Induced Mutagenesis Enhances Lodging Resistance and Photosynthetic Efficiency of Kodomillet (Paspalum Scrobiculatum)

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Received: 26 December 2019; Accepted: 27 January 2020; Published: 4 February 2020

Abstract: The present research was focused in the development of photosynthetically efficient (PhE) and non-lodging mutants by utilizing ethyl methane sulphonate (EMS) and gamma radiation in the kodomillet variety CO 3, prone to lodging. Striking variations in a number of anatomical characteristics of leaf anatomy for PhE and culm thickness for lodging resistance was recorded in M2 (second mutant) generation. The identified mutants were subjected to transcriptomic studies to understand their molecular basis. Expression profiling was undertaken for pyruvate phosphate dikinase (PPDK), Nicotinamide Adenine Dinucleotide Phosphate Hydrogen—(NADPH) and NADP-dependent malate dehydrogenase (NADP-MDH) in the mutants CO 3-100-7-12 (photosynthetically efficient) and in CO 3-200-13-4 (less efficient). For lodging trait, two mutants CO 3-100-18-22 (lodged) and CO 3-300-7-4 (non-lodged) were selected for expression profiling using genes GA2ox6 and Rht-B. The studies confirmed the expression of PPDK increased 30-fold, NADP-ME2 ~1-fold and NADP-MDH10 was also highly expressed in the mutant CO 3-100-7-12. These expression profiles suggest that kodomillet uses an NADP-malic enzyme subtype C4 photosynthetic system. The expression of Rht-B was significantly up regulated in CO 3-300-7-4. The study highlights the differential expression patterns of the same gene in different lines at different time points of stress as well as non-stress conditions. This infers that the mutation has some effect on their expression; otherwise the expression levels will be unaltered. Enhancement in grain yield could be best achieved by developing a phenotype with high PhE and culm with thick sclerenchyma cells.

Keywords: induced mutation; lodging resistance; photosynthetic efficiency; transcriptomics

1. Introduction

India is rich in agro-biodiversity and a large number of crop species are being cultivated. The growing population to a great extent depend on two crops, rice and wheat, for sustenance. In this context, the potentials of indigenous crop plants including millets are being gradually revisited for their genetic constitution which is becoming increasingly relevant in the changing agricultural scenario. Climatic and edaphic opponents are the present challenges in agricultural production. As a consequence, any innate
crop species with comparative advantages under these challenging environments need to be targeted [1].
One category that is expected to support the decline in agricultural production includes small millets
that contribute for the food security in dry and marginal lands where the major cereal crops like rice and
wheat are unsuccessful pertaining to yield.

The small millet group is represented by six crops, namely, finger millet, kodomillet, foxtail millet,
little millet, prosomillet and barnyard millet. Among the six crops, kodomillet (Paspalum scrobiculatum
L.) is indigenous to India [2]. Being a C₄ plant, it is gaining attention due to its suitability to changing
agro-climatic conditions as most of the arable land (69%) in India are arid and dry.

Lodging is a limitation in most of the small millet crops causing considerable losses in grain yield.
Cultivating lodging-resistant plants is the most productive way to shrink losses due to lodging. Also,
stem lodging disturbs the photosynthetic effectiveness of the canopy by affecting the grain filling which
was reported in rice [3]. By reducing rice canopy photosynthesis, rice grain yield and quality was reduced
by 60%–80% since these traits had negative associations with lodging. With a view to increasing the yield
potential, identification of the superior sources, assessment of variability for the different morphological
traits, increased photosynthetic efficiency and lodging resistance lines will add more value to the crop.

Nowadays, as opposed to the main cereals, there is an impetus to develop millets because of the
predominant nutritional level. It is been said that kodomillet has more free radicals than other millets [4].
In addition, it provides low cost protein, minerals and vitamins in the form of healthy food [5].

The present investigation is an assessment of the extent of genetic variability induced for two traits
viz., photosynthetic efficiency and lodging resistance, through physical (gamma rays) and chemical (ethyl
methane sulphonate, EMS) mutagens and to isolate productive mutants in M₂ (mutant second) generation.
The mutants of the desirable types for both the traits were identified and subjected to transcriptomic
studies to understand their molecular basis. For future genetic and genomic studies the transcriptomal
data provided in this crop will help improve the less studied, but nutritionally rich and sustainable crop.

2. Materials and Methods

2.1. Plant Material

The genetic variability was induced in Kodomillet variety CO 3 using different treatments of
physical mutagen (gamma rays) and chemical mutagen (EMS). The accessions were provided
by the Department of Millets, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural
University, Coimbatore.

2.2. Methodology for Generating Mutant Population and Identifying the Desirable Mutants

Mature and viable seeds (moisture 12.0%) of the kodomillet variety CO 3 were irradiated at 100,
200, 300 and 400 Gy with a radioisotope ⁶₀Co (Cobalt-60) which served as a source at the gamma
chamber installed at Bhabha atomic research centre, Kalpakkam. The percentage of moisture content
was determined based on the difference between fresh weight and dry weight of the seeds following
the International Seed Testing Association guideline [6]. About 500 seeds were packed in butter paper
covers and placed in the gamma chamber at different time intervals for each dose based on the half-life
of the source. Non-irradiated dry seeds were taken as control. For chemical treatments, presoaked (8 h)
seeds were mutaginized with different doses of EMS, viz., (0.2%), (0.3%), (0.4%) and (0.5%) at a room
temperature of 28 ± 1 °C. A sample of seeds soaked in distilled water for the respective duration was
utilized as control. Initially, the doses for chemical and physical treatments were determined based on
lethal dose (LD₅₀) values based on the probit analysis [7,8] from the germination and survival test [9]. 1000 seeds from each treatment were grown in the raised nursery beds established at the Department
of Millets with respective controls. The spacing was maintained at 45 cm (between the rows) and 10 cm
(between the seeds). A total set of 138 (58 gamma radiation and 80 EMS) self-pollinated fertile M₁
plants were harvested individually and healthy seeds from each harvested plant were sown in plant
progeny rows for growing M2 generation (Figure 1). A set of 102 mutants (43 gamma radiation and 59 EMS) selected in M2 generation were evaluated and validated.

The mutagenized population was screened for the deviations in phenotypic characters in comparison with the control plants for two characters 1) photosynthetic efficiency (PhE), and 2) lodging resistance. For PhE, traits like flag leaf length (cm), flag leaf breadth (cm), chlorophyll index, measured using the soil plant analysis development (SPAD) meter (SPAD-502, Minolta Co., Osaka, Japan) and stomatal distribution calculated from epidermal impressions of mature, fully expanded leaf abaxial surface [10] were recorded. The stomata length and width were measured at 40× magnification to the nearest micrometer. Stomatal density was measured by counting the number of stomata per field of view at a magnification of 40×. The following observations were made for lodging resistance, culm thickness (millimeter), culm strength by measuring the pushing resistance of the stem using a handy force-gauge meter, [11] (Figure 2). In microtome, cross sections of intermodal region from the unique mutants were studied for their difference in thickness (micrometres) of the culm and of the width in the sclerenchymatous cells. These quantitative phenotypic trait values were utilized for assessing the promising mutant line pertaining to PhE and lodging resistance which was further utilized for the transcriptome analysis.

Figure 1. Field view of the M2 (mutant second) generation.

Figure 2. Push-pull gauge used to measure the culm strength in the mutant population.
2.3. Stress Treatment, RNA Isolation, cDNA Synthesis and Quantitative RealTime—Polymerase Chain
Reaction (qRT-PCR) Analysis

The seeds of four extreme mutants (CO3-100-7-12—photosynthetically efficient; CO 3-200-13-4—less efficient; CO 3-300-7-4—non-lodged and CO 3-100-18-22—lodged) along with the wild type were grown in a plant growth chamber (PGC-6L; Percival Scientific Inc., Perry, USA) facility at the National Institute of Plant Genome Research, New Delhi, under the following conditions: 28 ± 1 °C day/23 ± 1 °C night/70 ± 5% relative humidity with a photoperiod of 14 h and a photosynthetic photon flux density of 500 µmol m⁻² s⁻¹. The plants were watered daily with one-third strength Hoagland’s solution. For abiotic stress treatments, 21-day-old seedlings were exposed to 20% polyethylene glycol (PEG) 6000 (dehydration). Whole seedlings were collected at 0 h, 6 h and 12 h post-treatments. Zero-hour samples were used as controls. All the tissues were immediately frozen in liquid nitrogen after harvesting and stored at −80°C until RNA isolation.

For all the samples i.e., control, photosynthetically efficient and non-efficient mutants, lodging resistant and lodged mutants, total RNA was prepared from the leaf samples using Trizol reagent [12] and treated with RNase-free DNase I (50 U/µL; Fermentas, Hanover, MD, USA). Quality and purity of isolated RNA was checked using a Nano Drop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) [OD260:OD280 nm absorption ratio (1.8–2.0)] and the integrity was ascertained by resolving on 1.5% agarose gel containing 18% formaldehyde. One microgram (1 µg) of total RNA was reverse transcribed to first strand cDNA by anchored oligodT priming and random priming using a Thermo Scientific Verso cDNA synthesis kit following manufacturer’s instructions. Quantitative realtime polymerase chain reaction (qRT-PCR) analysis was performed in StepOne™ Real-Time PCR Systems (Applied Biosystems, Foster City, USA) [13] using the primers mentioned in Table 1. The experiment was performed in three technical replicates for each biological duplicate. The amount of transcripts accumulated for each gene normalized to the internal control Act2 was analysed using the 2-∆∆Ct method [13]. The PCR efficiency was calculated as: Efficiency = 10(−1/slope) − 1 by the default software (Applied Biosystems, Foster City, USA).

Table 1. List of primers used for quantitative realtime-polymerase chain reaction (qRT-PCR) analysis.

| Primer Name | Primer Sequence | Number of Bases |
|-------------|-----------------|-----------------|
| SiPPDK2     | F: GGTCGCAAAGCATGGCCTAA | 20 |
|             | R: GAAGGCTCCCCACCATGTT    | 19 |
| SiNADP-ME2  | F: TGAGCCGTCTGGTGCAAAA   | 18 |
|             | R:GGCAAAGTCCTCAAACCTGAATGA | 23 |
| SiNADP-ME9  | F: AGATGGGCCCTTCTATTGGT   | 22 |
|             | R: GTAACGCGACTGCTCCTCATT  | 20 |
| SiNADP-MDH1 | F: GGCCTGACACCCCTATGTT    | 21 |
|             | R: TACATTGGCCTCAGCAT      | 20 |
| SiNADP-MDH4 | F: GCAGCAGTACGAGCGATTCA  | 20 |
|             | R: GCCCGCCGTGTGTCTTTCT    | 17 |
| SiNADP-MDH10| F: GTGGGAGAGGTTCTTGGACTTG | 22 |
|             | R: AGCATCCCCAAAATGCA      | 19 |
| SiNADP-MDH11| F: GGAATGGAGGCGAGCTGACTT  | 20 |
|             | R: CCCCTGTTCGCAAAATC      | 19 |
| Rht-B       | F: ATGAACCGCGAGTACGAGGA   | 20 |
|             | R: TCTGCCGCGGTGTCGCTGTTT  | 22 |
| SiGA2ox6    | F: CGCCCTCATTGCTCA        | 15 |
|             | R: ACGCTTCTTGATCTGTGTTG   | 21 |
3. Results and Discussion

3.1. Determination of Lethal Dose (LD$_{50}$) of Mutagens

Induced mutation is essential to increase the rate of genetic variability as spontaneous mutation is at a slow rate that hampers the breeders to utilize them in the crop breeding programs. The major gain in induced mutation is that multiple trait mutants can be isolated. Before the beginning of the mutation-breeding program, information on the relative effectiveness of the mutagens is essential, [14] to determine the correct dose/concentration of the mutagens. Probit analysis was carried out using seed germination values for both the mutagens (gamma rays and EMS) to determine the LD$_{50}$. The expected LD$_{50}$ value for the seeds treated with gamma rays was 300 Gy, in concordance with a previous study on radiation mutation [15,16] in kodo millet and for EMS it was observed to be 34.83 mM (0.4%). To comprehend, higher doses caused injury to the cell, which may be vital, and inhibited may cellular activities, eventually causing death of the cells. It had been noticed that, due to these chaos of the mutagens, seeds treated at high doses (500 Gy and 0.5% EMS) did not germinate, or their seedlings could not survive beyond a few days [16]. A wide range of variations was noticed among the M$_2$ generations for novel altered phenotypes in the traits viz., chlorophyll index, flag leaf length and breadth, stomatal number and length, culm thickness and culm strength.

3.2. Identification of Photosynthetically Efficient Mutant Lines

In order for plants to operate with efficiency, they need to balance the gaseous exchange from inside and outside the leaf to maximize carbon dioxide uptake for carbon assimilation and to attenuate water loss through transpiration. A total of seven mutants were selected for increased photosynthetic rate in the M$_2$ generation (Table 2) out of which the mutant CO 3-100-7-12 had a flag leaf length of 28.47 ± 0.50 cm which was comparatively less than the wild type, but there was an increase in the flag leaf breadth (1.33 ± 0.02 cm) over the wild type (1.17 ± 0.01 cm). It showed high chlorophyll index (47.97 ± 0.91) which possessed 79 ± 1.46 stomatas per unit leaf area accompanied by an increased stomatal length (10.27 ± 0.17µm) which was higher than the wild type. Stomatal number and length is related to specific stomatal conductance responsible for increased photosynthesis leading to higher yield. There were significant differences in number of stomata and length of stomatal apparatus among the selected mutants (Figure 3).

| S.No. | Mutants       | Stomatal Number | Stomatal Length (µm) | Chlorophyll Index | Flag Leaf Length (cm) | Flag Leaf Breadth (cm) |
|-------|---------------|-----------------|----------------------|-------------------|-----------------------|------------------------|
| 1     | CO 3-100-1-5  | 45.00 ± 1.46    | 9.03 ± 0.17          | 38.97 ± 0.91      | 22.77 ± 0.50          | 1.30 ± 0.02            |
| 2     | CO 3-100-7-12 (high efficient) | 79.00 ± 1.46    | 10.27 ± 0.17         | 47.97 ± 0.91      | 28.47 ± 0.50          | 1.33 ± 0.02            |
| 3     | CO 3-200-1-3  | 58.00 ± 1.46    | 7.99 ± 0.12          | 41.37 ± 0.70      | 18.10 ± 0.52          | 1.20 ± 0.01            |
| 4     | CO 3-200-4-1  | 71.00 ± 1.46    | 9.16 ± 0.12          | 35.20 ± 0.70      | 19.57 ± 0.52          | 1.17 ± 0.01            |
| 5     | CO 3-200-14-1 | 56.00 ± 1.46    | 8.85 ± 0.12          | 37.00 ± 0.70      | 20.27 ± 0.52          | 1.30 ± 0.01            |
| 6     | CO3-40.25-12-4 | 58.00 ± 1.33    | 9.90 ± 0.13          | 37.57 ± 0.60      | 32.33 ± 0.62          | 1.30 ± 0.03            |
| 7     | CO 3-40.25-30-2 | 61.00 ± 1.33    | 9.46 ± 0.13          | 41.97 ± 0.60      | 29.10 ± 0.62          | 1.57 ± 0.03            |
|       | CO 3 200-13-4 (low efficient) | 27.00 ± 1.46    | 9.18 ± 0.12          | 34.10 ± 0.70      | 28.17 ± 0.52          | 1.00 ± 0.01            |
|       | Wild type     | 55.00 ± 2.03    | 9.34 ± 0.49          | 35.62 ± 1.38      | 32.38 ± 0.40          | 1.17 ± 0.01            |

Stomata are the “doorkeepers” that regulate the volume of CO$_2$ infiltrating the intercellular air spaces of the leaf for photosynthesis. Earlier studies suggest that the increase in stomatal
density translates to an increase in stomatal conductance and 30% greater photosynthetic rate under high light conditions in *Arabidopsis* [17]. Increased stomatal density enhanced leaf photosynthetic capacity in *Arabidopsis* [18]. The mutant CO 3-200-13-4 had the least stomatal count (27 ± 1.46) per unit leaf area. Therefore, stomatal density may be a target trait for plant engineering to improve photosynthetic capacity.

![Stomatal distribution on the leaf epidermis of the identified extreme mutants along with control for photosynthetic efficient trait.](image)

**Figure 3.** Stomatal distribution on the leaf epidermis of the identified extreme mutants along with control for photosynthetic efficient trait.

### 3.3. Identification of Non-Lodging Mutant Lines

Lodging is usually referred to as a condition in which the stem of a crop bends at or near the surface of the ground, which could lead to the collapse of the canopy. Nine mutants showed superior lodging resistance in the M<sub>2</sub> generation until the time of harvest. The mutant CO 3-300-7-4 exhibited the thickest culm diameter of 4 ± 0.24 mm over the wild type (2.53 ± 0.12 mm). The bending of the crop stem at the ground surface was measured using the handy force gauge meter, and the strength of the highly resistant mutant (CO 3-300-7-4) was 39.73 ± 1.75 newtons, while the culm of the wild type exerted a strength of 24.53 ± 1.50 newtons (Table 3). The association of pulling force with other characters indicated that the mutant lines with high culm strength were taller than the wild and possessed thick culm, more productive tillers, and increased yield [19]. Not much work has been published on kodomillet relating to culm strength and lodging resistance. Nevertheless, rice has been used as a model crop for understanding the mechanical properties of culm in which the phenotypical traits such as pushing resistance [20,21] and stem diameter [22] were involved in evoking culm strength and for lodging resistance.

| S.No. | Mutants         | Culm Thickness (mm) | Culm Strength (newtons) |
|-------|-----------------|---------------------|-------------------------|
| 1     | CO 3-100-7-3    | 3.30 ± 0.07         | 34.73 ± 1.62            |
| 2     | CO 3-100-10-5   | 3.47 ± 0.07         | 22.08 ± 1.62            |
| 3     | CO 3-200-16-3   | 3.53 ± 0.05         | 29.73 ± 1.80            |
| 4     | CO 3-200-17-2   | 3.53 ± 0.05         | 24.18 ± 1.80            |
| 5     | CO 3-200-19-4   | 3.90 ± 0.05         | 39.63 ± 1.80            |
| 6     | CO 3-300-2-5    | 2.90 ± 0.24         | 43.65 ± 1.75            |
| 7     | CO 3-300-7-4 (highly stable) | 4.00 ± 0.24 | 39.73 ± 1.75 |
| 8     | CO 3-40.25-13-2 | 3.13 ± 0.05 | 30.27 ± 1.63 |
| 9     | CO 3-40.25-18-5 | 3.10 ± 0.05 | 34.49 ± 1.63 |
|      | CO3-100-18-22 (unstable) | 0.77 ± 0.07 | 6.88 ± 1.62 |
|      | Wild type       | 2.53 ± 0.12         | 24.53 ± 1.50            |

**Table 3.** Mutants screened for lodging trait in M<sub>2</sub> generation.
Based on culm thickness and culm strength, nine mutants (Table 3) were selected for non-lodging trait. The cross-sectional anatomy for the identified mutants was analyzed using scanning electron microscopy (SEM, Figure 4).

Thickness of sclerenchymatous cell (TSC) showed distinct variations that provided rigidity and culm strength. TSC of the mutants ranged from 8.61 μm to 16.01 μm, whereas the thickness of the wild type (CO 3) was 11.09 μm. The mutant CO 3-300-7-4 had the thickest TSC (16.01 μm). The mutant CO 3-100-18-22 showed the thinnest TSC (8.61 μm) (Figure 5). The information that culm strength is positively correlated with the thickness of sclerenchyma cell walls explained the better lodging resistance in barley [23].

Reduction in the mechanical strength of a plant may reflect alterations in cell wall structure, composition and fiber length. Therefore, cell-wall shape examined under SEM for the selected mutants revealed that the mutant’s (CO 3-300-7-4) sclerenchyma cell wall was thickened (Figure 6), in striking contrast with the wild-type sclerenchyma’s cell wall. The sclerenchyma of mutant CO 3-100-18-22 had the thinnest cell walls. Not much published evidence is available to substantiate this phenomenon in this crop.
which is then fixed to oxaloacetate, a C_4 pyruvate. The refixation of CO_2 is attained by ribulose-1,5-bisphosphate carboxylase (Rubisco) in the mesophyll cell. This C_4 acid intermediate is converted to malate by phosphoenolpyruvate carboxylase (PEPC) in the mesophyll cell. This C_4 acid intermediate is converted to malate by phosphoenolpyruvate carboxylase (PEPC) in the mesophyll cell. Thus, in contrast to C_3 cycle, photosynthetic efficiency of C_4 crops is higher (~50 folds) which confers several climate resilient adaptabilities including survival in high temperatures, high light intensities, radiations and drought conditions [27,28].

Secondly, the malate diffuses to bundle sheath where an NADP-malic enzyme (NADP-ME) performs decarboxylation to release CO_2 and pyruvate. The refixation of CO_2 is attained by ribulose-1,5-bisphosphate carboxylase (Rubisco) in the Calvin cycle. Thirdly, the pyruvate, orthophosphate dikinase (PORDK) converts pyruvate to phosphoenolpyruvate (PEP) in the mesophyll [26]. Thus, in contrast to C_3 cycle, photosynthetic efficiency of C_4 crops is higher (~50 folds) which confers several climate resilient adaptabilities including survival in high temperatures, high light intensities, radiations and drought conditions [27,28].

Recently, the complete C_4 enzyme repertoire of sequenced Poaceae genomes with emphasis on foxtail millet has been identified [29] (Figure 7).
Recently, the complete C4 enzyme repertoire of sequenced Poaceae genomes with emphasis on the expression of PPDK was observed in CO100-200-13-4 at 12 h post-treatment (more than 10-fold). At 12 h, the expression was down regulated in CO3 and upregulated in CO3-100-7-12 and CO3-200-13-4. Approximately 30-fold higher expression of PPDK was observed at 12 h post-treatment in CO3-100-7-12 (Figure 8) which suggests that the expression may confer higher enzyme activity to ensure the effective functioning of C4 photosynthesis. Similar observations were reported [27] where the expression of PPDK kept increasing in a bioenergy feedstock grass Miscanthus × giganteus during chilling stress. However, a decline in expression was observed in maize [30]. Over expression of maize PPDK in Arabidopsis showed a 4-fold upregulation in the expression, and the photosynthetic rates of transgenic plants had also increased [28].

3.4.1.1. Pyruvate, Orthophosphate Dikinase (PPDK)

The expression of PPDK in Control CO3, CO3-100-7-12 and CO3-200-13-4 at 0 hr. was unaltered; however, a significant upregulation was observed in CO3 and CO3-100-7-12 at 6 h post-dehydration treatment (more than 10-fold). At 12 h, the expression was down regulated in CO3 and upregulated in CO3-100-7-12 and CO3-200-13-4. Approximately 30-fold higher expression of PPDK was observed at 12 h post-treatment in CO3-100-7-12 (Figure 8) which suggests that the expression may confer higher enzyme activity to ensure the effective functioning of C4 photosynthesis. Similar observations were reported [27] where the expression of PPDK kept increasing in a bioenergy feedstock grass Miscanthus × giganteus during chilling stress. However, a decline in expression was observed in maize [30]. Over expression of maize PPDK in Arabidopsis showed a 4-fold upregulation in the expression, and the photosynthetic rates of transgenic plants had also increased [28].
3.4.1.2. Nicotinamide Adenine Dinucleotide Phosphate Hydrogen. (NADPH)

C₄ species are divided into subtypes, named for the primary decarboxylating enzyme that is localized to the bundle sheath. Maize, sorghum, and sugarcane use an NADP-dependent malic enzyme (NADP-ME subtype), whereas switch grass and teff (*Eragrostis tef*) use an NAD-malic enzyme (NAD-ME subtype) to generate a CO₂ pump in the bundle sheath cells. Most C₄ lineages are of the NADP-ME subtype [31].

In the present study, at 6 hr post-stress, NADP-ME2 was down regulated in CO 3-100-7-12 whereas NADP-ME9 did not show any difference as compared to control. At 12 h, NADP-ME2 was up regulated (~1-fold) in the better performing line, CO 3-100-7-12, whereas an incremental down regulation was observed in NADP-ME9. Both CO 3 and CO 3-200-13-4 showed increased levels of NADP-ME2, where CO 3-200-13-4 exhibited a 5-fold over expression in the 12 h sample. Similar elevated levels were observed for NADP-ME9; however, the relative expression levels were similar at both 6 h and 12 h samples in CO 3 and CO 3-200-13-4 (Figure 9). These expression profiles suggest that kodomillet uses an NADP-malic enzyme subtype C₄ photosynthetic system to fix carbon and, therefore, is a potentially powerful model system for dissecting C₄ photosynthesis.

3.4.1.3. Nicotinamide Adenine Dinucleotide Phosphate –Malate dehydrogenase (NADP-MDH)

In case of NADP-MDH1, there was no much difference in the expression levels across the lines, but a 2- to 3-fold up regulation was observed in CO 3 at 6 and 12 h samples. The expression was unaltered in CO 3-100-7-12, whereas a significant down regulation was observed in CO 3-200-13-4 at the 12 h sample. NADP-MDH4 exhibited a similar expression profile in CO 3, where an interesting observation of ~4-fold up regulation was noted in 6 h and 12 h samples. At 6 h post-stress, the level of NADP-MDH4 was almost similar in CO 3-100-7-12 and CO 3-200-13-4, but at 12 h, CO 3-200-13-4 showed a 1-fold up regulation. By contrast, NADP-MDH10 was highly expressed in CO 3-100-7-12 and CO 3-200-13-4 during stress as compared to control, and in case of non-stress condition (0 h), elevated expression was observed in CO 3-200-13-4 followed by CO 3-100-7-12 as compared to CO 3. These results indicate regulation of this enzyme is sensitive to dehydration in mutated plants. The gene NADP-MDH11 showed an initial up regulation at 6 h post-stress in CO 3-100-7-12 but down regulated at 12 h. In contrast, the transcript levels were increased with time in CO 3 and CO 3-200-13-4 (Figure 10) Similar differential expression of MDH genes was reported in wheat [32], *Arabidopsis* [33], maize [34], apple [35] and cotton [36].
were identified that were named GA2ox which showed a 20- to 70-fold increase in enzyme activity; however, the transgenic lines had aberrant GA2ox expression at the late-stage of dehydration stress. In CO 3-300-7-12, GA2ox expression was noticed in Figure 11. This could be due to the effect of mutation on the regulatory regions of the GA2ox gene expression. At 12 h post-dehydration treatment, the expression of GA2ox was increased in transgenic lines with null-positive effect on photosynthesis. All the over expression studies have demonstrated enhanced photoinhibition of photosynthesis due to an increase in the level of NADPH inside the chloroplast by the action of the C4-NADP-ME enzyme. These suggest that the cellular circuitry associated with NADP-ME is very complex in C4 plants and thus over expressing a single gene from C4 to C3 will show detrimental effects in the transgenic lines.

3.4.2. Expression Profiling of Lodging-Related Genes

Two genes were reported to be responsible for lodging resistance in rice and wheat, and interestingly, both were related to the plant growth hormone, gibberellic acid (GA). GAs control a variety of growth and developmental processes during the entire life cycle of plants. Several loss-of-function mutants in GA biosynthesis showed typical GA-deficient phenotypes, such as dwarfism, small dark green leaves, prolonged germination dormancy, inhibited root growth, defective flowering, reduced seed production, and male sterility. A major catabolic pathway for GAs is initiated by a 2β-hydroxylation reaction catalyzed by GA2 oxidases (GA2ox). In rice, 10 different classes of GA2ox were identified that were named GA2ox1 to GA2ox10, and in this study, the GA2ox orthologs of foxtail millet were first identified. However, foxtail millet possesses only seven GA2ox genes where the orthologs of GA2ox4, GA2ox9 and GA2ox10 were absent. A phylogenetic tree constructed to derive the evolutionary relationships between rice and foxtail millet GA2ox proteins showed that two distinct classes of these proteins exist in both the crops. The proteins GA2ox2, GA2ox5, GA2ox9 (ortholog absent in foxtail millet) and GA2ox6 formed a separate clade, and as shown by [39], these proteins contain the three unique and conserved motifs. At 12 h post-dehydration treatment, the expression of GA2ox6 was significantly reduced in the mutant CO 3-100-18-22, whereas an incremental decrease in gene expression was noticed in CO 3-300-7-4 (Figure 11). This could be due to the effect of mutation on the regulatory regions of the GA2ox6 or other gene(s) that could have a negative effect on GA2ox6 expression at the late-stage of dehydration stress.
were suggested to confer dwarfism by producing more active forms of growth repressors [40].

The transcript abundance of the Rht-B gene in all the three lines (Control CO 3, CO 3-100-18-22, CO 3-300-7-4) suggests interesting evidence of GA-regulated stress responsive machinery that might operate in these lines and also, the mutation would have occurred in such a way as to impede the average expression of Rht-B in CO 3-100-18-22 (Figure 12). In wheat, the mutant lines of Rht-B1b and Rht-D1b were suggested to confer dwarfism by producing more active forms of growth repressors [40].

The outcome of this study have shown striking variations for PhE and lodging resistance in a number of anatomical characteristics of flag leaf anatomy and culm thickness. The study highlights differential expression patterns of the same gene in different lines at different time points of stress as well as non-stress conditions. This infers that the mutation has some effect on their expression; otherwise the expression levels will be unaltered. Mutation in regulatory elements of each gene might have altered the motifs for recognition and binding of transcription factors, and this could have led to a differed transcriptional regulation of gene expression. Cloning and sequencing of the genes as well as their promoter regions can provide a clue about the variations that have been inserted through mutagenesis. However, the present data is very preliminary for drawing a solid conclusion, and further functional characterization is required to delineate the precise role of each gene in stress.

Figure 11. Relative expression profile of GA2ox6 gene analyzed using qRT-PCR under dehydration stress for 0 h (control), 6 h (early) and 2 h (late) in 21-day-old seedlings of CO 3, CO 3-100-18-22, and CO 3-300-7-4. The relative expression ratio of each gene was calculated relative to its expression in control sample (0 h). SiAct2 was used as an internal control to normalize the data. Error bars representing standard deviation were calculated based on three technical replicates for each biological duplicate.

Figure 12. Relative expression profile of Rht-B gene analyzed using qRT-PCR under dehydration stress for 0 h (control), 6 h (early) and 2 h (late) in 21-day-old seedlings of CO 3, CO 3-100-18-22, and CO 3-300-7-4.

4. Conclusions

The outcome of this study have shown striking variations for PhE and lodging resistance in a number of anatomical characteristics of flag leaf anatomy and culm thickness. The study highlights differential expression patterns of the same gene in different lines at different time points of stress as well as non-stress conditions. This infers that the mutation has some effect on their expression; otherwise the expression levels will be unaltered. Mutation in regulatory elements of each gene might have altered the motifs for recognition and binding of transcription factors, and this could have led to a differed transcriptional regulation of gene expression. Cloning and sequencing of the genes as well as their promoter regions can provide a clue about the variations that have been inserted through mutagenesis. However, the present data is very preliminary for drawing a solid conclusion, and further functional characterization is required to delineate the precise role of each gene in stress condition.
response and the impact of the mutation on the functioning of that particular gene. This also suggests that an improvement in grain yield could be best attained by developing a phenotype with high PhE and culm with thick sclerenchyma cells.

**Author Contributions:** Conceptualization, project administration R.R.; supervision R.M.; J.P.J., R.K.S. and J.G. performed the experiments. J.P.J. analyzed the data and wrote the manuscript. M.M.; M.P. data curation, validation, resources and supervision; J.P. formal analysis. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by UGC: Maulana Azad National Fellowship for Minority Students, F1-17.1/2016-17/MANF-2015-17-TAM-85427.

**Acknowledgments:** The authors profoundly acknowledge the Department of Millets, Tamil Nadu Agricultural University, Lab members of 103, NIPGR and the scientists at BARC, Kalpakam for the support rendered.

**Conflicts of Interest:** The authors declare no conflict of interest.

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