Phenotypic Characters and Expression of Cytokinin oxidase 2 (OsCKX2) Gene in Rice Genotypes with Enhanced Grain Filling Traits

Cielo Luz C. Mondejar1,2*, Maria Genaleen Q. Diaz1, Teresita H. Borromeo1, Tonette P. Laude1, Arlen A. Dela Cruz3 and Roel R. Suralta3

1University of the Philippines Los Baños, College, Laguna 4031, Philippines
2Philippine Rice Research Institute- Negros Branch Station (PhilRice Negros), Murcia, Negros Occidental 6129, Philippines
3PhilRice Central Experiment Station- Crop Biotechnology Center, Science City of Muñoz, Nueva Ecija 3119, Philippines

*Author for Correspondence; email: clcmondejar@philrice.gov.ph; ccmondejar1@up.edu.ph

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Poor grain filling is still a major problem in rice production. Recent attention in crop varietal improvement is focused on heritable delayed foliar senescence, enhanced root system, and fast-synchronous grain filling pattern. Nine genotypes were characterized for these three enhanced grain-filling traits to determine the role of these traits on grain yield. Based on heritable delayed foliar senescence, NSIC Rc436 and NSIC Rc512 were characterized as Type A, while PSB Rc18 and NSIC Rc514 as Type B. These genotypes had a significantly higher yield than IR64, owing to their high spikelets number. Data suggested a positive correlation between the delayed foliar senescence and grain number. NSIC Rc480, Arabon, Biniding (IR68), NSIC Rc512, Cuevas, and NSIC Rc436 were characterized with phenotypically superior root characters than IR64. A tendency of direct association was observed between the root characters and the percentage of filled grains and 500-grain weight. Only NSIC Rc480 was characterized with a fast-synchronous pattern, and its percentage of filled grains was significantly higher than the other tested genotypes. Integrating these enhanced grain filling traits in the selection criteria may improve poor grain filling in rice, which is a major constraint in improving the rice yield potential. Preliminary work on the expressions of Cytokinin oxidase 2 (OsCKX2), a gene implicated in the regulation of rice grain yield, was likewise conducted. Differences in the OsCKX2 expression was observed, which may entail a positive note on the exploration at the molecular level to identify the possible role of cytokinin in the gene regulation during rice grain filling.

Key words: cytokinin, Cytokinin oxidase/dehydrogenase 2 (OsCKX2), enhanced root system, fast synchronous grain filling pattern, heritable delayed foliar senescence, rice grain filling

Abbreviations: NPT—new plant type, DRO1—Deeper Rooting 1, QTL— quantitative trait loci, OsCKX2—Cytokinin oxidase 2, NIL—near-isogenic line, HI—harvest index, HYV—high yielding varieties, GSR—green super rice, PI—panicle initiation, DTF—days to flowering, DAF—days after flowering, and

INTRODUCTION

Tremendous efforts had been made by rice scientists in order to increase rice yield potential. Two significant growth periods which coincide with the introduction of semi-dwarfism and utilization of heterosis had been experienced since then (Peng et al. 1999). However, scientists have continued further research of the rice yield potential. Approaches include the development of a new plant type (NPT) using tropical japonica germplasm and the development of japonica/indica F1 hybrid rice for the tropical environments by IRRI scientists. The grain yield of NPT lines evaluated in replicated trials since 1994 did not significantly increase the grain yield because of low biomass production and poor grain filling (Peng et al. 2000; Lee and Ha 1999). In 1996, the traits from indica cultivars were introduced to NPT lines to broaden the genetic background of the NPT germplasm and refine the
Phenotypic and OsCKX2 Expression of Rice Genotypes Related to Grain Filling

Cielo Luz C. Mondejar et al.

original ideotype design, but this approach was not able to improve rice grain filling (Laza et al. 2003; Peng and Kush 1996). Biomass production significantly increased in the second-generation NPT rice; however, yield potential still did not improve, mainly due to the low grain filling percentage (Fu et al. 2011). Similarly, the major constraint in hybrid rice or utilizing the heterosis of japonica/indica F1 hybrid rice developed in IRRI was poor grain filling (Yang et al. 2002a; Peng et al. 2000). While IRRI was developing varieties through NPT breeding, Japan and China were also developing their super rice varieties, which were also aimed to increase the rice yield potential (Khush 1995; Cheng et al. 1998). China’s super rice varieties had a significant larger sink size which mainly resulted from a larger panicle size of primary and secondary branches (Fu et al. 2012; Wu et al. 2010, 2007). According to the reports, the yield advantage of super rice varieties was because of their high-rate flag leaf photosynthesis, slow leaf senescence, efficient carbohydrate remobilization, and high root activity (Wang et al. 2002; Zhai et al. 2002).

Rice yield is determined by the balance of a number of factors including sink size, source strength, and carbohydrate translocation. Improving sink size during grain filling period includes increasing grain weight and number of filled spikelets or the product of the total number of spikelets and percentage of filled spikelets. Leaf photosynthesis is one of the main aspects of source strength, and rice simulation models indicate that leaf photosynthesis, particularly flag leaves, plays an important role in determining crop yield (Yoshida and Horie 2009; Yoshida et al. 2008). Photosynthates generated after heading are responsible for 60 to 90% of the total carbon (C) accumulated in rice panicles at harvest (Mae 1997). Persistence of high photosynthetic capacity during grain filling has been considered as a key factor in increasing grain yield as reported by Abdelkhalik et al. (2005). Likewise, higher dry matter production is achieved by increasing the uptake of nitrogen (N) and water during grain-filling stage in paddy fields (San-oh et al. 2006; San-oh et al. 2004; Ookawa et al. 2003).

The existence of wide natural variation in root system architectures has been reported in various crops (O'Toole and Bland 1987). Root morphology and physiology are closely associated with the growth and development of above-ground part of plant; however, there are relatively less studies focusing on it (Yang et al. 2012). For the super rice variety Xieyou 9308, the agronomic traits including heading date, plant height, panicle length, grain yield per plant, number of spikelets per panicle, and grain setting density showed a significantly positive correlation with the root traits (Liang et al. 2011). A near-isogenic line (Dro1-NIL) that carries a functional allele of DEEPER ROOTING 1 (DRO1) derived from the deep-rooting cultivar ‘Kinandang Patong’ in the genetic background of the shallow-rooting parent variety ‘IR64’, which has a non-functional allele of DRO1, had been developed by Uga et al. (2009). The study conducted by Arai-Sanoh et al. (2014) showed that the grain yield of the Dro1-NIL was higher than IR64 in a paddy field. Higher grain yield was mainly due to the higher 1000-kernel weight and percentage of ripened grain. Dro1-NIL has better grain filling and higher harvest index (HI).

Variation in grain filling pattern and grain filling percentage had been reported by Yang et al. (2000). They reported three different types of grain filling patterns: fast synchronous, slow synchronous, and asynchronous. Fast synchronous grain filling pattern had the highest grain filling percentage while slow synchronous pattern had the lowest grain filling percentage, and genotypes with asynchronous pattern had intermediate grain filling percentage. The study of Yang et al. (2000) also demonstrates that the cytokinin contents in the roots, especially the translocating type of cytokinin, were closely related to the grains, and it is suggested that these types of cytokinins were synthesized in the roots and translocated to the grains where they regulate grain filling. Yang et al. (2002b) was able to determine what causes poor grain filling in rice by conducting an experiment using radioactive labeling to trace the assimilates during grain filling in hybrids. They found that only a small amount of carbon reserve was mobilized from source to sink and the rest of the carbon remained on the stems and leaves. This shows that poor grain filling was a result of poor translocation and partitioning of assimilates into grains rather than source limitation because of limited biomass production. The inability of the sink to fill despite the highest available source was also reported by Laza et al. (2003) as the cause of poor grain filling in NPT lines.

Most agriculturally important traits in crops such as yield and quality are controlled by several genes known as quantitative trait loci (QTLs) (Collard et al. 2005). In the past decade, efforts have been made to characterize QTLs for grain productivity. QTL analysis in rice has suggested that a locus responsible for grain yield, Gn1a, encodes cytokinin oxidase/dehydrogenase (OsCKX2) (Ashikari et al. 2005). Decreased expression of OsCKX2 in transgenic rice harboring antisense OsCKX2 cDNA resulted in increased grain number in the panicle.
Phenotypic and OsCKX2 Expression of Rice Genotypes Related to Grain Filling

Cielo Luz C. Mondejar et al.

(Ashikari et al. 2005). CKX is therefore regarded as a negative regulator of cytokinin metabolism. Consistent with this view, high-yielding rice cultivars which produce more grains in the panicles were found to have reduced or lost function of OsCKX2 and had higher cytokinins accumulated in the inflorescence meristems. Yang et al. (2000) observed varietal differences among tested genotypes on the content of zeatin (Z) and zeatin riboside (ZR), regarded as the translocating type of cytokinins. Genotypes with higher grain filling rate and percentage generally had higher Z and ZR contents in the grains and roots. Cytokinins have been implicated in communicating root nitrate availability to the shoot (Takei et al. 2002). Cytokinin application and modification of genes involved in cytokinin signaling influence chloroplast development, rice plant architecture, and yield (Hirose et al. 2007; Ashikari et al. 2005).

In this study, nine selected indica rice cultivars not previously characterized except for IR64 [a modern lowland indica cultivar widely used as a representative indica variety in research studies] were characterized with enhanced grain filling traits such as heritable delayed leaf senescence, superior root characters, and fast synchronous grain filling pattern. Agronomic traits, yield, and yield components were likewise determined in order to possibly define the role of these three enhanced grain filling traits on rice yield. Also, a work on the differential expression of cytokinin oxidase 2 (OsCKX2) in selected nine genotypes was conducted. This is a preliminary work in order to understand the metabolic cross talks during rice grain filling and to possibly explain how the grain filling enhancement traits such as heritable delayed foliar senescence, superior root characters, and fast synchronous grain filling pattern relate to one another and how these traits may improve rice grain filling.

MATERIALS AND METHODS

Plant Materials and Cultivation. Nine rice genotypes consisting of two traditional varieties, one farmer’s improved cultivar, four high yielding varieties (HYVs) also known as the Green Super Rice (GSR) lines, and two modern lowland indica cultivars widely grown in the Philippines were planted in plastic pots and grown in the greenhouse (Table 1) at PhilRice’s Central Experiment Station in Nueva Ecija from October 26, 2019 to March 15, 2020. The dimensions of the pots were 55 x 40 cm (d x h), and each pot could hold five plants. The pots were laid out in a complete randomized design. Each plant per pot represents the biological replication of each genotype. Each pot was filled with 200 kg of loamy-textured soil up to 30 cm in height, leaving 10 cm for floodwater. The soil was determined to have pH 7.5, 0.03% N, 13.5 ppm P, and 0.22 me K/100 g soil. The soil was first saturated almost uniformly before puddling by hand to the entire depth. Basal fertilizer treatment consisting of complete fertilizers (14-14-14) followed the recommended rate of 30-30-30 kg/ha N-P2O5-K2O. The seeds intended for planting were pre-germinated in a petri-dish for 7 days then transplanted at 15 x 15 cm spacing, allowing to plant 5 hills per pot (initially with 3 seedlings per hill, then reduced to 1 seedling per hill after 3 days, leaving behind the seedling with more or less uniform height). Water in the pots was maintained at saturation during the seedling stage and maintained at 3-5 cm at the following stages until 7 days before maturity. Additional N was applied at the rate of 10 kg N/ha using urea (46-0-0) at a weekly interval for 9 weeks while additional K using KCl was applied to all pots during panicle initiation and flowering at the rate of 15 kg/ha for each stage. Urea and KCl were dissolved in water for ease of application. Weeds, insects, and diseases were controlled.

Measurement of rice leaf greenness. Leaf greenness was measured using Chlorophyll A Chlorophyll meter (SPAD-502, Minolta Camera Co. Osaka, Japan). Top fully expanded leaves of all five plants in each pot were measured thrice a week after direct wet seeding up to the day before harvest. Three SPAD readings (dimensionless values, 650/940 nm wavelength transmittance ration) were conducted in each leaf around the mid-point of the leaf blade and 30 mm apart from one side of the midrib. Fifteen SPAD readings were averaged to represent the mean SPAD readings of each pot.

Root characterization. Roots were extracted from five plants of each genotype at harvest and were excised from the main stem and tillers. Images of roots were obtained using an Epson Dual Lens System (V700) scanner. The root length and diameter were measured using the software WinRHIZO™ Pro 2019a of Regent Instrument Inc. (https://www.agriculture-xprt.com/news/winhizo-2019a-801869) at 225 thresholds. The lengths of nodal and lateral roots were obtained based on their diameter ranges: ≤0.1 to 0.2 mm for the lateral roots and 0.2 to 10 mm for the nodal roots. Total root lengths were obtained as the sum of the length of nodal roots and lateral roots. The number of nodal roots was manually counted. Total root biomass was measured as the final weight of total root after oven-drying of root samples at 70°C until it attains constant weight.

Grain filling pattern characterization. The heading dates of individual panicles and the dates of individual spikelet opening were labeled and recorded. Three panicles of the main culm of three plants in each pot were labeled. For
each panicle, ten superior and ten inferior spikelets were labeled and checked every day to determine the grain filling pattern of each genotype according to the classification by Yang et al. (2000). The total number of spikelets and grain filling percentages at 7, 14, and 27 DAF (at maturity) of the labeled panicles were determined.

**Gene Expression Analysis.** For each genotype, the flag leaf from the main culm of the three plants were collected at 14 DAF as samples for gene expression analysis. The flag leaf of each plant represents the biological replication. The total RNA was extracted using RNeasy Plant Mini Kit (QIAGEN). The cDNA was synthesized using SensiFAST™ cDNA Synthesis Kit (Bioline). Both reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative polymerase chain reaction (qPCR) were conducted in this study. Primers used for RT-PCR and qPCR were: Actin (5'- CTCCCCCATGCTATCCTTCG-3' and 5'-TGAATGAGTAACCCACGCT-3') and OsCKX2 (5'- CGCCAACAAGTGGGACAGTAA-3' and 5'- GCCAGGTACTGCTTGTAGGC-3'). The cDNA sequence of the individual genes was obtained from the website of The Rice Annotation Project (https://rapdb.dna.affrc.go.jp/) while primers for individual genes were designed using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). In the RT-PCR, the basic PCR method was performed using Thermo Fisher Scientific Taq DNA Polymerase, GoTaq® Reaction Buffer, the primers previously mentioned, and newly synthesized cDNA (as the template DNA). The grey mean values of the region of interest were determined using LabImage 1D by Kapelan Bio-Imaging Solutions. The grey mean values of OsCKX2 were normalized using Actin as the internal control for normalization. Quantitative PCR was performed using innuMIX qPCR DSGreen Standard. The cycle threshold (Ct) value was determined using qPCR soft touch, which is a free software for TOWER G touch. Relative quantitation to compare the expression of OsCKX2 between varieties described as fold difference (2^{-\Delta \Delta Ct}) was computed using the Ct value of Actin as the internal control for normalization and the expression level of IR64 as a reference in the comparison of genotypes. Values were presented as mean with +/- standard deviation (SD) of three replicates of cDNA samples.

**Agronomic Traits.** Three panicles were collected for each tiller type: main, primary, and secondary. Panicle sizes between tiller types were compared. Days to flowering (DTF) was determined as the number of days when 50% of the spikelets within the panicle of the main tillers had flowered and DTF presented in the results was the average of five plants for each genotype. The number of panicles per plant was obtained as the average number of panicles of five plants of each genotype at harvest. Three 500 filled grains were counted for each genotype and 500-grain weight was determined as the average oven-dried weight of the samples. At harvest, grain yield (kg/ha) per plant was calculated using the formula: grain yield (kg/ha) = (total weight of filled grains at 14% M.C. (g) / total area of the pot (m²)) x 10 kg g⁻¹/ha m², where the total area of the pot was equal to 0.23758 m².

**Data Analyses.** Simple and multiple regression analyses were performed for SPAD readings using data from each growth stage and pooled data across growth stages. Agronomic and yield data were subjected to analysis of variance (ANOVA) and treatment means were compared using least significant differences (LSD) with an alpha level equal to 0.05 and presented in the results as pairwise mean comparison. Correlation analyses using Pearson’s product-moment correlation were also conducted to determine associations between phenotypic characters, agronomic traits, and normalized Ct value of OsCKX2 across genotypes.

**RESULTS**

**Rice Leaf Greenness.** Regression analysis of SPAD reading values for pooled data of all stages showed four sources of variations: linear, quadratic, cubic, and residuals ($R^2 = 0.69$ to 0.97) (Fig. 1). The regression analysis between chlorophyll meter (SPAD) readings across growth stages indicated no significant linear and quadratic regression but a significant negative cubic regression at the later stage of the rice plant. At the vegetative phase (2nd leaf to panicle initiation (PI) stage), the relative leaf greenness was increasing at linear. Then, an exponential increased in the graph can be observed at reproductive phase (between PI to heading or flowering stage). Inflection points where the graph of a function changes concavity [or algebraically where the second derivative is zero] were observed at PI or earlier. Graphs of the functions intersected with each other at PI for almost all of the genotypes except Biniding (IR68), which intersected with other genotypes at an earlier stage. Lastly, decrease in the leaf greenness was generally observed during the ripening phase (between heading to maturation). In the beginning, genotypes had leaf greenness ranging from 25 to 35 (Fig. 1). Genotypes can be grouped into two based on the observed trendlines of leaf greenness across growth stages. The 1st group which includes Arabon, Cuevas, Biniding (IR68), and NSIC Rc480 had initial values of ≥35 [higher than IR64], while the 2nd group consisting of NSIC Rc514, PSB Rc18, NSIC Rc436, and
NSIC Rc512 had < 30 initial SPAD reading [lower than IR64]. The peaks of the graphs of the 1st group were observed earlier than PI and the final readings of leaf greenness were lower than the other group (10 to 20) and not significantly different with IR64. On the other hand, the leaf greenness of the 2nd group continuously increased until the milk stage and had final values ranging from 35 to 45 at maturation, significantly higher than IR64. Additionally, the final SPAD reading values of the 2nd group can be further divided into two subgroups. NSIC Rc436 and NSIC Rc512 belong to the same subgroup where the decrease in the leaf greenness was more distinct than the other subgroup. The other subgroup includes PSB Rc18 and NSIC Rc514, with similar trendlines showing a slower decrease in leaf greenness than NSIC Rc436 and NSIC Rc512.

Root Characters. Analysis of variance showed significant differences (P < 0.05) among genotypes and no significant differences within genotypes on the root phenotypic characters (Table 2). NSIC Rc480 had the highest total root length which was significantly higher compared to three genotypes pre-characterized with deep rooting traits. Other genotypes with significantly higher total root length than IR64 aside from NSIC Rc480 and genotypes pre-characterized with deep rooting traits were NSIC Rc512 and NSIC Rc436. In contrast, PSB Rc18 and NSIC Rc514 were significantly lower than IR64. Lateral root length contributed 50 to 65% of the total root length. NSIC Rc480 had the highest lateral root lengths (Table 2). Arabon, Cuevas, Biniding (IR68), NSIC Rc512, and NSIC Rc436 remained significantly higher than IR64 while PSB Rc18 and NSIC Rc514 were significantly lower than IR64. For the nodal root length, NSIC Rc480 remained to be significantly higher than the other genotypes. PSB Rc18 had the same nodal root length as IR64, while NSIC Rc514 remained significantly lower than IR64. The average numbers of nodal roots of

Biniding (IR68) and Cuevas were significantly higher than NSIC Rc480. IR64 and PSB Rc18 had the same number of nodal roots, which was significantly higher than Arabon, NSIC Rc512, and NSIC Rc436. Moreover, NSIC Rc514 remained to have the least number of nodal roots among tested genotypes. Even though differences in the mentioned root characters were significant between genotypes, no significant difference can be observed on the total root biomass.

Grain Filling Pattern. Three grain filling patterns were observed among the test genotypes (Table 3), but only NSIC Rc480 was characterized as fast synchronous and...
The Philippine Agricultural Scientist Vol. 104 No. 1 (March 2021)

Phenotypic and OsCKX2 Expression of Rice Genotypes Related to Grain Filling

Cielo Luz C. Mondejar et al.

Table 2. Root lengths (total, lateral, and nodal) in centimeter, average number of nodal roots and total root biomass in grams of nine rice roots.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Total Root Length (cm)</th>
<th>Average No. of Nodal Roots</th>
<th>Total Root Biomass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSIC Rc480</td>
<td>35179 a</td>
<td>21179 a</td>
<td>14000 a</td>
</tr>
<tr>
<td>Arabon</td>
<td>28487 b</td>
<td>21705 b</td>
<td>11462 c</td>
</tr>
<tr>
<td>Biniding (IR68)</td>
<td>27421 c</td>
<td>14377 d</td>
<td>13044 b</td>
</tr>
<tr>
<td>NSIC Rc512</td>
<td>26024 d</td>
<td>16982 b</td>
<td>9041 e</td>
</tr>
<tr>
<td>Cuevas</td>
<td>25267 e</td>
<td>14534 cd</td>
<td>11093 d</td>
</tr>
<tr>
<td>NSIC Rc436</td>
<td>22564 f</td>
<td>13503 e</td>
<td>9061 e</td>
</tr>
<tr>
<td>IR64</td>
<td>16745 g</td>
<td>10078 f</td>
<td>6667 f</td>
</tr>
<tr>
<td>PSB Rc18</td>
<td>16404 h</td>
<td>9620 g</td>
<td>6784 f</td>
</tr>
<tr>
<td>NSIC Rc514</td>
<td>11388 i</td>
<td>6869 k</td>
<td>4519 g</td>
</tr>
</tbody>
</table>

In a column, means followed by the same letter are not significantly different at LSD 0.05.

only Biniding (IR68) was slow synchronous, while the other remaining genotypes were asynchronous. Analysis of variance for the percent filled spikelets at 7, 14, and 27 DAF were not significantly different within genotypes ($P < 0.05$). Between genotypes, percent filled spikelets showed significant differences at 7, 14, and 27 DAF. Likewise, the total number of spikelets showed no significant difference within one genotype but with significant differences between genotypes. At 7 DAF, genotypes with synchronous patterns [either fast or slow] had the highest percent filled spikelets but not significantly different with two genotypes characterized with asynchronous pattern namely NSIC Rc436 and NSIC Rc512. At 14 DAF, slow synchronous Biniding (IR68) had the same percent filled spikelets during 7 DAF. At maturity or 27 DAF, genotypes with fast synchronous patterns had significantly higher percent filled spikelets than the other genotypes. It was followed by asynchronous genotypes NSIC Rc436 and NSIC Rc512 with significantly higher percent filled spikelets than the other asynchronous genotypes, except for Biniding (IR68) which was also not significantly higher than the other genotypes with asynchronous grain filling patterns.

Expression of OsCKX2. OsCKX2 is responsible for the degradation of active cytokinins. Low expression of OsCKX2 can be assumed based on a high endogenous cytokinin concentration in the leaves at 14 DAF. Within the group of genotypes with enhanced root characters, the expression of OsCKX2 in Arabon was significantly higher than in Arabon while its expression in NSIC Rc480 was the same as in NSIC Rc480. The expression of OsCKX2 in Cuevas and Biniding (IR68) was significantly lower than in IR64. In the case of genotypes characterized with delayed foliar senescence, PSB Rc18 and NSIC Rc514 had a significantly higher relative expression level of OsCKX2 than IR64, while the OsCKX2 expressions in NSIC Rc436 and NSIC Rc512 were significantly lower than in IR64.

Agronomic Traits and Grain Yield. At 14 DAF, flag leaves from the main culm of the three plants in each genotype were cut as samples for molecular analysis. Two of these shoots were selected and the remaining two main culms with uncut flag leaves were compared to determine the effect of flag leaf cutting at 14 DAF on the panicle size of the main shoot or culm. Analysis of variance between the percent filled spikelets of panicles from main tillers with cut and uncut flag leaves at 14 DAF showed no significant difference within genotypes but with significant differences between treatments ($P < 0.05$). No significant difference was observed between cut and uncut flag leaves in the majority of genotypes with high percent filled spikelets at 7 DAF (Table 3), namely NSIC Rc480, NSIC Rc436, and Biniding (IR68) (Table 4). Genotypes with asynchronous pattern had significantly decreased percent filled spikelets with cutting of flag leaves at 14 DAF, except for NSIC Rc436 wherein no significant difference was observed and PSB Rc18 with

Table 3. Grain filling pattern, percent filled spikelets at three periods within ripening stage (7, 14 and 27 days after flowering or DAF), and total number of spikelets per panicle of nine rice genotypes.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Grain Filling Pattern</th>
<th>7 DAF</th>
<th>14 DAF</th>
<th>27 DAF</th>
<th>Total No. of Spikelets per Panicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSIC Rc480</td>
<td>fast synchronous</td>
<td>78.61 ab</td>
<td>82.27 cd</td>
<td>91.30 a</td>
<td>260 c</td>
</tr>
<tr>
<td>NSIC Rc436</td>
<td>asynchronous</td>
<td>74.97 b</td>
<td>88.21 ab</td>
<td>88.21 b</td>
<td>321 b</td>
</tr>
<tr>
<td>NSIC Rc512</td>
<td>asynchronous</td>
<td>73.68 b</td>
<td>86.53 b</td>
<td>87.35 b</td>
<td>231 d</td>
</tr>
<tr>
<td>IR64</td>
<td>asynchronous</td>
<td>60.37 c</td>
<td>81.34 cd</td>
<td>85.71 cd</td>
<td>194 f</td>
</tr>
<tr>
<td>Cuevas</td>
<td>asynchronous</td>
<td>59.86 c</td>
<td>86.52 bc</td>
<td>86.71 c</td>
<td>217 e</td>
</tr>
<tr>
<td>Arabon</td>
<td>asynchronous</td>
<td>54.04 d</td>
<td>62.42 f</td>
<td>86.98 c</td>
<td>187 g</td>
</tr>
<tr>
<td>NSIC Rc514</td>
<td>asynchronous</td>
<td>53.08 d</td>
<td>77.48 d</td>
<td>83.33 d</td>
<td>327 a</td>
</tr>
<tr>
<td>PSB Rc18</td>
<td>asynchronous</td>
<td>41.65 e</td>
<td>82.77 c</td>
<td>83.02 d</td>
<td>188 g</td>
</tr>
<tr>
<td>Biniding (IR68)</td>
<td>slow synchronous</td>
<td>76.21 ab</td>
<td>76.90 e</td>
<td>86.98 bc</td>
<td>196 f</td>
</tr>
</tbody>
</table>

In a column, means followed by the same letter are not significantly different at LSD 0.05.
significantly higher percent filled spikelets on cut flag leaves (Table 4). No significant difference was observed on the total grain weight between treatments even though there were significant differences on the percent filled spikelets, but plants with cut flag leaves had a lower total grain weight than plants with uncut flag leaves. PSB Rc18 had days to flowering (DTF) significantly longer than the other genotypes (Table 5). It was followed by NSIC Rc514 and NSIC Rc436, NSIC Rc512 and Arabon, and Cuevas, respectively with 72 to 76 DTF, while Biniding (IR68), NSIC Rc480, and IR64 had 67 to 69 DTF. The numbers of panicles were not significantly different with each other, with the mean ranging from 6 to 8. Genotypes with the range of 3 to 8 days from the day of the first to the last emergence of the panicle had only primary and secondary tillers: for example, NSIC Rc514, NSIC Rc512, PSB Rc18, NSIC Rc436, and Biniding (IR68). Meanwhile, other genotypes with a DTF range of 12 to 20 days produced tertiary tillers. However, the emergence of tertiary tillers was significantly late, indicating their inability to contribute mature grains at the time of harvest. The main culm had the highest total weight of filled grains among tiller types which can be attributed to the total number of spikelets per panicle (Table 5). A significant positive association ($P < 0.05$) can be observed between the total number of spikelets of primary and secondary tillers and the main culm ($r = 0.76$ and 0.58 for the primary and secondary tillers, respectively). The total number of spikelets of secondary tillers also had a significant positive correlation with primary tillers ($r = 0.62$). Panicles with a lower total number of spikelets had higher percent filled spikelets; for example, the secondary tillers of IR64 had the lowest total number of spikelets but had the highest percent filled spikelets among tiller types. The total weight of filled grains in a panicle was significantly lower in secondary tillers mainly because of the total number of spikelets. Biniding (IR68) and Cuevas had the highest 500-grain weight and were significantly higher than the other genotypes. Biniding (IR68) and Cuevas were followed by NSIC Rc512 and IR64, which were significantly different with the two genotypes. Meanwhile, PSB Rc18 was significantly lower than these four genotypes but significantly higher than NSIC Rc436.

Table 4. Percent filled spikelets and total weight of filled grains (g) at 10% moisture content of main culm between with cut and uncut flag leaves of each genotype.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>NSIC Rc480</th>
<th>NSIC Rc436</th>
<th>NSIC Rc512</th>
<th>IR64</th>
<th>Cuevas</th>
<th>Arabon</th>
<th>NSIC Rc514</th>
<th>PSB Rc18</th>
<th>Biniding (IR68)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent filled spikelets with flagleaf after 14 DAF</td>
<td>96.99 a</td>
<td>87.23 a</td>
<td>90.37 a</td>
<td>93.90 a</td>
<td>93.62 a</td>
<td>93.51 a</td>
<td>79.52 a</td>
<td>74.10 b</td>
<td>83.59 a</td>
</tr>
<tr>
<td>Percent filled spikelets without flagleaf after 14 DAF</td>
<td>91.30 a</td>
<td>84.66 a</td>
<td>84.40 b</td>
<td>84.65 b</td>
<td>80.50 b</td>
<td>82.87 b</td>
<td>71.85 b</td>
<td>82.82 a</td>
<td>89.79 a</td>
</tr>
<tr>
<td>Total weight of filled grains (g) at 10% M.C. with flagleaf after 14 DAF</td>
<td>3.81 a</td>
<td>4.73 a</td>
<td>4.49 a</td>
<td>2.80 a</td>
<td>4.47 a</td>
<td>2.95 a</td>
<td>2.62 a</td>
<td>2.51 a</td>
<td>3.79 a</td>
</tr>
<tr>
<td>Total weight of filled grains (g) at 10% M.C. without flagleaf after 14 DAF</td>
<td>3.49 a</td>
<td>4.47 a</td>
<td>4.04 a</td>
<td>2.56 a</td>
<td>4.32 a</td>
<td>2.58 a</td>
<td>2.37 b</td>
<td>3.28 a</td>
<td>3.72 a</td>
</tr>
</tbody>
</table>

In a column, means followed by the same letter are not significantly different at LSD 0.05.
and Araban. The NSIC Rc436 and Araban genotypes were significantly higher than NSIC Rc480. NSIC Rc514 had the least weight (at a significantly lower value) among the genotypes. Grain yield (kg/ha) at 14% M.C. of tested genotypes ranged from 3747 to 7137 kg/ha, with NSIC Rc512 having the highest yield which was significantly different from other genotypes. On the other hand, IR64 and Arabon had the least yield. Their grain yields were not significantly different from each other. Likewise, their yields were not significantly lower than Binding (IR68) and NSIC Rc436. Binding (IR68) and NSIC Rc436 were not significantly different from the remaining genotypes.

**DISCUSSION**

The SPAD-502 meter measures the transmittance of red (650 nm) and infrared (940 nm) radiation through the leaf and calculates a relative SPAD meter value that should correspond to the amount of chlorophyll present in the sample leaf (Minolta 1989). The SPAD meter is one of the most commonly used diagnostic tools to measure the crop N status of the rice crop. This was based on the observations that leaf area-based N concentration has a unique linear relationship with SPAD values of rice plants at all growth stages (Peng et al. 1995). In the Philippines, the threshold SPAD value of 35 works well for transplanted *indica* DS rice varieties with productive tillers from 450 to 500 m² according to Balasubramanian et al. (2000). In this study, SPAD-502 with a threshold of 35 was used to characterize the delayed foliar senescence traits in rice varieties with the assumption of a higher intensity color of leaves during the ripening stage of rice.
crop in the basis of the report of Yuan et al. (2016) on the close relationship between SPAD values and the extracted chlorophyll a and b content. The results observed in the SPAD reading values showing four sources of variations are similar to the result of the regression analysis for pooled data for all stages conducted by Esfahani et al. (2008). The increasing intensity of color (increasing N) with time represents continuous uptake and translocation to various sink partitions, becomes slow, or changes in slope until it reaches a peak where a balance of in-flow and partitioning might happen. Then, the partitioning accelerates to the extent that N in the leaves is translocated to the more demanding parts, so the SPAD reading goes below the threshold.

Inflection points were generally observed during panicle initiation. Changes in the color intensity can be observed, which may be related to the changes in the rate of N assimilation due to the redistribution of assimilates which happens at this period. Panicle initiation is said to be the first stage in the reproductive phase of growth and the start of panicle formation. At this stage, chlorophyll accumulates in the internodes in preparation for the

<table>
<thead>
<tr>
<th>Leaf Greenness</th>
<th>Percent Filled Spikelets of the Main Culm</th>
<th>Total Filled Spikelets of the Main Culm</th>
<th>Total No. of Filled Spikelets</th>
<th>Weight of Filled Grains</th>
<th>500-Grain Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>at anthesis</td>
<td>at 9 DAF</td>
<td>at 15 DAF</td>
<td>at 27 DAF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>coef</td>
<td>-0.1839</td>
<td>0.2266</td>
<td>0.0478</td>
<td>0.0072</td>
<td>-0.0997</td>
</tr>
<tr>
<td>p-value</td>
<td>0.132</td>
<td>0.3873</td>
<td>0.2727</td>
<td>0.029</td>
<td>0.0037</td>
</tr>
<tr>
<td>n</td>
<td>0.1927</td>
<td>0.2047</td>
<td>0.5922</td>
<td>0.0026</td>
<td>-0.1194</td>
</tr>
<tr>
<td>at 7 DAF</td>
<td>at 9 DAF</td>
<td>at 15 DAF</td>
<td>at 27 DAF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>coef</td>
<td>-0.2667</td>
<td>0.1671</td>
<td>0.4205</td>
<td>0.037</td>
<td>-0.4314</td>
</tr>
<tr>
<td>p-value</td>
<td>0.3873</td>
<td>0.2727</td>
<td>0.004</td>
<td>0.004</td>
<td>0.0041</td>
</tr>
<tr>
<td>n</td>
<td>0.1399</td>
<td>0.1399</td>
<td>0.4343</td>
<td>0.4343</td>
<td>0.4343</td>
</tr>
<tr>
<td>at 14 DAF</td>
<td>at 9 DAF</td>
<td>at 15 DAF</td>
<td>at 27 DAF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>coef</td>
<td>-0.1916</td>
<td>0.2433</td>
<td>0.439</td>
<td>0.3768</td>
<td>-0.1194</td>
</tr>
<tr>
<td>p-value</td>
<td>0.2074</td>
<td>0.1073</td>
<td>0.2398</td>
<td>0.1207</td>
<td>0.0107</td>
</tr>
<tr>
<td>n</td>
<td>0.1782</td>
<td>0.1782</td>
<td>0.3902</td>
<td>0.3902</td>
<td>0.3902</td>
</tr>
<tr>
<td>at 27 DAF</td>
<td>at 9 DAF</td>
<td>at 15 DAF</td>
<td>at 27 DAF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>coef</td>
<td>0.2074</td>
<td>0.1073</td>
<td>0.2398</td>
<td>0.1207</td>
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</tr>
<tr>
<td>n</td>
<td>0.1782</td>
<td>0.1782</td>
<td>0.3902</td>
<td>0.3902</td>
<td>0.3902</td>
</tr>
</tbody>
</table>

Table 7. Pearson’s product-moment correlation between rice leaf greenness, root characters, relative expression of OsCKX2 (Ct value), and yield component characters.
Phenotypic and OsCKX2 Expression of Rice Genotypes Related to Grain Filling

Arabon, Cuevas, and Biniding (IR68) had higher initial SPAD reading values, which may be because these genotypes had deep rooting traits which served as the basis for their pre-selection. Likewise, NSIC Rc480 had a similar trendline to these three genotypes, which can be explained by its superior root characters. NSIC Rc480 was an approved variety for rainfed and drought-prone rice environments and an enhanced root system was one of the main criteria for selection in this type of ecosystem because of this character’s adaptive ability under stress environments (Kim et al. 2020). Initial assimilation of N of these genotypes may be faster than the others since these genotypes may have well-developed roots that initiate an earlier start of assimilation. After panicle initiation, this group of genotypes had stopped assimilation [reflected their N assimilation of SPAD readings] or had continued assimilating N but had failed to register an increasing greenness in the leaves, which may be because of the redistribution of assimilates to other parts. NSIC Rc436 and NSIC Rc512 had superior root characters than NSIC Rc514 and PSB Rc18, which may explain their higher initial SPAD reading values than those of NSIC Rc514 and PSB Rc18.

NSIC Rc480 was the only genotype characterized by a fast synchronous grain filling pattern and significantly higher percent filled spikelets than the other genotypes. Other genotypes with relatively high percent filled spikelets at maturity (27 DAF) were the genotypes with high percent filled spikelets at an earlier period of grain filling (7 DAF) irrespective of the total number of spikelets. However, the pattern of distribution of the assimilates cannot be completely described since only the grain filling pattern of the main culm was observed and not the other tiller types which, based on the results of this study, had a different character pattern between genotypes. The significant positive associations of the total number of spikelets of the main culm to other tiller types resulted in the selection of varieties with high total number of spikelets in the main culm. However, genotypes with a high total number of spikelets in the main culm may be superseded by some of the genotypes with a significantly higher total number of spikelets in the primary and secondary tillers, especially when the percentage of filled spikelets is not significantly different across tiller types.

In this study, RNA was extracted from flag leaves at 14 DAF. This is based on previous reports that flag leaves provide the most important source of photosynthetic energy during reproduction (Asadur Rahman et al. 2013; Abou-Khalifa et al. 2008; Misra 1987; Yoshida 1981). Moreover, Arai-Sanoh et al. (2014) had determined that cytokinin flux in the flag leaves during 14 days after heading was at its peak. Since the individual spikelets in a panicle of the rice plant did not open at the same time, the flowering date was determined as the date when 50% of the spikelets within the panicle had already opened. OsCKX2 is responsible for the degradation of active cytokinins, which means that a high expression of OsCKX2 can be assumed based on a low cytokinin concentration in the leaves or vice versa. Chlorophyll loss in leaves has been correlated with leaf cytokinin flux by Soejima et al. (1995), implying that changes in cytokinin content which are also associated with the grain-filling rate (Yang et al. 2000) may be involved in the onset of leaf senescence. Among the genotypes with heritable delayed foliar senescence, Type A genotypes (NSIC Rc436 and NSIC
Phenotypic and OsCKX2 Expression of Rice Genotypes Related to Grain Filling

Cielo Luz C. Mondejar et al.

Rc512) had a low relative expression level, while Type B genotypes (PSB Rc18 and NSIC RC514) had a significantly high relative expression level. The heritable delayed foliar senescence trait of Type A genotypes may be related to cytokinin. These genotypes had superior root characters. Cytokinins are being synthesized in the roots and transported to the shoot (Kamada-Nobusa et al. 2013; Sakakibara 2006; Van Staden and Davey 1979). However, among genotypes with superior root characters, Arabon had a significantly higher relative expression of OsCKX2. Root oxidation activity which is necessary to maintain root biomass, root and shoot growth, and ion uptake was not investigated in this study. Characterizing the roots using these traits may give a complete picture on how the roots contribute to the shoot growth. Cytokinin concentrations in different time points (for example: before, during and after grain filling stage) were not measured in this study. Their measurements in different parts of the plants such as the roots, stem, leaves, and grains as well as the differential expression of genes in different parts of the plant were also not conducted in this experiment. Another study which may focus on these will explain the differences in the expression of OsCKX2 at 14 DAF between Types A and B heritable delayed foliar senescence and how endogenous cytokinin may contribute to the enhancement of rice grain filling.

In general, genotypes characterized by a heritable delayed foliar senescence had DTF or days to maturity longer than the other genotypes. These two traits may be related to each other; however, another study should be conducted to determine the relation of these traits. In contrast, these genotypes only had primary and secondary tillers. Developing rice varieties with optimum numbers of primary and secondary tillers and without tertiary tillers are being emphasized today (Mohanan and Mini 2008), which possibly explains the no tertiary tiller of newly released HYVs, namely NSIC Rc514, NSIC Rc512, and NSIC Rc436. A negative correlation was observed between the root characters and rice leaf greenness. This may be because genotypes in this study were pre-selected for their specific traits which resulted in an incidental (but not cause-and-effect) relationship of root traits with SPAD values. However, data suggests that traits with an observed significant linear relationship with the root characters had a negative association with leaf greenness and traits found to be correlated with leaf greenness or vice versa. For example, the root characters were found to have a positive association to percentage filled spikelets and 500-grain weight, and these two yield components were observed to be negatively associated with leaf greenness. On the other hand, leaf greenness had the tendency towards a positive association with the total filled spikelets, which was observed to be negatively associated with the root characters. Likewise, the six qRL6.1 near-isogenic lines (NILs) carrying a functional allele of qRL6.1 from Kasalath (a major QTL for root length of rice seedlings) in the genetic background of IR64 had improved root characters than IR64 but were observed to have a significantly less total number of spikelets than IR64 (Mondejar et al. 2020). However, another experiment should be conducted to confirm the relationships of the traits because the study only had a limited number of samples, and the traits are influenced by many genetic and environmental factors.

One of the significant findings of this study was that NSIC Rc512, characterized by a Type A heritable delayed foliar senescence and superior root characters and displayed a low expression of OsCKX2, had the highest grain yield which was significantly higher than the other genotypes. A tendency towards a positive correlation was found between the normalized Ct value of OsCKX2 and percentage filled spikelets at the early grain filling stage of the main culm, weight of filled spikelets of the primary tillers, and 500-grain weight. This may describe the route of assimilate at the earlier stages of grain filling. The source of assimilate supply for the filling of grains at this period may come from the redistribution of C reserve and the continuous N assimilation of the rice crop signaled by senescence. The redistribution on the basis of its root characters, and further supplies may come from the remaining C reserve of the genotype or its continuous assimilation and redistribution on the basis of its root characters.

According to recent studies, a reduced expression of Gln1a, the gene encoding OsCKX2, in the inflorescence meristem of Habataki elevates cytokinin content, resulting in a high grain number per panicle (Kim et al. 2016). However, the study of Kim et al. (2016) also presents that indica varieties belong to types of Gln1a alleles that are different from japonica varieties, and that the Gln1a-type 3 allele was not effective in indica cultivars (Kim et al. 2018). Therefore, in order to explain how the traits relate to one another and how they may improve grain partitioning in rice, more explorations of the molecular basis to understand the metabolic cross talks during grain filling are needed.

CONCLUSION

Four genotypes were characterized by a delayed foliar senescence. NSIC Rc436 and NSIC Rc512 were Type A, while PSB Rc18 and NSIC Rc514 were Type B. Type A genotypes were characterized by having superior root traits as compared to Type B. PSB Rc18 and NSIC Rc514
had a shallow rooting system similar to IR64. Genotypes with heritable delayed foliar senescence had a significantly higher yield than IR64, owing to the high total number of spikelets in the main shoots. Moreover, data indicated a tendency towards a direct association between the heritable delayed foliar senescence trait and the grain number. NSIC Rc480, Arabon, Biniding (IR68), NSIC Rc512, Cuevas, and NSIC Rc436 were characterized by phenotypically superior root characters than IR64. Superior root characters were found to have a positive correlation with percent filled spikelets and 500-grain weight. Only NSIC Rc480 was characterized by a fast-synchronous grain filling pattern and was observed to have an improved grain filling percentage. This information is very useful in developing varieties with the breeding objective of increasing the rice yield potential. Including these traits in the criteria for selection during varietal development may improve poor grain filling, which is one of the limitations when it comes to further improving the yield potential of rice. However, another study should be conducted to confirm the relationships of these traits because these traits are influenced by many genetic and environmental factors and because this study only tested nine genotypes. The observed correlations between the expression of OsCKX2 on some of the yield component parameters may entail a positive note on the possible exploration of the molecular and biochemical basis in order to explain the role of cytokinin in the expression of genes related to rice grain filling. Moreover, cytokinin concentrations in different time points (for example: before, during, and after grain filling stage) were not measured in this study. Their measurements in different parts of the plants such as the roots, stem, leaves, and grains as well as the differential expression of genes in different parts of the plant were not tested. Another experiment focusing on these may explain the differences in the expression of OsCKX2 in the tested genotypes and other cytokinin-responsive transcriptomes, which may also explain the possible role of cytokinin in the regulation of genes related to rice grain filling.

REFERENCES CITED


MONDEJAR CLC. 2020. Expression of Cytokinin oxidase 2 (OsCKX2) gene in rice genotypes characterized with enhanced grain filling traits. [MS thesis]. Los Baños, Laguna: University of the Philippines Los Baños. 106p. (Available at the UPLB Library)


