_os05g4190.1), which is an important precursor to activate the functionality of MDH, was identified to be upregulated. The overexpression of this gene strongly suggests that maize MDH is metabolically active in rice as indicated by the increase in demand of its thioredoxin precursor. Differentially expressed genes which code for protein-modifying gene products totaled to 11 where six are downregulated.

Most of the genes were identified to have functions pertaining to lectin kinases and functions in cell communication in response to pathogenesis which supports the role of MDH in plant pathogen response (Wang and Bouwmeester 2017). Energetics of transformed rice events appear to have been altered as well due to the five out of the 11 genes with upregulated ATP production -related functions such as calmodulin-binding complexes and ATP-binding complexes. Genes relating to protein degradation were also identified to be differentially expressed in MDH events. Six genes were downregulated with functions pertaining to acyl-aminoacyl peptidase and hydrolases, among others. Five upregulated genes relating to the said function were also identified to be members of the peptidase superfamily. These results suggest that the changes in the level of expression of the genes coding for these proteins, as revealed by the transcriptome, could infer to the changes in the proteome

of the transformed lines compared to the wild type.

Singular Enrichment Analyses (SEA) using the AgriGO V2.0 online tool revealed significant concerted effects of the genes which were differentially expressed in MDH overexpressing lines (Fig. 1b). A total of eight out of the 107 annotated genes were identified to enrich the catabolic processes (GO:0009056). Six out of the eight were identified to be related more specifically to the monosaccharide metabolic process (GO:0005996) and is ultimately narrowed down to the glucose catabolic process (GO:000607).

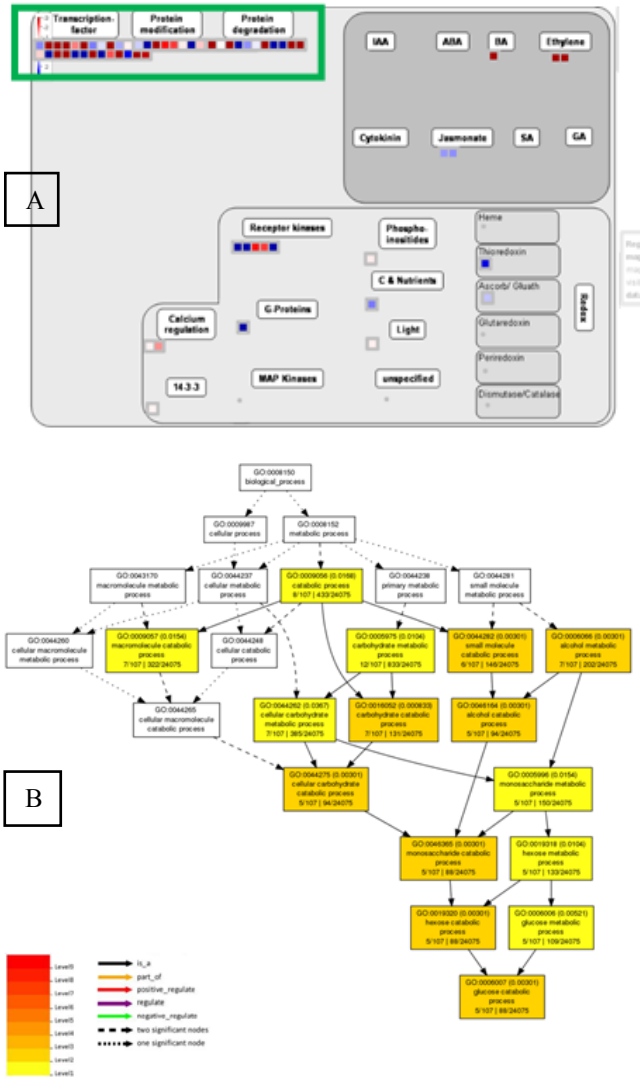

Fig. 1. Pathway visualization and Singular Enrichment Analyses (SEA) via MapMan and AgriGO V2.0. Genes were identified to have functions as transcription factors as well as post translational modifications such as protein modification and degradation as depicted by the colored boxes (Fig. 1A.). The most significant concerted effect of a subset of all differentially expressed genes is towards catabolism of carbohydrates as indicated by SEA (in yellow and orange colored boxes) (Fig. 1B).

Genes relating to C_4 function were individually identified. As mentioned, thioredoxin (loc_os05g40190.1) which regulates MDH activity in the chloroplasts (Wolosiuk et al. 1977) was found to be strongly upregulated. Photorespiratory gene glycolate oxidase (loc_os04g53214.2) was also identified to be downregulated which suggests a positive effect of the presence of MDH on the efficiency of carbon fixation and the lower incidence of the photorespiratory pathway (Esquivel et al. 1998).

Metabolite Analysis

Carbohydrate content in terms of soluble (glucose, fructose and sucrose) and insoluble (starch) sugars were significantly different in MDH 22 and MDH 48 lines (Table 1) compared to the wild type IR64. The MDH 22 event contained significantly less glucose (0.36 ± 0.06), fructose (0.30 ± 0.07) and sucrose (0.42 ± 0.10) compared to the wild type's glucose (0.73 ± 0.12), fructose (0.64 ± 0.09), and sucrose (2.00 ± 0.27) contents as presented in milligrams per gram of fresh weight (mg/g FW). However, event MDH 48 exhibited significantly higher amounts of starch (1.89 ± 0.68 mg/g FW) compared to the wild type (0.55 ± 0.17 mg/g FW). The results obtained were related to the characterized transcriptome of the MDH transformed events.

A connection can be established between the list of genes which are differentially expressed to the resulting phenotype (i.e., carbohydrate content). The resulting lower amount of simple sugars can be attributed to the overexpression of maize MDH. The higher rates of conversion of oxoglutarate to malate delivered a high surge of potential raw materials (i.e., small carbon compounds) to be metabolized. These had led to an increase in the turnover of these sugars to starch which explains the lower recovered amounts in MDH 22. This result is similar to the study of Centeno et al. (2011) who worked on the function of MDH in the ripening of tomatoes where they also observed a decrease in glucose, sucrose, and fructose as MDH activity increases. In a contrasting study by Hebbelmann et al. (2012), higher sucrose content was identified in *Arabidopsis thaliana* lines with knocked-out endogenous MDH. The findings of the knockout study and the results from this experiment strongly link the effect of malate dehydrogenase activity to the utility of simple sugars such as sucrose to starch synthesis rather than its catabolism.

Regarding the increased starch content in MDH 48 lines, no previous studies correlate the direct relationship of malate to starch content. However, it is of great importance that the increase in starch in MDH 48 and the decrease in simple sugars in MDH 22 are observed to be

Table 1. Simple and complex sugars in MDH 22, MDH 48, and wild type IR64. Glucose, fructose, and sucrose content was identified to be significantly less in MDH 22 events while starch was identified to be significantly higher in MDH 48 events compared to the wild type.

Carbohydrate Content (mg/g fresh weight) from youngest fully expanded leaves taken from 6-wk-old plants				
MDH Events	Glucose	Fructose	Sucrose	Starch
MDH 22	0.355 ± 0.060*	0.301 ± 0.068*	2.009 ± 0.273**	0.424 ± 0.095
MDH 48	0.958 ± 0.054	0.797 ± 0.058	3.376 ± 0.733	1.890 ± 0.675**
Wild type (IR64)	0.731 ± 0.120	0.642 ± 0.092	3.580 ± 0.248	0.546 ± 0.165

* Significance threshold: 0.05
** Significance threshold: 0.001
MDH – malate dehydrogenase

binary. This could be inferred to the effect of the maize MDH to processes involved in starch metabolism such as the transition of simple sugars (as indicated by the lower simple sugar content in MDH 22) to be converted to starch and starch accumulation (as indicated by the increased starch content in MDH 48). These results concur with the findings of Centeno et al. (2011) and Tiessen et al. (2002) who independently pointed out that malate, being the final product of the conversion of oxoglutarate to malate via MDH redox reactions, indirectly plays a role in starch synthesis via unknown mechanisms. The results of this study may suggest the possible role of MDH in starch synthesis by inhibiting the degradation of simple sugars and rather, by storing them in the form of starch.

The combined results from the transcriptome analyses (i.e., downregulation in genes with functions related to carbohydrate catabolism) and metabolite characterization (i.e., increase in accumulated starch in MDH 48 and decrease in simple sugars in MDH 22) support previous studies on MDH. In an experiment by Tarpley et al. (1994) on sorghum, it was emphasized that starch accumulation requires a change in the rate of sucrose degradation (Casanova-Katny et al. 2005). Calviño et al. (2011) analyzed the transcriptomes from the varieties studied by Tarpley et al. (1994) and reported that sucrose- and fructose-degrading enzymes have lower expression in varieties that have higher starch content such as the sweet varieties compared to non-sweet sorghum. The decrease in the expression of genes for carbohydrate catabolism in transformed rice conferring maize MDH overexpression explains the observed starch accumulation. It should be noted, however, that the specific biochemical processes leading to such are yet to be discovered.

Phenotype

The phenotypes of MDH 22 and MDH 48 were analyzed against the wild type and have shown significant differences in terms of plant height (Table 2). Plant height was identified to be significantly higher in MDH 22 and MDH 48 events at 96.5 and 98.0 cm, respectively, compared to the wild type with a recorded plant height of 94.3 cm. Tiller number was also higher in MDH 22 and MDH 48 at 16.44 and 16.40, respectively, compared to the tiller number of wild type rice (13.70). No significant differences were observed in leaf number and greenness.

Shoot biomass and expected grain yield were also analyzed against the wild type control (Table 3). The transformed lines had significantly higher shoot biomass at 24.26 and 24.28 g compared to the wild type shoot biomass of 21.13 g. Despite the consistently higher values

Table 2. Phenotype characterization of rice lines with MDH overexpression. The results indicate that the transformed lines have higher plant height and tiller number with no significant differences in terms of leaf number and greenness.

	Phenotype Variables			
MDH Events	Plant Height (cm)	Tiller No.	Leaves per Main Tiller	Chlorophyll Content (in SPAD Readings)
MDH 22	96.50 ± 3*	16.44 ± 2.6*	4	44.15 ± 0.10
MDH 48	98.00 ± 3*	16.40 ± 2.7*	4	41.95 ± 1.67
Wild type (IR64)	94.30 ± 3	13.70 ± 2.9	4	42.74 ± 1.81

*a=0.05
MDH – malate dehydrogenase

Table 3. Postharvest agronomic traits of MDH 22 and MDH 48. Presence of exogenous MDH from maize in rice led to a slightly higher shoot biomass compared to the wild type. However, no specific trend was observed in total grain yield in kg/ha.

	Postharvest Characteristics	
MDH Events	Average Shoot Biomass per Plant (fresh weight, g)	Total (Filled and Unfilled) Grain Yield (kg/ha)
MDH 22	24.26 ± 7.4*	5 525.00 ± 2 061.30
MDH 48	24.28 ± 8.3*	4 734.25 ± 2 154.85
Wild type (IR64)	21.13 ± 4.1	5 451.00 ± 2 828.74

*a=0.05
MDH – malate dehydrogenase

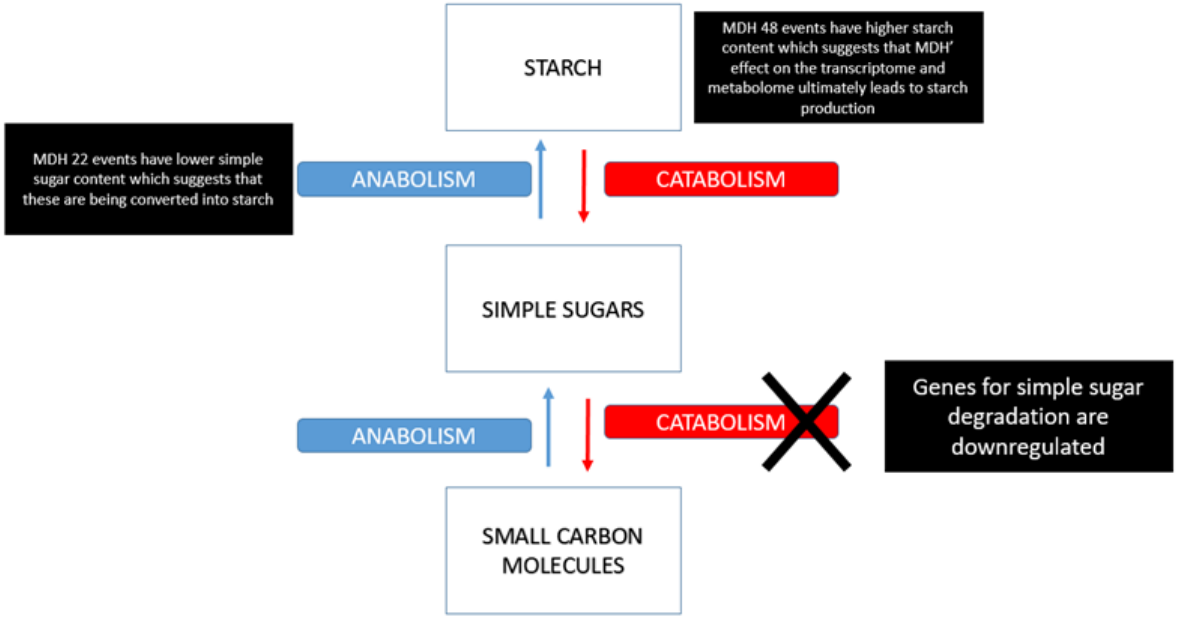

Fig. 2. The effect of malate dehydrogenase (MDH) on carbohydrate metabolism in IR64 rice varieties. MDH from maize resulted in the downregulation of genes coding for enzymes involved in sugar degradation as suggested by the transcriptome analysis. These further reflect the results of metabolite characterization. Increase in starch content is attributed to the decrease in the expression of genes related to sugar catabolism since simple sugars become more available as the rate of degradation decreases as inferred from the transcriptome.

in MDH 22 and MDH 48 rice lines, no significant difference was observed in terms of total grain yield with 5,525 and 4,734 kg/ha, respectively, compared to the wild type's total grain yield of 5,451 kg/ha. The results of the phenotypic analyses may directly associate the activity of maize MDH in rice with the observed increase in height and shoot biomass. The consistent trend observed on the two variables concurs with several correlating studies on plant height and biomass yield observed in sorghum (Murray et al. 2008; Zhao et al. 2009). The positive effect of the maize enzyme on the height and biomass of transformed rice can be connected to the effect of the gene insertion and expression on the transcriptome leading to the accumulation of starch which, in turn, cascaded further into the increase in plant height and biomass.

Data on the phenotypic variables were consistent with the previous controlled field tests conducted on the variables but due to having the highest number of replicates, the data from this experiment was presented.

CONCLUSION AND RECOMMENDATION

Despite the well-established effect of MDH on the pathways responsible for growth and survival, the enzyme is among the most enigmatic in terms of its full effect on agronomic traits such as height, yield, and biomass, among others. This study established that the expression of maize MDH results in differential expression of genes with significant enrichment to carbohydrate metabolism in rice. This resulted in lower degradation rates of simple sugars in transformed IR64 rice plants which led to an increase in raw material reserves necessary for the synthesis of starch (Fig. 2). This, in turn, led to the higher amount of starch the role of which is highly linked to the increase in plant height as observed in the phenotype of fully-grown rice transgenics. The continuity of the results from the transcriptome all the way to the resulting phenotype aligns the contribution of the maize-derived MDH to rice via alterations, more specifically on carbohydrate metabolism.

With respect to inducing the C_4 pathway in rice, the role of the enzyme in this initiative becomes more elaborate. In the process of fulfilling its primary function by converting oxaloacetate to malate, it was identified that the enzyme also became the probable cause for the metabolic changes observed which could be the main reason behind the significant difference in a few identified agronomic traits. Even though the results are still inconclusive when it comes to the transformed lines being close to having the C_4 type of photosynthesis, the changes stemming from the effect of maize MDH overexpression

holds promise to the potential of C_4 enzymes in crop improvement.

The next phase of the study is to study the heritability of the traits along with the effects on the phenotype if heterozygosity is considered, knowing that the individuals used in the study are homozygous for the trait. This will open breeding strategies to ensure the continuity of the effects in succeeding generations.

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