**Characterization and evaluation of transgenic indica rice overexpressing *SoSPS1* gene in greenhouse trials**

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**Abstract**

Rice is the most popular staple food in the world and it is reported to feed approximately half of the world population. Previous study showed that overexpression of sugarcane *SoSPS1* gene enhanced sucrose synthesis activity, growth and productivity of the transgenic rice. This study was directed to characterize and evaluate the transgenic rice lines grown in biosafety greenhouse. Seven lines of transgenic rice were germinated for a month and transplanted in pot for evaluation of the growth and productivity. The growth of the transgenic lines was initially similar at 30 days after planting (DAT) compared to wild-type (WT) rice. However, in the next stage the transgenic lines were grown faster and significantly higher plant height and tiller number at 60 and 90 DAP. As the consequences, the flowering time take a longer time compared to WT. Furthermore, number of panicles, percentage filled grains and grains number per panicle were significantly increased in the transgenic lines. The higher sucrose synthesis provides a higher sucrose partitioning into rice seed and biomass accumulation in the transgenic rice. Therefore, grain yield and biomass accumulation were significantly increased in transgenic lines compared to WT rice. The transgenic rice increased 1.5-fold rice production and it is really appropriate for the purpose of the rice self-sufficient.

 **Introduction**

Rice is an important plant that used as the primary source of carbohydrates and energy for the Indonesian population. The annual rise rice consumption in domestic corresponds directly to the nation's expanding population, and need to escalate the level of rice production (Sen et al., 2020; Yu et al., 2020). Mostly the world’s rice (*Oryza sativa* L.) is grown and consumed by small-scale farmers in Asia and the Pacific (Fukagawa & Ziska, 2019; Zhang et al., 2020).

Biotechnology is one of the methods to improve plant growth and productivity. The biotechnology with genetic transformation plays a significant role in the development of new plant characters and variations (Yin et al., 2021). The conventional breeding required lengthy breeding cycles, more laborious, costly and time consuming. The genetic transformation for introducing a new DNA trait that inherited in subsequent generation offers an opportunity to increase plant growth and productivity (Ahmar et al., 2020).

Sucrose is one of major photosynthetic carbon assimilation product that synthesized in leave or source tissue and transported through phloem to all parts of plant or sink tissues (Chibbar et al., 2016). The primarily functions of sucrose is for source of carbon and energy of plant organs that incapable of conducting photosynthesis (Lemoine, 2000). Sucrose has been postulated to effect on carbon partitioning in plants, and higher sucrose synthesis may lead to increase sink activity like plant growth and productivity (Rolland et al., 2006). In addition, sucrose is recognized for its involvement in regulating diverse developmental and metabolic processes within plants and plays a crucial role in the plant defense against pathogenic infections (Tauzin & Giardina, 2014). Therefore, the sucrose can promote the growth and development of leaves, stems, roots, and various other plant components (Ciereszko, 2018; Lunn, 2008; Ruan, 2014).

Sucrose metabolism involve several enzymes activity such as sucrose-phosphate synthase (SPS), sucrose synthase (SuSy) and invertase. Sucrose-phosphate synthase is believed as a key enzyme for sucrose synthesis from UDP-glucose (UDPG) and fructose-6 phosphate (F6P), and controlling sucrose content in plant. The gene encoding for SPS proteins have been cloned from various plant, including from sugarcane (Sugiharto et al., 1997). Genetic transformation of gene for SPS increased sucrose content in tomato (Nguyen-Quoc et al., 1999), plant growth and biomass accumulation in transgenic poplar (Maloney et al., 2015) and *Brachypodium distachyon* (Falter & Voigt, 2016). Our studies have confirmed that overexpression of *SoSPS1* gene from sugarcane elevated sucrose content, growth and productivity in rice (Mulyatama et al., 2022) and sugarcane (Anur et al., 2020).

In this study, the transgenic rice lines overexpressing *SoSPS1* were selected and grown in a biosafety greenhouse at University of Jember, Indonesia. The transgenic rice lines were germinated pot tray, selected using PCR analysis, and the positive transgenic rice lines were transplanted in the pots. The growth and productivity of the transgenic lines were observed and compared to the non-transgenic counterpart. The results showed that growth and production of transgenic were increased in plant biomass, grain yield, and plant height compared to the non-transgenic rice. Consequently, the transgenic rice lines required longer time for flowering.

**Materials and Methods**

***Plant growth condition and experimental design***

The third generation of transgenic rice seeds and non-transgenic counterpart (Ciherang cultivar) were germinated in pot tray for three weeks. After selection of the transgenic rice seedling using PCR analysis, the positive transgenic lines were transplanted into pot containing 10 litter of soil media. The soil media were prepared by homogenation and muddy of the soil. The experiment was carried out in Completely Randomized Design (CRD) with seven transgenic rice lines T3, T4, T5, T6, T8, T9, T11, and one non transgenic rice with three times replications. The plants were grown in biosafety greenhouse under sun light illumination (± 25000 lux), maintain at temperature range 24-28o C, and relative humidity between 60-80%.

The rice plants were grown under submerged conditions by regularly watering until harvest and supplied with nitrogen, phosphate and potassium fertilizer at 15, 30 and 45 days after planting (DAP). The nitrogen, phosphate and potassium were provided as urea, SP-36, and KCl at amount 2.7, 0.9, and 0.9 g per pot, respectively. After grain filling, the watering was reduced according to the soil condition.

***DNA Isolation and PCR analysis***

Approximately 0.3 g of rice leaves were ground in liquid nitrogen, followed by extraction of the genomic DNA using Plant Genomic DNA Kit (Tiangen, China). The concentration of DNA was measured at 260 nm by NanoVue Spectrophotometer (GE Healthcare, USA) and stored in -20oC. The presence of the inserted gene was analyzed by PCR using the leaves genomic DNA and a pair of primer for detection of *npt*II gene as the marker gene. The nucleotide sequences of the primer were nptII-R (5'-GTCGCTTGGTCGGTCATTTC-3') and *npt*II-F (5'-GTCATCTCACCTTGCTCCTGCC-3'). PCR analysis was performed in a T100 Thermal Cycler (Bio0Rad, USA) using a mixture containing 12.5 µL of 2 x Promega GoTaq G2 Green Master Mix (Thermofisher scientific, USA), 1µL primer (10µmol/L), and adjusted with nuclease free water up to 25µL. PCR reaction was conducted at 95°C for 5 min, followed by 35 cycles at 95°C for 30 s, 53°C for 30 s, 72oC for 1 min, and final extension at 72°C for 5 min. The amplified PCR product was run on 1% agarose gel, stained with ethidium bromide, and documented on GelDoc (Major Science, USA).

***Determination of plant growth and productivity***

To determine of plant growth and productivity the characters such as plant height, number of tillers, number of grains per panicle, 1000 grain weight, grain weight and biomass per pot were measured. The plant growth was determined by measurement of plant height at 30, 60, 90, and 120 DAP. Plant height (cm) was measured from the surface of the growing medium or the base to the tip of the tallest plant. Flowering time is a prerequisite for crop production, was determined when the first flower is appeared. Number of tillers was determined by counting the number of rice tillers originating from the primary rice stem at 30, 60, 90, and 120 DAP. Other productivity characters such as number of panicles, number of grains per panicle, panicle length (cm), percentage of grains per panicle were determined at harvest. Harvesting of rice plants were carried out at 120 DAP, when the rice grains have reached physiological maturity, and that characterized by the grain being fully ripe and the panicles starting to bend. After removing the panicle and seeds, the dry weight biomass was determined after drying the biomass at 105oC for 3 days or constant weight. The seeds were removed from panicles and used for determination of 1000 grain weight and grain weight per pot.

***Statistical Analysis***

Statistical analysis was conducted to evaluate the differences between the data using analysis of variance (ANOVA). Duncan's Multiple Range Test (DMRT) at the 5% level was used for further analysis to determine statistical significance. A p-value of 0.05 was considered for determining statistical significance.

**Results**

***PCR Analysis***

PCR analysis was used to verify that the targeted genes were inserted in the genomic of transgenic rice. Detection of *npt*II gene as marker gene was used to verify the transgenic lines, and not the gene encoding for SPS protein due to the transgenic rice lines contain similar endogenous SPS protein (Mulyatama et al., 2022). PCR amplification of *npt*II gene using specific primer pair detected corresponding DNA bands with molecular size 700 kb in all transgenic lines (Fig. 1). This result indicated that seven transgenic lines, T3, T4, T5, T6, T8, T9, and T11 are positively expressing targeted *SoSPS1* gene.

 M WT T3 T4 T5 T6 T8 T9 T11

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700 bp

Figure 1. Visualization of DNA-PCR products in agarose gel (1% agarose) electrophoresis using specific *npt*II primers and rice genome DNA as the template. The PCR products at 700 bp was expected *npt*II DNA. M: DNA marker (1 Kb Ladder); T3, T4, T5, T6, T8, T9, T11 were transgenic rice lines.

***Analysis of morphological traits***

*Plant Height*

Measurements of plant height were conducted three times during the rice growth at 1-, 2- and 3-months after planting (MAP) (Table 1). The plants height of transgenic lines was similar and not significantly different compared with non-transgenic counterpart at early growth. The growth of transgenic lines was started to increase at 2 MAP and significantly increased in T6 and T9 transgenic lines. Furthermore, the plants height was significantly higher in all transgenic lines compared to WT (non-transgenic line) at 3 MAP. The range of plants heights were 103.7 to 111.3 cm in transgenic line and only reached 90.7 cm in WT (Table 1). These results indicated consistently a higher growth in the transgenic lines overexpression of *SoSPS1* gene.

*Number of tillers*

Tillers number started to increase after 10 DAP and continued until the plants reached the maximum vegetative phase (50–60 DAP). The tillers generated after the maximum vegetative period slowly grown and eventually die, but healthy rice tillers were capable producing panicles.

The number of tillers were not significantly different in the first month (1 MAP), but the number were significantly increased thereafter in all transgenic lines compared to WT at 2 and 3 MAP (Table 1). The increasing tiller numbers were in agreement with development of plant height. Furthermore, the transgenic lines T4 and T6 had the higher tiller number, 52.3 and 54.3 at 2 MAP, respectively and continued to increase at 3 MAP.

Table 1. Plant height and tiller number of transgenic rice lines and non-transgenic (WT) at 1, 2 and 3 MAP. Values are means ±SD for three independent plants, and the different lowercase letters denote significant differences (p ≤ 0.05).

|  |  |  |
| --- | --- | --- |
| **Lines** | **Plant Height** | **Number of tiller** |
| **1 MAP** | **2 MAP** | **3 MAP** | **1 MAP** | **2 MAP** | **3 MAP** |
| WT | 75,67 ± 3,21 | 79,67 ± 3,51 c | 90,67 ± 2,52 c | 22,00 ± 1,00 a | 35,67 ± 3,51 c | 34,33 ± 2,52 d |
| T3 | 69,67 ± 3,06 | 83,00 ± 2,65 abc | 111,33 ± 3,06 a | 18,33 ± 1,53 b | 36,33 ± 2,08 c | 40,00 ± 3,00 c |
| T4 | 70,67 ± 4,16 | 83,67 ± 1,53 abc | 108,33 ± 2,08 ab | 20,00 ± 2,00 ab | 52,33 ± 0,58 a | 56,33 ± 3,51 a |
| T5 | 65,00 ± 2,65 | 82,00 ± 4,00 bc | 109,00 ± 4,36 ab | 18,00 ± 2,00 b | 41,33 ± 2,08 b | 41,00 ± 2,00 c |
| T6 | 73,33 ± 1,53 | 87,67 ± 1,53 a | 106,00 ± 2,65 ab | 22,67 ± 2,52 a | 54,33 ± 3,21 a | 50,00 ± 3,61 b |
| T8 | 66,67 ± 2,89 | 83,67 ± 1,53 abc | 106,33 ± 3,21 ab | 21,33 ± 1,53 ab | 45,33 ± 1,53 b | 37,67 ± 2,52 cd |
| T9 | 69,00 ± 4,58 | 86,33 ± 1,53 ab | 103,67 ± 2,89 b | 19,67 ± 2,52 ab | 45,67 ± 3,06 b | 46,67 ± 1,53 b |
| T11 | 67,00 ± 6,56 | 84,33 ± 2,08 abc | 106,67 ± 1,15 ab | 22,67 ± 1,53 a | 42,67 ± 3,06 b | 41,33 ± 2,08 c |

*Flowering Time*

The flowering time was different between transgenic lines and WT. Compared to WT plant, transgenic lines displayed significantly longer flowering time. Flowering of WT was faster at 69.7 DAP than all transgenic lines at 77.7 to 79.7 DAP (Fig. 3).

Figure 2. Flowering time of wild type (WT) and transgenic rice lines (T3 – T11) overexpression *SoSPS1* gene. The flowering time was observed when the first flower is appeared. Data are presented as means ± SE of three independent plant and the different lowercase letters denote significant differences (p ≤ 0.05).

*Number and length of panicles*

The productivity of rice is determined by number of panicles that contains filled grain. The count of filled grains in panicles provide the yield of rice. The number of panicles were significantly increased in transgenic lines T4, T6 and T9, although the others transgenic lines were not significantly increased (Fig 4.a). The panicles number per clump were 45, 43, and 43.7 for T4, T6, and T9, respectively, and only 20 panicles in WT. In addition, all the transgenic lines only displayed a little longer in panicle length compared to WT plant, but they were not significantly different (Fig 4b), The longest panicle was showed 21.5 cm in T8 transgenic lines and 20 cm in WT plants.

Figure 3. The number (left) and length (right) of panicle in wild type (WT) and transgenic rice lines (T3-T11) overexpression *SoSPS1* gene. Data are presented as means ± SE of three independent plant and the different lowercase letters denote significant differences (p ≤ 0.05).

***Grain yield and biomass productivity***

*Number of grains per panicle*

An increase in the number of grains per panicles contributes to the higher production of grains yield. To determine the rice grain yield, the number of grains per panicle, the percentage of filled grains, and the weight of grains per plant were measured (Table 2). The number of grains per panicle were significantly greater in all transgenic lines compared to WT rice. The greatest grains number was found in T5 transgenic lines (165.7), increased more than 150% compared to WT which only reached 99.7 grains number per panicle. In line with the grain numbers, percentage of filled grains were also significantly greater in all transgenic lines. The percentage in T9 transgenic lines was 93.2%, increased more than 150% compared to WT plant (60.7%). Almost 40% grain seeds per panicle were unfilled grain in WT rice. The higher in panicle number per clump as well as the percentage of filled grains indicate higher the rice productivity.

*Grain yield and biomass productivity*

Grains harvesting was conducted by removing the grains from panicles and after drying the 1000 grain weight and grain weight per plant were measured. The 1000 grain weight were similar between the transgenic lines and WT rice (Table 2). Range of the 1000 grain weight were 22.4 - 23.09 g. This result revealed that there is no significancy of size of grain yield between transgenic rice and WT rice. In contrast, grain weight per clump were significantly greater in transgenic rice compared to WT rice. The greatest grains weight was T9 transgenic line reach 105.93 g, while the lowest grain weight was WT rice achieve only 52.24 g per clump. The grains weight per clump is the total weight of grains produced per plant. The grains weight per plant can be used as an indicator to estimate rice production by converting to hectare. The transgenic rice lines were estimated to produce the grains yield range 14.09 to 16.95 ton per Ha, around twice comparted to WT (8.4 ton per Ha), when the rice grown in the field with spacing distance of 25 x 25 cm and rice population 160.000 clumps. These results indicate that rice production was significantly higher in transgenic lines compared to WT rice.

Table 2. Grain yield and biomass accumulation in transgenic lines and WT rice

Values are means ± SD for three independent plants, and the different lowercase letters denote significant differences (p ≤ 0.05).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Lines** | **The grains number per panicle**  | **Percentage filled grains (%)** | **1000 grain weight (g)** | **Grain weight per plant (g)** | **Biomass weight (g)** |
|  |
| WT | 99,75 ± 2,78 f | 60,70 ± 2,94 c | 23,09 ± 0,42 a | 52,24 ± 3,51 d | 131,16 ± 3,49 f |  |
| T3 | 157,50 ± 2,50 bc | 82,79 ± 4,02 b | 22,76 ± 0,33 a | 88,08 ± 3,95 c | 169,75 ± 1,08 e |  |
| T4 | 141,92 ± 3,25 e | 87,76 ± 0,65 ab | 22,50 ± 0,88 a | 105,01 ± 1,89 a | 198,82 ± 4,11 a |  |
| T5 | 165,75 ± 3,19 a | 87,00 ± 3,09 b | 22,63 ± 0,57 a | 93,98 ± 4,17 bc | 183,02 ± 2,58 c |  |
| T6 | 159,83 ± 3,47 b | 87,23 ± 1,61 b | 22,82 ± 0,32 a | 97,45 ± 1,59 b | 189,41 ± 3,20 b |  |
| T8 | 156,00 ± 2,38 bcd | 87,87 ± 3,84 ab | 22,36 ± 0,60 a | 93,39 ± 2,91 bc | 183,02 ± 2,98 c |  |
| T9 | 152,58 ± 3,51 cd | 93,18 ± 3,40 a | 22,80 ± 0,12 a | 105,93 ± 3,20 a | 198,86 ± 3,35 a |  |
| T11 | 151,17 ± 1,70 d | 85,31 ± 3,97 b | 22,65 ± 0,96 a | 89,59 ± 4,02 c | 177,00 ± 3,76 d |  |

*Biomass accumulation*

The cultivation of rice results in two major types of biomass rice, straw and husk that have potential role for biomass energy. After harvest, the rice straw was dried and measured the weight as biomass accumulation, excluded the husk. The rice biomass as expressed by rice straw accumulation depends on various factors such as varieties, soils and nutrient management and weather. As expected, the dry biomass accumulation in all transgenic lines were significantly increased compared to WT since the plant height and number of tillers are higher in transgenic lines. In line with grains yield, the highest biomass accumulation was found in T9 transgenic lines, reach 198.86 g per clump, while in WT rice reached only 131.2 g (Table 2). This biomass accumulation will reach 31.82 ton per Ha in transgenic line, increased 1.52-fold compared to WT that only accumulated the biomass 20.98 ton per Ha.

*The relation between growth and productivity*

Grains yield and biomass accumulation are the complex trait and highly dependent on the agronomical characters such as plant height and tiller number. The rice grains yield was significantly positive correlated with the tiller number and plant height (Table 3). Furthermore, rice biomass accumulation was also significantly with tiller number and plant height. The grains yield as well biomass accumulation also have positive significantly correlated with number of panicles, number of seeds per panicle and percentage of filled grain, although they were negatively corelated with 1000 grain weight (Table 3).

Table 3. The correlation coefficients between the rice grain yield and biomass accumulation with tiller number, plant height, panicle number, percentage of filled grain and 1000 grain weight.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Characters** | **Tiller number**  | **Plant height**  | **Panicle number**  | **Grain number per panicle** | **Percent filled grains**  | **1000 grains weight**  | **Grains weight per plant**  | **Biomass weight per plant**  |
| Tiller number  | 1,00  |   |   |   |   |   |   |   |
| Plant height  | 0,38  | 1,00  |   |   |   |   |   |   |
| Panicle number  | 0,71 \* | 0,03  | 1,00  |   |   |   |   |   |
| Grains number per panicle  | 0,30  | 0,83 \* | 0,08  | 1,00  |   |   |   |   |
| Percentage of filled grains  | 0,52 \* | 0,69 \* | 0,47 \* | 0,83 \* | 1,00  |   |   |   |
| 1000 grains weight  | -0,21  | -0,64 \* | -0,22  | -0,60 \* | -0,56 \* | 1,00  |   |   |
| Grains weight per plant  | 0,69 \* | 0,72 \* | 0,56 \* | 0,79 \* | 0,95 \* | -0,60 \* | 1,00  |   |
| Dry biomass per plant  | 0,72 \* | 0,65 \* | 0,61 \* | 0,75 \* | 0,93 \* | -0,61 \* | 0,98 \* | 1,00  |

**Discussion**

The characterization and evaluation of transgenic rice overexpressing sugarcane *SoSPS1* gene revealed that the growth and productivity were significantly increased compared to the WT counterpart. These results confirmed previous finding that overexpression of *SoSPS1* gene enhanced SPS activity, growth and productivity in sugarcane (Anur et al., 2020) and rice (Mulyatama et al., 2022). In this study, the transgenic rice lines were grown in biosafety greenhouse for determination of their growth and productivity. The biosafety greenhouse was used to avoid or prevent unintentional transmission of the recombinant DNA or hereditary materials to environments. The results showed that plant height and tiller number were significantly higher in transgenic lines compared to WT rice (Ciherang variety) (Table 1). A higher plant height and tiller number were significantly positively correlated with rice productivity (Table 2 and 3). Similarly, overexpression of maize *SPS* gene increased tuber weight and total yield in transgenic potato (Ishimaru et al., 2004). On the other hand, the transgenic rice lines required longer flowering time compared to WT rice (Fig 2.), which might be because of different genetic elements in the plants (Cho et al., 2017; Shim & Jang, 2020). Collectively, these results proved that growth and productivity were increased in transgenic rice overexpressing *SoSPS1* gene and that the productivity estimate to reach 16.95 ton grain yield per Ha.

In higher plants, sucrose is the major product of photosynthesis and translocated to sink tissue through the phloem. The grains filling is determined by a complex sink-source balance and affected by sugar translocation (Chen et al., 2019).The sucrose is transported long distance through phloem specific transporter and increasing the sucrose transporter led to increasing grain yield in transgenic rice (Wang et al., 2015). The sucrose is hydrolyzed by sucrose synthase (SuSy) to form ADP glucose and fructose which is substrate for biosynthesis of starch in seed (Lim et al., 2006). Overexpression of SuSy has enhanced grains yield and weight of transgenic rice in the field experiment (Fan et al., 2019). Therefore, enhanced sucrose synthesis increases grains yield in transgenic rice. A poor percentage of filled grain in WT rice reduced grains yield (Table 2), might be caused by lower of sucrose content and consequently lower starch content and grains yield (Jiang et al., 2021).

Increasing SPS activity modulates sucrose content and cleavage to release the substrate for cellulose synthesis (Anur et al., 2020). The increasing SPS activity has greater impact on cellulose synthesis and improved fiber quality in transgenic cotton (Haigler et al., 2007). Enhanced Susy activity provides ADP glucose and improves cell wall cellulose synthesis in transgenic rice (Fan et al., 2019). Furthermore, overexpression of *SoSPS* gene from sugarcane has been reported to improve biomass yield in transgenic *Brachypodium distachyon* (Falter & Voigt, 2016). In line with the reports, overexpression of *SoSPS1* gene increased biomass accumulation in transgenic rice (Table 2).

Rice is one of the major staple food crops over the world, including Indonesia. The increasing human population has an impact on increased food demand and the world population will continue to grow which demands to increase in food production. The availability of rice cultivars with higher productivity will ensure the rice self-sufficiency in the future. Thus, utilization of the transgenic rice that estimated to reach 1.5-fold increasing in rice production is really appropriate for the purpose of the rice self-sufficient. In addition, the increasing biomass accumulation is an important trait for lignocellulose feed stock and its utilization in biomass fermentation for ethanol production.

**Conclusions**

In this study, characterization and evaluation of transgenic indica rice overexpressing *SoSPS1* gene was conducted in greenhouse trials. The plant height and tiller number were significantly higher in the transgenic lines compared to WT rice. Furthermore, number of panicles, percentage of filled grain and grains number per panicle were significantly also increased in the transgenic lines. The grain yield and biomass accumulation were significantly increased in the transgenic lines and positively corelate with growth tiller number, plant height, grin number per panicle, but negatively corelate with 1000 grain weight.

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