QTLs for Shoot Length and Chlorophyll Content of Rice Seedlings Grown under Low-Temperature Conditions, using a Cross between Indica and Japonica Cultivars

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Abstract: Quantitative trait loci (QTLs) affecting the shoot length and chlorophyll content of seedlings grown at a low temperature (16ºC) for 4 weeks were analyzed using inbred lines derived from a cross between an indica rice cultivar, Habataki, and a japonica rice cultivar, Arroz da Terra. Culm length of the inbred lines, measured at the harvesting stage, was also analyzed. The QTLs linked to increased shoot length (qSL1) and culm length (qCL1) on the Arroz da Terra allele were detected in the same region of chromosome 1, where the gibberellin 20 oxidase-2 gene (identical to Semi Dwarf1: SD1) located. Other QTLs (qSL3 and qSL8) that were linked to increased shoot length on Arroz da Terra and Habataki alleles, but had no effects on the culm length, were detected on chromosomes 3 and 8, respectively. The alleles of qSL3 and qSL8 might improve early shoot growth without increasing the risk of lodging caused by a long culm length. A novel QTL (qCC1) linked to increased chlorophyll content of seedlings grown at low temperatures through the effect of an Arroz da Terra allele was detected on chromosome 1, but it differed in position from the QTLs for shoot length.

Key words: Chlorophyll content, Chlorosis, Culm length, Low temperature, Quantitative trait locus (QTL), Rice (Oryza sativa L.), Shoot length.

A low temperature is the main limiting factor for rice production, especially in cold temperate zones and high altitude areas. A low temperature has negative effects on rice seedlings, including reduced rate of seedling growth and increased leaf chlorosis (Mackill and Lei, 1997; Andaya and Mackill, 2003). These negative effects are particularly serious for rice sown by the direct seeding method. In direct seeding, it is important to increase the percentage of established seedlings. When seedlings reach the phase enabling normal plant growth, starting photosynthesis and taking root in soil, they are defined as established seedlings. However, a low temperature increased seedling mortality and reduced percentage of surviving seedlings which often results in poor seedling establishment (Zhao and Takahashi 1999; Ogiwara and Terashima 2001; Iwata et al., 2010). Therefore, improving tolerance to low temperatures at the early seedling stage is an important objective in rice breeding.

There are 2 major rice subspecies, indica and japonica, which differ in their tolerance to low temperatures. Of them, indica originated in the tropical area, and is considered to be more sensitive to low temperatures than japonica, which is adapted to the temperate area (Mackill and Lei, 1997; Zhao and Takahashi 1999; Ohsumi et al., 2012). Indica cultivars generally have bigger heads and are used as mother plants to improve yielding capacity (Nagata et al., 2002; Ando et al., 2008; Terao et al., 2010). However, the sensitivity to low temperatures at the seedling stages limits the utilization of indica-based cultivars in cold temperate areas. Thus, improvement of low-temperature tolerance of indica-based cultivars is required, and hence quantitative trait locus (QTL) analysis and subsequent identification of genes affecting the seedling growth under a low temperature conditions are necessary.

One important agronomic trait in the direct seeding is the long shoot length, because short shoots could not emerge from paddy water surface, which cause the seedling mortality (Peterson et al., 1978). QTLs for shoot length under a low temperature condition have been reported previously (Redona and Machkill, 1996; Zhang et al., 2005a and b; Zhou et al., 2007). Multiple QTLs are detected even in the same mapping populations. Moreover, most of the QTLs are located on different loci depending on the mapping population. These results suggest that shoot length under low temperatures is a complex trait controlled by multiple genes. Two main
traits, germination speed and rapid early growth of shoot are thought to be important to gain longer shoot length. For seed germination in cold conditions, the low temperature germinability gene, \( q_{LTG3-1} \) (Fujino et al., 2008), might be a key; this gene seems to have the potential to improve seedling growth in direct seeding, particularly when ungerminated seeds are sown on the paddy fields (Fukuda et al., 2014). However, primed or germinated seeds are often sown to improve seedling vigour in the direct seeding method (Furuhashi et al., 2010; Yamauchi 2012). In this case, the growth rate of seedlings is more important than germinability for improving seedling establishment. Additionally, a low correlation between low-temperature germinability and cold tolerance at the seedling stage has been reported (Bertin et al., 1996; Wang et al., 2011). Furthermore, the germinability rate of \( indica \) cultivars in cold conditions is not low (Miura et al., 2001; Baruah, et al., 2009; Wang et al., 2011), but the growth rate at the seedling stages is limited under low temperatures (Ohsumi et al., 2012). Therefore, identification of the genes that affect the seedling growth rate after germination at low temperatures is important for the improvement of \( indica \) cultivars. However, the genes that effectively improve low-temperature seedling growth rate have not been identified.

For the trait that affects seedling growth rate in cold conditions, chlorophyll contents of leaves are suggested to be important. Zhao and Takahashi (1999) reported that the shoot length and chlorophyll contents of leaves had the positive correlation among cultivars in direct seeding. Moreover, Takahashi and Kaimori (1978) reported that \( indica \) cultivars showed chlorosis symptoms in cold conditions and the low chlorophyll contents of leaves reduced the seedling growth rate. Previous studies have examined QTLs involved in cold-induced leaf chlorosis (Andaya and Mackill, 2003; Lan et al., 2010; Zhang, et al., 2014). The traits of cold-induced leaf chlorosis are thought to be controlled by multiple genes. Previously, 2 genes, PPR (Lan, et al., 2010) and \( NUSI \) (Kusumi et al., 2011), which control seedling chlorosis under a low temperature, have been identified. Additionally, a QTL for low-temperature seedling chlorosis, \( q_{CTS4} \), has been fine mapped (Andaya and Tai 2007). However, the positions of the QTLs for tolerance to chlorosis do not correspond to those of low-temperature seedling growth rate, and it is unclear whether the genes responsible for the cold-induced chlorosis positively affect the low-temperature seedling growth rate.

In this study, we identified QTLs that affect shoot length under low temperature conditions using backcrossed inbred lines (BILs) produced with an \( indica \) cultivar, Habataki, as the recipient parent, and a \( japonica \) cultivar, Arroz da Terra, as the donor parent. Arroz da Terra is known to have strong seedling vigor even under low temperature conditions (Ogiwara and Terashima, 2001; Iwata et al., 2010; Fukuda et al., 2014). We also measured the chlorophyll contents of leaves of BILs under low temperature conditions, to elucidate whether the QTLs for the chlorophyll contents correspond to that of shoot length. On the other hand, long length of plants at the harvesting stage increases loading resulting in decreased yield, though the long shoot length at the early seedling stage is important for seedling survival on direct seeding. Therefore, we analyzed the culm length of the BILs at the harvesting stage, and compared them with shoot length at the seedling stage, to detect the QTLs that increased only the shoot length but the culm length.

### Materials and Methods

#### 1. Plant materials

Ninety one lines of Habataki // Habataki / Arroz da Terra BILs (the BC\(_1\)F\(_4\) generation and the BC\(_1\)F\(_5\) generation) were used in the experiments. The BC\(_1\)F\(_4\) plants were developed by the single seed descent method from 91 BC\(_1\)F\(_1\) plants (Fukuda et al., 2012b). Culm length and the number of days from sowing to heading of the BC\(_1\)F\(_4\) plants were measured as follows. Parent cultivars and the BC\(_1\)F\(_4\) seeds were sterilized for 24 hr in 0.25 g L\(^{-1}\) Ipconazole (Kureha Co, Tokyo, Japan) and 0.25 g L\(^{-1}\) Cu(OH)\(_2\). Then seeds were soaked in 30°C water for 2 days to germinate, and sown in nursery boxes on 14 April in 2010. Nursery boxes were placed at 30°C in the dark for 2 days. Then seedlings were grown in a greenhouse for 34 days and seedlings were transplanted into the experimental paddy fields on the Daisen campus of the NARO Tohoku Agricultural Research Center (Daisen, Akita, Japan, 39°29’N, 140°29’E) on May 18. Heading date was recorded for each BC\(_1\)F\(_4\) plants when the first panicle emerged from the sheath of the flag leaf. For Habataki and Arroz da Terra cultivars, 10 plants per cultivar were grown, and days to heading were defined until half of the 10 plants headed. Culm length was measured on August 21 (15 – 39 days after heading), and seeds were harvested on September 10 (35 – 59 days after heading). The harvested seeds (the BC\(_1\)F\(_5\) seeds) were stored at 6°C for 19 – 21 months until they were used for the analysis of the shoot length and chlorophyll contents.

#### 2. Analysis of the shoot length and chlorophyll contents

Parental cultivars and BC\(_1\)F\(_5\) seeds were used for the analysis of shoot length and chlorophyll contents under low temperature conditions. Seeds were pre-germinated and prepared in the following manner: seeds were sterilized in 60°C water for 10 min and soaked in 30°C water for 2 days to germinate. One germinated seed was sown in each compartment (1.6 cm diameter × 2.5 cm depth with a distance of 1.9 cm between the compartments) of the seedling pots filled with commercial nursery soil (“Honens nursery soil No.1”, Honen Agri Co. Niigata, Japan) to 5
mm below the top edge of the compartments, and covered with the nursery soil to a depth of 5 mm. Seedling pots were placed at 30°C in the dark for 2 days in an incubator until the coleoptiles emerged on the surface of the soil. Then seedlings were grown in a growth chamber for 4 weeks at 16°C under a 12 hr artificial light with 19 – 50 μmol m⁻² s⁻¹ of photosynthetically active radiation and a 12 hr dark photoperiod. After 4 weeks of growth, the shoot length and chlorophyll content of the third leaves were analyzed. Experiments were replicated 3 times, and 7 plants per line were used in one replication. The mean values of the shoot length in the three replications were used for the QTL analysis. The chlorophyll content of the third leaves was analyzed as the following sentences. The value \( r \) indicates the correlation coefficient in BC₁F₄ plants.

### Results

1. **Culm length of BC₁F₄ plants at the harvesting stage**

The object of this study was to detect the QTL that increased the shoot length at seedling stage under low temperature conditions. However, increase of the culm length by the QTL increasing shoot length, would not be beneficial, because the long culm causes loading. Therefore, to detect the QTL that affect culm length, we analyzed the culm length of BC₁F₄ plants at first. Additionally, we analyzed the number of days required from sowing to heading, because the growth periods of plants were suggested to affect the culm length. The number of days from sowing to heading of the first panicles of Habataki and Arroz da Terra plants was 110 and 90 days, respectively, and from 97 to 114 days for BC₁F₄ plants (Fig. 1A). The culm lengths of Habataki and Arroz da Terra plants were 80 ± 3 cm and 62 ± 3 cm, respectively (mean values with standard deviations, \( n = 10 \)), and 59 – 120 cm for BC₁F₄ plants (Fig. 1B). The culm length of BC₁F₄ plants was not significantly correlated with the number of days from sowing to heading (Fig. 1C).

2. **Shoot length and chlorophyll content of BC₁F₄ seedlings**

After the analysis of culm length of BC₁F₄ plants, we analyzed the shoot length of BC₁F₄ seedlings under low temperature conditions, which was important for the seedling establishment. Moreover, we measured the chlorophyll contents of BC₁F₄ seedlings, and analyzed the correlation between the chlorophyll contents and the shoot length. After 4 weeks of incubation at 16°C, the third leaves of Habataki seedlings showed chlorosis symptoms (Fig. 2); the mean value of their chlorophyll content was 0.20 ± 0.23 μg mg⁻¹ fresh weight (FW) (with the standard deviation, \( n = 3 \)). The leaves of Arroz da Terra seedlings remained green, and their mean chlorophyll content was 1.45 ± 0.17 μg mg⁻¹ FW (with the standard deviation, \( n = 3 \)).

### References

- Wintermans and De Mots (1965).
- McCouch et al., 2010.
- Wang et al., 2002.
- Lander et al., 1987.
- Thomson and Henry (1995).
- Wiharto et al., 2010.
The chlorophyll content of the third leaves of BC$_2$F$_5$ seedlings was 0.03 – 0.59 µg mg$^{-1}$ FW (Fig. 3A). The mean value of the shoot length of Habataki and Arroz da Terra seedlings was 8.5 ± 2.1 cm and 18.6 ± 2.9 cm, respectively (with standard deviations, n = 21), and 7.5 – 12.7 cm for BC$_2$F$_5$ seedlings (Fig. 3B). In the BC$_2$F$_5$ seedlings, the chlorophyll content of the third leaves and shoot length were significantly and positively correlated at the 5% level (Fig. 3C).

3. QTL analysis

QTL analysis was conducted for the number of days from sowing to heading and culm length in BC$_1$F$_4$ plants. Furthermore, QTL analysis for the shoot length and chlorophyll contents in BC$_2$F$_5$ seedlings was conducted, then the chromosomal positions of detected QTLs were compared. No significant QTL related to the number of days from sowing to heading in BC$_1$F$_4$ plants was detected. One QTL (qCL1) that increased culm length of BC$_1$F$_4$ plants at the harvesting stage was detected on the Arroz da Terra allele near the marker RM1068 on chromosome 1 (Table 1 and Fig. 4). Additionally, the analysis of the shoot length of BC$_2$F$_5$ seedlings detected 3 QTLs (qSL1, qSL3 and qSL8) on chromosomes 1, 3 and 8. Among them, qSL1 and qSL3 increased shoot length via the Arroz da Terra allele, while qSL8 increased shoot length through the Habataki allele. The most effective QTL explaining the highest phenotypic variation ($R^2$) of shoot length, qSL1, was located in the same chromosomal region as qCL1, which affects culm length. One QTL (qCC), which increased chlorophyll content of BC$_2$F$_5$ seedlings, was detected near the marker RM3598 on chromosome 1.

Table 1. QTLs for culm length of BC$_1$F$_4$ plants, and shoot length and chlorophyll contents of BC$_2$F$_5$ seedlings.

| Traits          | Chr | Nearest marker | LOD  | Additive effect | $R^2$ (%) |
|-----------------|-----|----------------|------|-----------------|-----------|
| Culm length     | 1   | RM1068         | 22.23| 26.9            | 75.5      |
|                 | 1   | RM1068         | 8.78 | 0.78            | 31.8      |
| Shoot length    | 3   | RM1371         | 4.23 | 0.67            | 15.5      |
|                 | 8   | RM4997         | 3.20 | −0.48           | 10.2      |
| Chlorophyll content | 1   | RM3598         | 5.85 | 0.07            | 21.1      |

Chr. indicates the chromosome on which the QTL is located. Additive effect is the effect of substituting an Arroz da Terra allele for a Habataki allele. Positive value indicates that Arroz da Terra has the allele to increase the trait value. $R^2$ indicates variation explained by each putative QTL.
4. Relationship between culm length and shoot length

Correlation analysis between culm length of BC$_1$F$_4$ plants and shoot length of BC$_1$F$_4$ seedlings was conducted to clarify the relationship between the plant height at the harvesting stage and the seedling stage. The culm length of BC$_1$F$_4$ plants and the shoot length of BC$_1$F$_4$ seedlings were significantly and positively correlated at the 1% level (Fig. 5). We also compared the shoot length and the culm length among the genotypes for the nearest markers to LOD peaks of the QTLs, $qSL1$, $qSL3$ and $qSL8$ (Fig. 6). The shoot length and culm length were significantly higher in the lines that had the Arroz da Terra allele of $qSL1$ than those that had the Habataki allele (Fig. 6). The lines that carried the Arroz da Terra allele of $qSL3$ had longer shoots than those that carried the Habataki allele. On the other hand, the lines that carried the Arroz da Terra allele of $qSL8$ had shorter shoots than those that carried the Habataki allele. The genotypes of $qSL3$ and $qSL8$ did not show any significant differences in culm length between the BC$_1$F$_4$ lines that carried the Habataki allele and those that carried the Arroz da Terra allele.

Discussion

The most effective QTL in increasing the shoot length via the Arroz da Terra allele, $qSL1$, was detected in the same region as $qCL1$, which increased culm length (Table 1 and Fig. 4), suggesting that the $qSL1$ region of the Arroz da Terra allele increased the plant growth both at seedling and harvesting stages. The region corresponding to $qSL1$ contained genes that affect the plant height: $D61$ (Yamamuro...
Al., 2005; Yano et al., 2012). The Habataki cultivar was known as a semi-dwarf gene (Sasaki et al., 2002). OsGA20ox2 is the enzyme involved in the biosynthesis of gibberellin (Sasaki et al., 2002). Loss of the function of OsGA20ox2 is known to shorten the plant height both in the seedling and heading stages (Sasaki et al., 2002; Ashikari et al., 2005. Yano et al., 2012)

Fig. 6. Mean value of the shoot length of BC1 F4 plants in each genotype with standard deviations. The genotype of Habataki homozygous (Ha) and Arroz da Terra homozygous (Ar) in the QTLs are indicated. The genotypes of qSL1, qSL3 and qSL8 were classified by SSR markers RM1068, RM1371 and RM4997, respectively. ** and * indicate significant differences between Ha and Ar at the 1% and 5% levels, respectively, detected by a t-test.

reported to have the nonfunctional allele of OsGA20ox2, which had the 383-bp deletion in the coding region (Ashikari et al., 2005). The Arroz da Terra cultivar has the functional allele of OsGA20ox2 without the deletion (data not shown). Therefore, OsGA20ox2 is thought to be a key component of the QTLs for shoot and culm length in this study. The previous study suggested that OsGA20ox2 affected the shoot length at 25°C conditions (Yano et al., 2012). In this study, qSL1 containing OsGA20ox2 affected the shoot length at 16°C, suggesting that this region affects the shoot growth rate in the low-temperature conditions as well as in the warm conditions. Whether the other genes that affect plant height (D61, D10, THIS1, Psd1, CIGR and SPS) are linked to the QTL in this study remain unclear, and further fine mapping is necessary to resolve this issue.

Although the Arroz da Terra allele of qCL1 increased the culm length of the BC,F plants, Arroz da Terra plants had a shorter culm than Habataki plants at harvesting stage (Fig. 1B). This might be caused by the shorter growth period of the Arroz da Terra cultivar, because its heading date was 20 days earlier than that of the Habataki cultivar (Fig. 1A). On the other hand, the culm length of the BC,F plants was not significantly correlated with the number of days to heading among the BC1 F4 plants. The difference between the earliest heading date and latest heading date in the BC,F plants was 17 days. Moreover, most of the plants (79%) in the BC,F headed within a week, 101-108 days from sowing (Fig. 1A). In the conditions that the differences of the days to heading were relatively small, the other factors, such as OsGA20ox2, might strongly affect the culm length rather than the growth period.

Though the Arroz da Terra allele of qCL1 increased the culm length of the BC,F plants, Arroz da Terra plants had a shorter culm than Habataki plants at harvesting stage (Fig. 1B). This might be caused by the shorter growth period of the Arroz da Terra cultivar, because its heading date was 20 days earlier than that of the Habataki cultivar (Fig. 1A). On the other hand, the culm length of the BC,F plants was not significantly correlated with the number of days to heading among the BC,F plants. The difference between the earliest heading date and latest heading date in the BC,F plants was 17 days. Moreover, most of the plants (79%) in the BC,F headed within a week, 101-108 days from sowing (Fig. 1A). In the conditions that the differences of the days to heading were relatively small, the other factors, such as OsGA20ox2, might strongly affect the culm length rather than the growth period.

The low-temperature germinability gene, qLTG3-1, is located on chromosome 3 (Fujino et al., 2008), but localized at a different position than qSL3 (Fig. 4). In this study, seeds were previously germinated and seedling growth rate was analyzed after the germination stage. As a result, a different QTL linked to the low-temperature germinability gene was detected. Additionally, OsGA20ox1, an isoform of OsGA20ox2, known to affect the initial growth rate of the shoot (Abe et al., 2012; Yano et al., 2012), has been located on chromosome 3. However, it was
localized in a position different from that of \( qSL3 \) detected in this study (Fig. 4). On the other hand, the position corresponding to \( qSL3 \) contained several genes that caused dwarf phenotypes: OsDWARF4 (Sakamoto et al., 2006), \( LRK1 \) (Yang et al., 2013), \( d162(t) \) (Zhang et al., 2011), OsAPC6 (Kumar et al., 2010) and SSD1 (Asano et al., 2010). Although these dwarf genes shorten the culm length of rice, \( qSL3 \) did not affect the culm length. Therefore, the genes involving \( qSL3 \) might not be the same as those known dwarf genes. In previous QTL analysis, Ranawake et al. (2008) detected a QTL linked to increased shoot length through the \( japonica \) allele at the corresponding position for \( qSL3 \), using a cross between a \( japonica \) cultivar, Hyogo-Kitanishiki, and an \( indica \) cultivar, Hokuriku 142, during 11-day cold stress at 4 °C followed by a 4-day recovery period at 35°C. So far, it is not clear whether the previously reported QTLs were identical to the QTL, \( qSL3 \), we presently report. However, the increase in growth rate common in the \( japonica \) allele suggests that the QTLs describe the same locus. If the QTLs detected in this study are identical to the QTLs reported previously, the QTL might be effective not only for Habataki and Arroz da Terra, but also for lines with a wide range of genetic backgrounds. Further, our research was performed under a continuous low temperature of 16°C, suggesting that these QTLs were effective even without a warm period. Additionally, in plants grown at 20°C, Fukuda et al., (2012a) detected the QTL linked to increased root weight on the \( japonica \) allele at a region corresponding to \( qSL3 \), using lines which carries chromosomal segments of an \( indica \) cultivar, Kasalath, in a background of a \( japonica \) allele. Although this QTL has not yet been identified as \( qSL3 \), it is possible that \( qSL3 \) is responsible for both the over-ground and under-ground growth linked to the \( japonica \) allele.

No genes previously linked to shoot length were reported at the corresponding position on \( qSL8 \) of chromosome 8, suggesting that novel genes affected the shoot growth in \( qSL8 \). Zhang et al., (2005b) detected a QTL that increased the shoot length via the \( indica \) allele using a cross between a \( japonica \) cultivar, Lemont, and an \( indica \) cultivar, Teqing grown at 20°C and 25°C. Both the previously reported QTL and \( qSL8 \) detected in this study increased the shoot growth through the \( indica \) allele, supporting the hypothesis that the same locus might be responsible for these effects. As this QTL increased the shoot growth through the \( indica \) allele, it might be useful to improve the seedling vigor of \( japonica \) cultivars.

Several QTLs linked to cold damage, including leaf chlorosis, have been previously reported (Andaya and Mackill, 2003; Lan et al., 2010). However, the relationships between the QTLs for tolerance to cold damage and the shoot growth rates were not yet clear. We showed an obligate but significant positive correlation between the chlorophyll contents of the leaves and shoot length in the present study (Fig. 3C). This suggests that the higher chlorophyll contents may have contributed to the enhancement of shoot growth, possibly through photosynthetic carbon assimilation. On the other hand, the QTL linked to chlorophyll contents (\( qCC1 \)) was detected in a different region than the QTLs for shoot length (Fig. 4). This suggests that the effect of \( qCC1 \) might not be sufficiently strong to enhance the shoot growth enough to be detected in the QTL analysis. No genes or QTLs for control of seedling chlorosis under low temperatures have been reported at the corresponding position on \( qCC1 \). Therefore, the gene underlying \( qCC1 \) might be a novel gene controlling low-temperature-induced chlorosis.

Both the shoot length and chlorophyll content of BILs showed relatively low values, similar to the Habataki cultivar, and no lines reached the same level as the Arroz da Terra cultivar (Fig. 3). This suggests that the effect of an Arroz da Terra allele in the genetic background of the Habataki cultivar may not be strong enough to increase the shoot length or chlorophyll content to the same level as found in the Arroz da Terra cultivar. Pyramiding of multiple positive but weak QTLs is necessary to breed cultivars that have strong seedling vigor even under low temperatures.

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