Research Paper

Normalization method for canopy temperature as an indirect indicator of yield potential in wheat breeding programs

Shizen Ohnishi^{*1)}, Masashi Kasuya^{1,2)}, Tatsuya Sonoda¹⁾ and Hironobu Jinno^{1,3)}

¹⁾ Hokkaido Research Organization Kitami Agricultural Experiment Station, Kunneppu, Tokoro-gun, Hokkaido 099-1496, Japan

²⁾ Hokkaido Research Organization Kamikawa Agricultural Experiment Station, Pippu, Kamikawa-gun, Hokkaido 078-0311, Japan

³⁾ Hokkaido Research Organization Central Agricultural Experiment Station, Naganuma, Yubari-gun, Hokkaido 069-1395, Japan

Canopy temperature (CT) is often related to potential yield and is a possible yield indicator in breeding programs. However, it is difficult to evaluate genetic variations of CT accurately in large-scale investigations, such as breeding programs, because CT is strongly affected by environmental conditions. In this study, to precisely evaluate these genetic variations, we determined the environmental factors that affect CT measurement and proposed a convenient normalization method to minimize their influence. We measured the CT of CT-high or CT-low cultivars in the field under various conditions. We found that as the sun and shade levels were alternated, the CT changed within seconds; the position in the field also critically affected the CT. However, even under these conditions, the differences between cultivars became clearer if CT was normalized by neighboring lines. Additionally, we revealed that CT measurements between 12:00 and 15:00 maximized the difference between cultivars. Using our normalization technique under the favorable conditions specified can help breeders select high-yield lines using CT in breeding programs.

Key Words: canopy temperature, wheat, yield, breeding.

Introduction

The potential yield (yield in the absence of abiotic and biotic stresses) is one of the most important aims in wheat breeding programs. However, in the early generations of these programs, selection by yield is frustrated by the limited available space for each line, low amount of seed, poor accuracy levels, and increased labor required for evaluating the numerous lines.

When the potential yield is advanced in breeding programs, stomatal aperture-related traits such as stomatal conductance are often improved simultaneously (Fischer *et al.* 1998, Fischer and Rebetzke 2018, Morrison *et al.* 1999, Takai *et al.* 2010, Tanaka *et al.* 2010). Canopy temperature (CT) is a stomatal aperture-related trait that reflects the transpiration rate through the stomata by vaporization heat, and many studies have reported the relationships between CT and yield in wheat under various environmental conditions (Bilge *et al.* 2008, Fischer *et al.* 1998, Gautam *et al.* 2015, Karimizadeh and Mohammadi 2011, Mason and Singh 2014, Rebetzke *et al.* 2012). Some of these reports have suggested the use of CT applications or stomatal

aperture-related trait measurements in breeding programs (Condon *et al.* 2007, 2008, Ginkel *et al.* 2008).

CT is a promising approach for potential yield selection in breeding programs, as it can be conveniently and quickly measurable using a hand-held spot thermometer, which is inexpensive and straightforward to use. A thermo-camera attached to an unmanned aerial vehicle (UAV) can also be used, potentially provide a high-throughput method for determining CT (Deery *et al.* 2016, 2019). However, in large-scale experiments, measurements are affected by various factors such as environmental fluctuations, causing the heritability of large-scale measured CT to be low (Deery *et al.* 2016). This low confidence has led breeders to hesitate to adopt CT for indirect yield selection.

Additionally, in Japan, where weather is changeable and humidity is high during the crop growing season, accurate CT measurement is challenged by inconsistent solar radiation intensity due to clouds, and small differences in CT among cultivars due to low saturation deficit. Thus, precise and quick measurement of CT is key to its utilization for breeding.

A novel winter wheat cultivar 'Kitahonami' (*Triticum aestivum*, abbreviated as Kit) in Hokkaido, in the northern part of Japan, showed higher yields than the former leading variety 'Hokushin' (abbreviated as Hok) by 18% on average (Yanagisawa *et al.* 2000, 2007). It is possible that stomatal aperture-related traits are improved in Kit compared

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*Corresponding author (e-mail: ohnishi-shizen@hro.or.jp)

to Hok, but no known studies have assessed the CT of Japanese wheat cultivars having different genetic backgrounds and environmental growing conditions than those of the main wheat production areas in the world.

In this study, by measuring CT with a hand-held spot thermometer, we first confirmed the CT variations between the Kit and Hok cultivars, which are dramatically different in potential yield. Second, we revealed that microclimate changes and location in the field potentially affect CT measurements. Finally, we proposed a powerful normalization method for large-scale CT measurements in breeding programs and determined favorable survey conditions.

Materials and Methods

Plant material and field management

We conducted all field tests at Kitami Agricultural Experiment Station (Kitami AES) in Hokkaido, Japan (43.75° N, 143.72° E), under a warm temperature, moderate radiation, andosol soil, and rain-fed condition. Seasonal total precipitation from April to July is approximately 255 mm on average, and there is no water stress without irrigation.

Hok and Kit are soft red winter wheat used for Japanese noodles, which are bred for Hokkaido, in the northern part of Japan. Kit was first bred in 2006 (Yanagisawa *et al.* 2007) and is the current leading variety in Hokkaido. Hok was the previous predominant cultivar released in 1995 (Yanagisawa *et al.* 2000).

Populations KC-5098 (23030/K-87) and KC-5156 (K-87/23132) were developed in the wheat breeding program of the Kitami AES. In the 2011 sowing season, 23030 (with a high amount of aboveground biomass) and K-87 (semi-prostrate, elected, with dark green leaves) were crossed and the progenies were maintained as KC-5098, and in the 2012 season, K-87(described previously) and 23132 (with an extremely elected leaf) were crossed and the progenies were maintained as KC-5156, for breeding high-yield lines. The yield of 23030 and K-87 was higher than that of Hok but lower than Kit, whereas the yield of 23132 matched that of Kit. There is no information regarding the CT of the parents when the crosses were performed, but a subsequent observation revealed that the CT of 23030, K-87, and 23132 was lower than that of Hok but higher than Kit. After F_1 plants were harvested, both crosses were maintained as populations (1400 plants per population) without selection before spike-progeny lines were developed. In the 2015 sowing season, 154 spike-progeny lines derived from F_4 (KC-5098) and 152 lines derived from F_3 (KC-5156) were sown at the breeder's early-generation line selection plot (60 cm single rows of 1.5 m long) accompanied by Kit, Hok and the parental lines as controls. This experiment was entitled L1, and its overview is shown in Fig. 1 and Table 1. Forty-three lines for KC-5098 and 49 lines for KC-5156 were re-sown 2016 (L2), with generally the same design as L1 (Table 1). L3

Fig. 1. Schematic arrangement of line selection field plots (A) and CT distribution over the field, on June 28 of experiment L1 (B). Sibling lines derived from crosses KC-5156 and KC-5098 were placed in blocks I to IV, and V to VIII respectively (A). Among sibling lines, ‘standard block’ was integrated which consists of Kit, Hok, K-87 and a parent (A). Color map was generated from CT of plot. The number in each plot directs plot number (A) and CT of the plot (B). Dash in plot means missing data due to too poor growth (B).

consisted of Kit and Hok alternately sown 10 times in the 2016 season (Table 1), in order to investigate the effect of time of day.

We performed yield trials (Table 1) with a 5.4 m² plot consisting of six 4.5 m rows with 1.2 m widths and 0.2 m inter-row spacing (Y1 and Y2). Trial Y1 consisted of only Kit and Hok, with three replications in the 2014 to 2016 sowing season. Trial Y2 consisted of Kit and the elite breeding lines which bred at the Kitami AES for white salted noodles (three replicates). Nine elite breeding lines, K1996, K2003, K2004, K2005, K2006, K2007, K2008, K2009, and K2010, with various genetic backgrounds were selected based on the results of a one- or two-year yield trial, as well as resistance to yellow mosaic and Fusarium head blight, flour yield, and flour color.

Both line selection and yield trial plots were sown in mid to late September, and grains were harvested after maturity in August. Weeds and diseases were controlled by

Table 1. Schematic review of field experiments

Experiment	Plot type	Plot size ^a	Sowing year	Details
Y1	Yield trail	20 cm, 6, 4.5 m	2014 to 2016	Only Kit and Hok are sown
Y2	Yield trail	20 cm, 6, 4.5 m	2019	Kit and 9 elite breeding lines
L1	Line selection	60 cm, 1, 1.5 m	2015	Represented in Fig. 1
L2	Line selection	60 cm, 1, 1.5 m	2016	Generally the same as L1
L3	Line selection	60 cm, 1, 1.5 m	2016	Only Kit and Hok are sown alternately

^a raw width (cm), raw number per plot, plot length (m).

herbicides and pesticides, respectively. Starter fertilizer was applied to both line selection (50 kg/ha N, 90 kg/ha P₂O₅, and 60 kg K₂O) and yield trial plots (57 kg/ha N, 175 kg/ha P₂O₅, and 70 kg K₂O), and additional nitrogen was introduced for yield trials in April (50 kg/ha N) and pre-boot (50 kg/ha N), which achieved nearly maximum yield (8 t/ha) without lodging.

CT measurement

We measured CT with a hand-held spot thermometer (KEW-5515; KYORITSU, Tokyo, Japan) in the average mode, which provides the average of multiple measurements (0.5-second intervals) while triggering the switch. The thermometer was held at 20 degrees below the horizon and 15 cm above the canopy, where we scanned 50 cm along the row over approximately 8 seconds, avoiding the beginning edge of the plot (approximately 30 cm). We obtained approximately 16 measurements (8 seconds of measurements at 0.5-second intervals in average mode) within the 50 cm length by 8 cm diameter area (**Supplemental Fig. 1**). In all experiments, CT was measured at two different stages: pre-boot (approximately 7 days before boot to just before boot) and grain filling (post-anthesis to just prior to the start of leaf senescence). At the beginning of each measurement, except for the measurements performed during the 2014 sowing season, the temperature, relative humidity, cloudiness level, and wind velocity were recorded. For experiment Y1, CT was measured 12 times (approximately every 4 days) for both pre-boot and grain-filling for each sowing season, and averaged at each growing stage for each season. In experiments Y2, L1, and L2, CT was measured 17, 2, and 6 times (at 4, 1, and 3 days, respectively) at pre-boot, and 21, 14, and 13 times (at 3, 7, and 6 days, respectively) at grain filling, and averaged at both stages. For all four experiments, the measurements were taken between 13:00 and 15:00. For experiment L3, 6 to 10 measurements were conducted a day between 9:00 and 16:00 for 4 days at grain-filling, in order to investigate the effect of time of day. In experiment Y2, we measured CT with a thermography camera in addition to the hand-held spot thermometer in order to compare the results. In this experiment, CT was measured 3 times (at 3 days) at grain filling, and then averaged. The thermography camera (ZemuseXT2, FLIR Systems, Wilsonville, USA) was attached to an unmanned aerial vehicle (Matrice 200 V2,

SZ DJI Technology, Shenzhen, China), and thermal images were taken vertically with a thermal image resolution 2.5 cm/pix. Using ImageJ software (<https://imagej.nih.gov/ij/>), the average temperature of each plot was calculated from the thermal images.

To determine CT temporal changes, we measured the CT sequentially from the first to the last plot of Y1 in the 2014 sowing season on May 30 (pre-boot stage). After recording data from the last plot, we returned to the first plot and re-measured. While the measurements were repeated, the degree of sunshine was monitored. The time before the first measurement and the time after the last were recorded for each cycle, and the time course was estimated by dividing the total duration by the number of measurements repeated. The measurement was performed on a cloudy day beginning at 11:00 when the sun emerged from the clouds. While measuring CT, we recorded the timing of the sun blockage by the clouds and its re-emergence by visual observation.

Analysis

For Kit and Hok, broad-sense heritability was calculated by $[V_{KH} - (V_K + V_H)/2]/V_{KH}$, where V_{KH} is the variance of Kit and Hok, and V_K and V_H are the variances within Kit and Hok, respectively (Mori 2000). For the breeding population, broad-sense heritability was calculated by $[V_{KC} - (V_K + V_H + V_P)/3]/V_{KC}$, where V_{KC} is the variance among sibling lines derived from KC-5098 or KC-5156, V_K , V_H , and V_P are the variances within Kit, Hok, and K-87, respectively. If heritability was below zero, it was regarded as zero. Statistical analysis was performed using Excel 2019 (Microsoft, Redmond, WA, USA).

Normalization of CT

In this study, we used two normalization methods. The first was ‘subtraction from the specific line’, where the normalized CT is calculated as a deduction from a specific standard line that is placed among the tested lines at a certain frequency, specifically [CT of target] – [CT of standard cultivar] (**Fig. 2A**). In this experiment, a breeding line (K-87) was chosen as a standard and placed in ‘standard block’ with Kit and Hok (**Figs. 1A, 2C**). The second normalization is ‘subtraction from the average of the neighboring lines.’ In this approach, normalized CT is calculated by subtracting the average of neighboring lines (6, 10, and 14 lines in this study) from the normalization target, namely

Fig. 2. Schematic image of CT normalization methods CT subtraction from the specific standard line (A) and CT subtraction from the average of neighboring lines (B). Single square shows single plot (0.6 m width and 1.4 m length). Solid plots mean target lines for normalization, gray painted plots show standard or neighboring lines used for normalization. Plots without text indicate sibling lines derived from cross KC-5156 or KC-5098 (Fig. 1). For Kit and Hok, neighboring lines always contain specific lines (Kit, Hok, K-87, and parent) as those line are placed in standard blocks (C). Therefore, for subtraction from the average of neighboring lines for Kit and Hok, neighboring lines are shifted outside of the standard block (C). Arrow head indicates measuring order of plots. At the normalization by CT subtraction from standard line (A), K-87 is used as the standard in this experiment. In this case, the distance between target and standard was ranged from 1 (next to target) to 3 plots for Kit and Hok because Kit, Hok, and K-87 are all included in the standard block (Fig. 1).

[CT of target] – [average of neighboring lines around the target] (Fig. 2B, 2C); neighboring lines are genetically dif-

ferent depending on the target. However, they are sibling lines in a population in breeding programs, and the average of neighboring lines is expected to be a considerable value.

Results

Difference of CT between Kit and Hok

As mentioned in the introduction, the current leading variety Kitahonami (Kit) yields 18% more than the former, Hokushin (Hok). We compared the CT of Kit and Hok (Table 1, Y1), and as is shown in Table 2, Kit had a consistently lower value than that of Hok by 0.5°C, independent of year and plant growth stage (pre-boot and grain-filling). Notably, the CT of Kit was lower than Hok even when the yield of Kit did not exceed that of its counterpart in the 2016 sowing season (Table 2).

Factors affecting accurate measurement of CT in large scale examination

Measurement of CT is affected by various environmental factors, and we attempted to reveal these factors using Kit and Hok, which have clearly different CTs. First, we estimated the effect of sunshine by examining CT changes per minute in experiment Y1 (Table 1). As shown in Fig. 3, we observed an immediate reduction in CT when the sun was shaded, and a quick elevation after the radiation was recovered. The changes in CT were approximately 3.0°C, which was substantial considering the difference between cultivars was only about 0.5°C (Table 2, Fig. 3). However, interestingly, while CT increased or decreased according to the changes in sunlight, the CT difference between Kit and Hok was mostly conserved (Fig. 3).

Second, in order to identify problems that occur in large-scale measurements, we investigated the CT of large-scale line selection plots in 2015 (L1 in Table 1, Fig. 1A). We measured the CT of each plot in order of plot number starting from plot number 1 (Fig. 1A). In this experiment, we found that the position of the plot in the field strongly affects CT. As shown in Fig. 1B, CT tended to be higher in the western part of the field and lower in the east. Although

Table 2. Difference of CT between cultivars at experiment Y1

Sowing year	Cultivar	Maturity	Yield (t/ha) [Percentage of Hok]	CT (°C) [Difference from Hok]	
				Pre-boot	Grain-filling
2014	Kit	Jul. 25	9.50 [108]	20.3 [−0.56]	22.1 [−0.46]
	Hok	Jul. 23	8.77	20.9	22.6
	p-value ^a		0.00**	0.04*	0.12
2015	Kit	Jul. 28	8.29 [109]	21.7 [−0.57]	22.8 [−0.47]
	Hok	Jul. 25	7.63	22.2	23.3
	p-value ^a		0.12	0.00**	0.02*
2016	Kit	Jul. 24	6.97 [99]	21.9 [−0.65]	22.3 [−0.51]
	Hok	Jul. 22	7.07	22.5	22.8
	p-value ^a		0.46	0.01**	0.00**

^a p-value of t-test between Kit and Hok.

Fig. 3. Chronological change of CT depending on a sun shaded condition. The measurement began when the sun emerged from the clouds and the timings of the sun blockage by clouds and re-emergence is recorded (the vertical bars at the beginning and ending of the sun shaded period). Open square and solid diamond direct CT of Kit and Hok respectively. Vertical bars for each plot represent standard error of the mean ($n=3$). Though the difference between cultivars is held over time, CT changed dramatically depending on the sun/shade condition and time course.

the distribution of CT was somewhat altered in relation to the actual day of measurement, the general trend was observed throughout the majority of the season. In the next season, 2016, the CT of line selection plots in another field was measured (L2 in Table 1), and the opposite variation was confirmed; CT was higher on the east side in this field

(data not shown). The position effect of CT could be estimated up to 4–6°C (Fig. 1B), which was considerably larger than the genetic differences of CT, at approximately 0.5°C (Table 2).

Normalization method for CT in the large scale

As a model for large-scale CT measurement for breeding, we investigated the CT of Kit and Hok embedded in line selection plots L1 (Fig. 1A). In this examination, in addition to Kit and Hok, we measured the other lines planted in the field, in order of plot number (Fig. 1A). The large number of measurements returned CT difference, on average between Kit and Hok (Fig. 4A); however, the CT of both cultivars varied widely and often overlapped (Fig. 4A). The position in the field (indicated in Fig. 1B) is likely responsible for the variations, and the temporal changes in the environment (Fig. 3) could have widened these, considering the measurements occurred over approximately 90 min.

As discussed above, when measuring CT in a large number of plots, the removal of environmental variations is essential. Therefore, we used two normalization methods that are described in the Materials and Methods section. We normalized the CT of experiment L1 at grain filling using these two methods. The wide variations were reduced as shown in Fig. 4B, by subtraction from standard ([CT of target] – [CT of standard cultivar]) and were dramatically reduced as described in Fig. 4C, by subtraction from neighboring plots ([CT of target] – [average of neighboring lines around the target]). Additionally, if the number of neighboring lines used for the average was changed, the normalization effects did not change significantly (Fig. 4C–4E).

Further evaluations of the normalization method were

Fig. 4. Differences of CT between Kit and Hok with various normalization approaches. Single dot means single plot. Measured CTs during grain filling in experiment L1 are used. Horizontal bars represent CT average of each cultivar. Raw data of CT (A), subtraction from a standard line (B), and subtraction from average of neighboring lines (C, D, and E). The number of neighboring lines is represented with ‘ n ’ (C, D, and E). The p -value of Student’s t -test was calculated.

Table 3. Difference of CT heritability^a among normalization methods for Kit and Hok

Experiment	Stage	CT raw data	CT subtraction from standard line	CT subtraction from average of neighboring lines		
				n = 6 ^b	n = 10 ^b	n = 14 ^b
L1	Pre-boot	0.00	0.00	0.06	0.00	0.00
	Grain-filling	0.00	0.47	0.74	0.73	0.62
L2	Pre-boot	0.00	0.22	0.27	0.30	0.35
	Grain-filling	0.04	0.18	0.33	0.31	0.23

^a Broad sense heritability calculated by $[V_{KH} - (V_K + V_H)/2]/V_{KH}$. V_{KH} : variance of Kit and Hok, V_K , and V_H : variance within Kit and Hok, respectively.^b Number of neighboring lines for average calculation.**Table 4.** Difference of CT heritability^a between normalization methods for breeding populations in L1

Cross	Stage	CT raw data	CT subtraction from average of neighboring lines		
			n = 6 ^b	n = 10 ^b	n = 14 ^b
KC-5098	Pre-boot	0.00	0.45	0.20	0.05
	Grain-filling	0.00	0.55	0.47	0.48
KC-5156	Pre-boot	0.00	0.50	0.34	0.27
	Grain-filling	0.00	0.71	0.65	0.68

^a Broad sense heritability calculated by $[V_{KC} - (V_K + V_H + V_P)/3]/V_{KC}$. V_{KC} : variance among sibling lines derived from KC-5098 or KC-5156, V_K , V_H , and V_P : variance within Kit, Hok, and K-87, respectively.^b Number of neighboring lines for average calculation.

performed with broad-sense heritability. **Table 3** shows the broad-sense heritability of the CT of Kit and Hok using two normalization methods. Except for the 2015 season pre-boot, in which no genetic difference was observed between Kit and Hok, normalization by ‘subtraction from average of neighboring lines’ produced larger heritability than normalization by ‘subtraction from standard’. Additionally, when the normalization method was applied to sibling lines derived from breeding populations, the normalization method also improved heritability (**Table 4**).

Favorable conditions for CT measurement

To use CT in a breeding program, it is important to know the favorable conditions for measurement. First, we investigated the effect of the time of day. In this experiment, we measured the CT of experiment L3 (**Table 1**), in which Kit and Hok were alternately sown. We repeatedly measured CT during various periods of the day, and the data was evaluated using the broad-sense heritability of Kit and Hok. The experimental conditions are described in detail in **Supplemental Table 1**. Heritability was high, at more than 0.3, between 12:00 to 15:00, and less than 0.3 between 9:00 and 12:00 (**Fig. 5A**) and after 15:00. These observations indicate that CT measurements between 12:00 and 15:00 are suitable for detecting genetic differences in CT.

Second, we evaluated the effects of other environmental conditions. In this experiment, the time of measurement was fixed between 13:00 and 15:00, and the CT of Kit and Hok was measured on various days in which air temperature, relative humidity, cloudiness level, and wind velocity were different (**Supplemental Table 1**). Although none of the conditions were significantly correlated with heritability

Fig. 5. Broad sense heritability of CT with Kit and Hok at various measuring conditions. Measured CT at various times of the day over 4 days at field test L3 in **Table 1** (A). Measured CT at various weather conditions of experiment L2 in **Table 1**, CT was measured at 13:00–15:00 on 9 days (B). Correlation coefficient and p-value are shown (B).

(data not shown), cloudiness exhibited a negative correlation ($r = -0.39$, $p = 0.11$) (**Fig. 5B**).

Relationship between CT and yield among breeding lines

As described above, we conducted all experiments with two cultivar Kit (high yield and low CT) and Hok (medium yield and medium CT). However, it is unclear whether the relationship between yield and CT will be applicable to other breeding lines at the Kitami AES. Most researchers studying CT use thermography for measurements, which can capture the entire canopy at once. However, we adopted an inexpensive hand-held spot thermometer for

Fig. 6. Correlations between CT and yield at field trial Y2. Correlation between yield and CT by the hand-held spot thermometer measured at pre-boot (A), and grain-filling (B). Correlation between CT measured with hand-held spot thermometer and CT with thermography (C). CT is an average of 4 days of pre-boot (A), and 3 days of grain-filling (B, C). Date and time when CT were measured are different between (B) and (C). Each correlation coefficient and p-value of test for no correlation is shown (A, B, C).

breeding, which captures a specific part of the canopy (deduced to be 8 cm wide and 50 cm long in our measuring conditions, [Supplemental Fig. 1](#)). Therefore, we measured the CT of yield trials of elite breeding lines (Y2 in [Table 1](#)) in 2018 at the Kitami AES using both the hand-held spot thermometer and thermography. Correlations between CT and yield were observed (CT at pre-boot [Fig. 6A](#) and CT at grain-filling [Fig. 6B](#)) with the hand-held spot thermometer. Additionally, we confirmed the correlation between CT measured with the hand-held spot thermometer to that with thermography ([Fig. 6C](#)).

Discussion

We confirmed that the CT of Kit was lower than that of Hok, corresponding to differences in yield. Additionally, such a correlation between CT and yield was confirmed among elite breeding lines bred at the Kitami AES for white salted noodle use. However, in large-scale measurements, such as in breeding programs, we revealed that CT is strongly affected by environmental factors, specifically changes in weather conditions and the position in the field ([Figs. 1B, 3, 4](#)). Accordingly, we proposed a normalization method for large-scale CT measurement using the standard lines, which are integrated among plots of breeding lines at appropriate intervals. We confirmed that such normalization was moderately successful if the standard lines were close to the normalized plot ([Fig. 4B](#)). However, judging from the distribution of CT in the field ([Fig. 1B](#)), frequent insertion of standard lines is necessary, requiring significant labor for seed preparation and field spacing.

The alternative normalization method we proposed is the subtraction of the average of neighboring lines. This normalization approach appears unreasonable since normalizations were made by genetically different lines depending on the target plot; however, it efficiently removes environmental effects and clarifies the genetic differences of CT ([Fig. 4C–4E](#), [Tables 3, 4](#)). The omission of integration of additional standard lines is an advantage of this normaliza-

tion in breeding programs. This method could be promising for selecting better lines within a breeding population, since normalizations are made from sibling lines that are expected to express the same value for CT. However, in this normalization method, two lines from different populations that would be normalized by different sibling lines should be more carefully compared, as they would have different expected values of CT.

Prashar *et al.* (2013) measured the CT of a potato breeding plot using thermal images. They tested normalization methods and concluded that the subtraction from the average of all plots in a single thermal image was superior. There were differences in the measuring instrument and distribution of plots used for normalization in this experiment, but the same concept was identified as our recommended method.

If the normalization method is employed, the number of plots used for is a concern. Our experiment showed stable normalization effectiveness independent of the number of plots ([Fig. 4C–4E](#), [Table 3](#)). However, normalization with 6 plots achieved stronger normalization than 10 or 14 plots ([Table 4](#), partially in [Table 3](#)). Using fewer plots has the advantage that there is the ability to measure closer plots, which are expected to be more similar in condition; however, utilizing a smaller number of plots creates an increased risk of skewing the average by including an abnormal value. Considering these and our experimental results, normalizations by 6–10 plots appear to be appropriate.

We detected a strong effect of the position in the field on CT. Namely, the CT gradually decreased depending on cardinal direction in the field. We observed this phenomenon in two seasons and in two different fields. In both cases, such tendencies were observed throughout the season. In the 2015 sowing season, CT was higher on the west side ([Fig. 1B](#)), and in 2016 it was higher on the east side (data not shown). These observations show that the position of the sun is not related to this tendency. A possible cause is the ground-water level, as water stress at the lower ground-

water side could increase CT, although such graduations were not generally observed directly after rainfall (data not shown).

Our results showed that CT measurements between 12:00 to 15:00 are suitable for our experimental conditions (genetic and environmental background) in terms of heritability. Deery *et al.* (2019) also reported that heritability is high between 11:00 and 15:00 for other genetic and environmental backgrounds. Based on these observations, CT should be measured from approximately 12:00 to 15:00 for selection of low CT lines.

In our experiment, we could not determine the critical conditions other than time of day. However, as shown in **Fig. 5B**, cloudiness degree showed a negative correlation with heritability ($r = -0.39$, $p = 0.11$, $n = 18$). Since high solar irradiance induces stomatal opening, promotes transpiration, and results in decrease of CT, therefore, sunny condition may be favorable to evaluate the genetic variation precisely. Further physiological experiments are needed to elucidate this hypothesis, because we could not discuss the direct effect of CT on photosynthesis through this experiment.

We monitored CT at two separate stages, pre-boot and grain-filling (**Tables 3, 4, Fig. 6**). Condon *et al.* (2008) reported that the contribution to yield could differ between CT at pre-boot and grain-filling under multiple regression analysis. In our experiment, only a single case of CT measured at pre-boot and grain-filling correlated equally with yield (**Fig. 6**). However, in experiment L1 in **Table 3**, the heritability of CT at pre-boot was close to 0, in contrast to grain-filling. Such observations imply that CT at the two stages should be dealt with separately. The CT-measured stage (or both) being adopted as an indirect indicator of yield is an interesting and important issue for more extensive studies in the future.

We revealed CT differences between cultivars and elite breeding lines, corresponding to differences in yield (**Table 2, Fig. 6A, 6B**). We also presented a suitable measurement condition and normalization method for measuring CT. Our results could entice more breeders to use CT for breeding potential yield in wheat and other crop breeding programs.

Author Contribution Statement

SO and HJ designed the experiments. SO, MK, and TS performed the experiments. SO analyzed the data and wrote the manuscript.

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