Physiological and Metabolic Studies of Temperature Effects on the Juvenile Growth of the Sheath Blight-Resistant Rice Genotype 32R

（イネ紋枯病抵抗性系統32Rの初期生育における温度反応に関する生理・代謝特性研究）

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（イネ紋枯病抵抗性32R系統の初期生育における低温感受性に関する生理・代謝特性研究）

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CHAPTER 1

General Introduction

Rice is the staple food for more than half of the world's population and is the world's second most important cereal crop belongs to the grass family (Poaceae) of the plant kingdom (Gnanamanickam, 2009). Cultivated rice includes two species, *Oryza sativa* (Asian rice) and *Oryza glaberrima* (African rice). African rice is cultivated in West Africa, whereas Asian rice is grown worldwide. *Oryza sativa* includes two subspecies: the sticky, short grained *japonica* and the non-sticky, long-grained *indica*. *Japonica* is usually cultivated in temperate zones of East Asia, upland areas of Southeast Asia and high elevations in South Asia, while *indica* is mainly cultivated in lowland, mostly submerged in tropical Asia (http://ricepedia.org). Global rice production in 2015 is 742.6 million tons and is lower than 0.4% in comparison with that in 2014 (http://www.fao.org). In addition, global food demand is estimated to rise up to 110% by 2050 (de Souza *et al*., 2015),

particularly because of world's population growth that will reach 9 billion people (Alexandratos and Bruinsma, 2012). Therefore, the food security is a challenging problem for the world. To meet increasing global food demand and consumption, rice production must be enhanced. To increase rice production, high-yielding cultivars have been introduced in many countries. However, rice is subject to many diseases that often place major biological limitations on its production. Of these, rice sheath blight disease (ShB) is one of the most prevalent causing great damages to rice yield and quality worldwide (Lee and Rush, 1983).

Rice sheath blight disease, caused by *Rhizoctonia solani* Kuhn, is a serious fungus that exists primarily as vegetative mycelium and sclerotia in plant debris, floats to the surface of floodwater, germinates and infects the rice plants (Zheng *et al*., 2013). *R. solani* was first reported in Japan in 1910. This disease causes yield losses up to 50% when all the leaf sheaths and leaf blades of susceptible cultivars are infected. After initial infection on lower leaf sheaths, mycelia move up the plant by hyphae and develop new infection structures and bigger lesions over the entire plant (Lee and Rush, 1983). There are many *indica* resistant cultivars to ShB, such as Tetep, Tadukan, Teqing, Saza, Marsi, Tauli, Brimful, Jasmine 85, ZYQ8, Minghui 63, LSBR-5 and LSBR-33, are against *R. solani* (Groth and Nowick, 1992; Li *et al*., 2003; Sato *et al*., 2004; Wasano, 1988). Of these, Tetep, a primitive cultivar from Vietnam, offers excellent protection against the ShB pathogen and

reduces infection area by extracting the oxidized phenolic compound (Groth and Nowick, 1992; Wasano *et al*., 1983). Wasano *et al*. (1985) conducted the crossing between Tetep and CN_4-4-2 (*japonica*), developing from Chugoku 45 and Nipponbare (Nb) and selected two genotypes, 32R and 29S. Wasano and Hirota (1986) reported that 32R showed more resistance to ShB than the parent - Tetep and 29S showed more susceptibility than Nb. In addition, Gaihre and Nose (2013) reported that the yield of the F_1 generation (by crossing 32R and Nb, 12.5 MT/ha) is greater than the parents, while the yield of 32R (7.9 MT/ha) is lower than that of Nb (9.6 MT/ha) and 29S (8.8 MT/ha). Another factors that affect the development of *R. solani* is high temperature (28-32°C) (Lee and Rush, 1983).

Climate change is a challenge to food and agriculture systems. It fronts an original threat to global food security and sustainable development (http://www.fao.org). The ancient Greeks (370–286 B.C.) documented that cereals cultivated at higher altitudes had lower disease occurrence than cereals cultivated at lower altitudes, but recently it was reported that the environment can affect the host plant growth and susceptibility; pathogen reproduction, scattering, survival and activity; as well as host-pathogen interaction (Ghini *et al*., 2008). The major climate change factors probably to affect plant disease as follows: increased atmospheric CO_2, heavy and unseasonal rains, increased humidity, drought, cyclones and hurricanes and warmer winter temperatures (Luck *et al*., 2011). One (or all) of these

climate factors change can affect the distribution and biology of plant pathogens with positive or negative effects (Fuhrer, 2003). As an example, temperature is one of climate factors that has risen by about 1.0°C over the last century in Japan, especially average winter temperature in Hokkaido have increased by 1.3°C over the last century (Case and Tidwell, 2014; Cruz *et al*., 2007). The rising temperature is leading to an increase in the number of hot days (days with maximum temperatures higher than 35°C) and less extreme cold days in Japan (Case and Tidwell, 2014). The Intergovernmental Panel on Climate Change (IPCC) reported that rice production will be decreased up to 40% in irrigated lowland areas of central and southern Japan (Cruz *et al*., 2007), and rising temperature can also provide better growth condition for disease (Case and Tidwell, 2014). In addition, temperature has been known to affect the plant performance (Nagai and Makino, 2009). Recently, it was reported that the ShB is expanding in the northern Japan, thus the increased risks of diseases, particularly ShB, to rice plants should be considered in the forecasting the effects of climate change on rice yield (Kobayashi *et al*., 2006).

The growth and development of plants are affected by temperature surrounding the plant (Hatfield and Prueger, 2015). Grime and Hunt (1975) showed that growth of species under unfavorable conditions tends to have low relative growth rate (RGR: increase in dry weight per unit biomass present and per unit of time), whereas growth of species under favorable conditions tends to have inherent

high RGR. They assumed that these variations in RGR were adapted to temperature conditions. However, Lambers and Poorter (2004) argued that not RGR itself but rather one of the growth parameters associated with RGR is the target of selection. Of these, the net assimilation rate (NAR: dry weight increase per unit leaf area and time) and the leaf area ratio (LAR: leaf area per unit total plant dry weight) relate to RGR. NAR is a physiological parameter and is the net result of carbon gain due to photosynthesis and carbon losses, mainly due to respiration. LAR is a morphological parameter (Evans, 1972; Quero *et al*., 2008). In many studies of the relationship between RGR and its underlying on temperature stresses indicated that LAR is one of the most important factors explaining inherent variations in RGR between species, whereas differences in NAR is secondary important (Poorter and Remkes, 1990; Poorter and Werf, 1998). In contrast, a study of temperature effects on plant growth and photosynthesis of rice and wheat, Nagai and Makino (2009) indicated that NAR plays an important role in the different RGR between rice and wheat. The responses of NAR to temperature can be described relating with temperature where the highest RGR is reached (Hunt and Halligan, 1981). Several studies indicated that if the range of temperature change is small, the effect of temperature on NAR is often not identified, because the optimal temperature is not so much different (Bruhn *et al*., 2000; Stirling *et al*., 1998). The importance of different plant growth is related to traits of RGR, and RGR depended on growth temperature (Loveys *et al*., 2002). In addition, early stage of plant growth, two important parameters are plant dry

weight and leaf area. Total dry weight is a measurement of photosynthetic accumulation of biomass corrected for respiratory loss over time. Leaf area provides a measurement of expressing the photosynthetic potential. The growth response of plant during early stage can be estimated relating to maturation stage in many plant (Jiao *et al*., 2005).

In rice, the growth is divided into three stages: (i) the juvenile stage, from germination to panicle initiation; (ii) the reproductive stage, from panicle initiation to flowering; and (iii) the maturation stage, from flowering to maturity. The rate of rice growth is not constant, because it depends on the environmental factors, particularly temperature. The optimum temperature for plant growth is different between *japonica* and *indica* rice. The optimum temperature for *indica* is from 25 to 35°C, while for *japonica* is from 18 to 33°C (Hardjawinata, 1980). Temperature affects difference depending on growth stages. At the early growth stage, the temperature affects yield via affecting the panicle number per plant, spikelet number per panicle, and the percentage of ripened grains (Yoshida, 1981). The observations in growth of 32R genotype indicated that the seedling growth of 32R during cold season showed lower than those of 29S. The 32R and 29S were developed from the crossing of Tetep and CN_4-4-2 (Wasano *et al*., 1985) and continuously screened for ShB resistance and susceptibility for over 20 years. The genetic relationship analysis indicated that 32R had 45% similar to Tetep, while 29S had 91% similar to Nb

(Gaihre, 2015). Recently, Gaihre and Nose (2013) indicated that the 32R is ShB-resistant and high yield potential. Therefore, 32R may be useful candidate for the development of a new ShB-resistant rice cultivar under temperate area with a high yield through the use of QTL pyramiding. However, before 32R can be cultivated in temperate zone, its cold sensitivity should be examined.

On the other hand, temperature is one of abiotic factors and affects the physiological activity at all spatial and temporal scales. In many cases, the rate of photosynthesis obtained maximum when plants grow under optimum temperature, and drops off with increasing slope as temperatures rise above the thermal optimum. When growth temperatures change, the thermal optimum of photosynthesis can also change. Several species, particularly those in more equitable habits, can be acclimated to temperature change as shifts in the thermal optimum of photosynthesis and enhanced assimilation rates at the new growth temperature (Atkin *et al*., 2006; Sage and Kubien, 2007). Other species, such as specialists for extreme environments, show less potential to acclimate and exhibit similar thermal responses in warm or cool growing conditions (Atkin *et al*., 2006). Temperature affects the photosynthesis via the activities of Rubisco and/or ribulose bisphosphate (RuBP) regeneration (Sage and Kubien, 2007). At low temperatures, the prominent limitation of photosynthesis is the regeneration of RuBP. At high temperatures, the

limitation of photosynthesis is assuming: i) heat instability of Rubisco activase and ii) depression of the chloroplast electron transport rate (ETR) (Yamori *et al*., 2013).

Furthermore, the physiological activities during the plant growth and development depend on the responses of the metabolic status. The response of plant metabolites to growth temperatures is a complex process (Fernie *et al*., 2011; Guy *et al*., 2008; Usadel *et al*., 2008). This process was assumed relating to function of roots and leaves in a plant. Leaves essentially have a photosynthetic function, whereas roots take up water and nutrients. The responses of growth and physiological function of roots to unfavorable conditions often precede those of leaves. Thus, leaves and roots may contrast to the functions that a plant required (Huang *et al*., 2012). Many compounds, such as amino acids (AAs), were synthesized in leaves but allocated and used in large amounts in roots under abiotic stress. Gargallo-Garriga *et al*. (2014) indicated that the concentration of sugar, AAs and nucleosides (Nus) were lower in leaves than in roots of two grass species (*Holcus lanatus* L. and *Alopecurus pratensis* L.) during drought stress. Recently, metabolic profiling is considered a major tool in studying plant stress responses (Guy *et al*., 2008; Shulaev *et al*., 2008). There were the first two groups has conducted the metabolic studies under cold stress condition by using gas chromatography/time of flight-mass spectrometry (GC/TOF-MS). Cook *et al*. (2004) compared metabolic changes during cold stress in two ecotypes of

Arabidopsis thaliana, Wassilewskija-2 (Ws-2) and Cape verde islands-1 (Cvi-1), which relate to cold tolerant and sensitive, respectively. Of 325 (75%) metabolites have been up-regulated in Ws-2, whereas 256 (79%) metabolites have been up-regulated in Cvi-1 in response to cold stress. These metabolites include the AAs and sugars. Other group of Kaplan *et al*. (2004) conducted metabolic analysis of *Arabidopsis* over the time course under cold and heat stress conditions. Cold stress influenced metabolism much more profoundly than heat stress. Of 143 and 311 metabolites or mass spectral tags were changed in response to heat and cold stresses, respectively. These metabolites include amino acids derived from pyruvate and oxaloacetate, polyamine precursors and compatible solutes. Recently, metabolic studies were also used to generate new rice cultivars in the context of identification of valuable gene functions (Baker *et al*., 2006; Oikawa *et al*., 2008).

In the previous study, a QTL of ShB resistant encoding cytokinin-*O*-glucosyltransferase located in the long arm of chromosome 7 was identified in 32R (Gaihre and Nose, 2011; Costanzoanzo *et al*., 2011; Gaihre, 2015). The cytokinin-*O*-glucosyltransferase was assumed to catalyze interaction between phytohormones and amino acids (Li *et al*., 2015; von Saint Paul *et al*., 2011). In addition, as well known, the cytokinin is the phytohormone relating to nitrogen metabolism (Sakakibara *et al*., 2006). Recently, Gargallo-Garriga *et al*. (2014) reported that the reason for opposite metabolic responses between shoots and/or leaves and roots is

different functions: metabolic shoots and/or leaves were deactivated during abiotic stress to reduce the intake of water and nutrients, while metabolic roots were stimulated to boost the uptake of water and nutrients. However, no study has been done on the profiling of amino acids in rice root of 32R in response to cold stress.

Recently, the method of metabolic analysis is often performed by using high-performance liquid chromatography (HPLC) or GC-TOF/MS (Williams *et al*., 2007). However, both HPLC and GC-TOF/MS remain some challenges, such as larger amount of protein or peptide requirement in HPLC and difficult comprehensive analysis of all amino acids in GC-TOF/MS (Ramautar *et al*., 2015), and many amino acids can not detected by GC-TOF/MS compared with calibrate electrophoresis- time of flight-mass spectrometry (CE-TOF/MS) (Williams *et al*., 2007). Thus, the need for faster, cheaper and more sensitive analysis methods has determined researchers to consider different methodologies. The CE-TOF/MS is a novel strategy to analyze and differentially display metabolic profiling of cationic and anionic metabolites. The CE-TOF/MS method required underivatization prior to injection and used minimal sample preparation. More recently, many studies demonstrated the potential of CE-TOF/MS system in the analysis of the plant metabolome (Maruyama *et al*., 2014; Ramautar *et al*., 2015; Yamakawa and Hakata, 2010).

This study aimed to investigate how the rice seedlings respond to different temperature conditions that result in the physiological and metabolic studies of temperature effects on the ShB rice genotype 32R. At first, effects of temperature on growth and photosynthesis in the juvenile seedling stage of 32R were examined in comparison with those of 29S and Nb. By this study, the differences in growth and photosynthesis between the ShB-resistant and -susceptible rice genotypes were investigated as shown in Chapter 2. Further investigation, effects of cold stress on the accumulation of soluble proteins and free amino acids of 32R relating to root growth was also conducted in comparison with 29S. The results of this study indicated that the accumulation of soluble proteins and free amino acids under cold stress were different between 32R and 29S as shown in Chapter 3. Furthermore, to deeply understand the amino acid (AA) metabolism in root of 32R in the response to cold stress, metabolic profiling of cationic metabolites in root of 32R using CE-TOF/MS method in comparison with that of 29S has been analyzed. The results of this study indicated that what kinds of metabolite participate in the cold-tolerant responses and how metabolites differ from root of 32R with that of 29S under cold stress as shown in Chapter 4. In the General Discussion, the results of these studies on plant responses to temperature effects, especially metabolic changes in the roots of the contrasting sheath blight resistant rice genotypes under cold stress were summarized and discussed. In addition, a proposal for metabolic network strategy to develop useful rice cultivars having superior qualities and particularly for the

clarification of the functions of valuable genes and to enhance their resistance response with temperature and diseases was presented.

CHAPTER 2

Effects of temperature on growth and photosynthesis in the seedling stage of the sheath blight-resistant rice genotype 32R

1. Introduction

Rice sheath blight caused by *R. solani* Kuhn is one of the most serious fungal diseases reducing rice yield. A rice yield loss as large as 50% may occur when plants are infected with *R. solani* (Lee and Rush, 1983). Many studies on the resistant mechanism of this disease have been conducted. Previous studies indicated that the activity and gene expression of various key enzymes in the glycolytic, pentose phosphate and phenylpropanoid pathways increased after *R. solani* infection in 32R compared to 29S (Mutuku and Nose, 2010; Mutuku and Nose, 2012). ShB-resistant genes are scattered in various chromosomes: 1, 4, 5 and 7 (Gaihre and Nose, 2011). Although many attempts to understand the mechanisms of ShB-resistance in rice have been studied, the evidences remain controversy.

Climate change is a major factor that alters plant performance (Nagai and Makino, 2009). The RGR is a consequence of variations in a wide variety of parameters related to physiology, morphology and chemical composition (Quero *et al*., 2008). Studies reported that NAR plays a more important role in determining the variation in RGR (Loveys *et al*., 2002; Nagai and Makino, 2009), meanwhile Villar *et al*. (2005) reported that the LAR plays a more important role in determining the variation in RGR under contrasting temperatures. On the other hand, climate change also causes the limitations of photosynthesis via the activities of Rubisco and/or RuBP regeneration (Sage and Kubien, 2007). At low temperatures, the prominent limitation of photosynthesis is the regeneration of RuBP. At high temperatures, the limitation of photosynthesis is assuming: i) heat instability of Rubisco activase and ii) depression of the chloroplast ETR (Yamori *et al*., 2013). In addition, Sage *et al*. (2008) indicated that the rates of initial slope of photosynthetic CO_2 response has been used to determine Rubisco activase limitations. Thus, effects of climate change, especially contrasting temperatures, on the rates of growth and photosynthesis of plant remain dispute.

The 32R and 29S were developed from the same parents, Tetep (*indica* ShB resistance) and CN_4-4-2 (*japonica* ShB sensitivity) and continuously screened for ShB resistance for over 20 years (Wasano *et al*., 1985). The CN_4-4-2 was developed from Chugoku 45 and Nb. Many aspects of 32R, such as metabolic pathways (Mutuku and Nose, 2012), proteomics analysis (Miyagi *et al*., 2006) and QTL analysis (Gaihre and Nose, 2011), have been studied. Although the

yield of 32R (7.9 MT/ha) is lower than that of Nb (9.6 MT/ha) and 29S (8.8 MT/ha), the yield of the F_1 generation (by crossing 32R and Nb, 12.5 MT/ha) is greater than the parents (Gaihre and Nose, 2013). In addition, Kobayashi *et al*. (2006) reported that the epidemic of the ShB would expand in rice grown under elevated CO_2 concentrations in the Northern Japan, thus the increased risks of ShB to rice plants are necessary to consider in the forecasting the effects of climate change on rice yield. The 32R is ShB-resistant and high yield potential, but the observations identified that the seedlings growth of 32R during cold season showed lower than those of 29S. Therefore, a new rice variety with ShB resistance, high yield and cold resistance is expected to overcome the above conditions, in which the 32R is a useful candidate for developing a new variety for temperate areas under climate change.

This study aimed to examine the rates of growth and photosynthesis of 32R under contrasting temperatures, which provides evidences for breeding a new cultivar with high yield and ShB resistance using QTL pyramiding. Seedlings at the 4^{th} leaf stage were exposed to 14/14, 19/14, 25/20, 31/26, 37/32 and 37/37°C (day/night) for 5, 10 and 15 days. The aim of this study is to address the questions: (i) what is the difference between the ShB-resistant and susceptible rice genotypes in terms of their responses to the rates of growth and photosynthesis? (ii) Are there any relationships between the temperature responses of individual leaf and whole-plants?

2. Materials and Methods

2. 1. Experimental design

The seeds of 32R, 29S and Nb as described by Mutuku and Nose (2010) were sterilized with a 0.1% solution of the insecticide Sumichion (Yashima Chemicals Industry Co., Ltd., Japan) and a 0.5% solution of the bactericide Tekurido C (Kumiai Chemicals Industry Co., Ltd., Japan) for one day and then soaked in the tap water for two days at 25/20°C. Twelve seeds were sown in a plastic pot (20 × 26 × 10 cm) filled with a 1:1 ratio of peat moss to vermiculite. The pots were placed in growth chambers (KG-50 HLA, Koito Industries Co., Ltd., Japan) with a 14-h photoperiod and watered sufficiently to maintain wet but not saturated soil until the completion of germination. The seedlings were then supplied with water daily, which was maintained at a height of 10 mm from the soil surface. The chamber temperature was maintained at 25/20°C with a relative humidity of 70% and photosynthetic photon flux density (PPFD) of 400 to 420 μmol m^{-2} s^{-1} at plant level. After the initial 14 days of growth, the plants were fertilized weekly with a half-strength concentration of the basal nutrient solution (Makino *et al*., 1988) with slight modifications. All of the seedlings at the 4^{th} leaf stage were placed at a day/night temperature of 14/14, 19/14, 25/20, 31/26, 37/32 and 37/37°C for 5, 10 and 15 days post-exposure (dpe), and the other conditions during temperature treatment were similar to pre-temperature treatment. All experiments were done with three replications.

2. 2. Growth analysis

The 4th harvests were conducted during the experiment. The first harvest was pre-treated. The following harvests were conducted sequentially at the interval of 5 days, at which time the seedlings had been exposed for 5, 10 and 15 days at contrasting growth temperatures. Four harvests were used to determine for the three growth periods.

For each harvest, six seedlings per genotype per treatment were selected at random. During the harvest, the soil was completely removed from the roots. The leaves, stems and roots were then separated. The leaves were scanned with a scanner (Canon MP640, Canon Inc., Tokyo, Japan), and determining leaf area by LIA32 software (http://hp.vector.co.jp/authors/VA008416/index-e.html). Leaves, stems and roots were oven-dried at 80°C for at least 48h, and recorded their dry weight. RGR, NAR and LAR were determined separately for each genotype as below:

$$RGR = \frac{1}{W}\frac{\Delta W}{\Delta t} = \frac{\ln W_2 - \ln W_1}{t_2 - t_1} \quad (1)$$

where W_1 and W_2 are total dry weights of the whole plant including roots at times t_1 and t_2.

$$NAR = \frac{1}{A}\frac{dW}{dt} = \left(\frac{W_2 - W_1}{t_2 - t_1}\right)\left(\frac{\ln A_2 - \ln A_1}{A_2 - A_1}\right) \quad (2)$$

where A_1 and A_2 are total leaf areas of the whole plant at times t_1 and t_2.

$$LAR = \frac{A}{W} = \frac{1}{2}\left(\frac{A1}{W1} + \frac{A2}{W2}\right) \quad (3)$$

RGR can be also calculated by multiplying NAR and LAR,

$$RGR = NAR \times LAR = \left(\frac{1}{A}\right)\left(\frac{\Delta W}{\Delta t}\right)\left(\frac{A}{W}\right) \quad (4)$$

We calculated the mean of the three growth periods for each genotype to compare effects of contrasting growth temperatures.

2. 3. Photosynthesis measurements

The rates of photosynthesis were determined in the fully expanded, youngest leaf of seedlings at 5, 10 and 15 dpe by using a portable gas exchange system (LI-6400, Li-Cor, USA), and measured based on the responses of irradiation, leaf temperature and CO_2 concentration. For the responses of irradiation, PPFD on the leaf surface was controlled in six steps in descending order from 800 to 0 μmol m^{-2} s^{-1}. During measurement, leaf temperature was controlled at 30°C, vapor pressure different between the leaf and air (VpdL) was

maintained at 1.7 ± 0.3 kPa and the CO_2 reference was maintained in ambient air (370 μmol mol^{-1}). To evaluate the responses of leaf temperature, the leaf temperature was changed in increasing order from 15 to 35°C with an interval of 5°C. During measurement, the CO_2 reference, irradiation and VpdL were maintained at 370 μmol mol^{-1}, 400 μmol m^{-2} s^{-1} and 1.7 ± 0.3 kPa, respectively. To evaluate the responses of CO_2 concentration, CO_2 concentration was controlled in six steps in descending order from 800 to 0 μmol mol^{-1}. During measurement, irradiation, leaf temperature and VpdL were maintained at 1000 μmol m^{-2} s^{-1} and 30°C and at 1.7 ± 0.3 kPa, respectively. All of the measurements were initiated at 9:00 and completed at noon. After photosynthetic measurements were completed, the leaf samples were immediately immersed in liquid nitrogen and then stored at -80°C until use.

2. 4. Chlorophyll and Rubisco content measurements

Frozen leaves (0.2 g) were ground with a chilled mortar and pestle with 0.2 g quartz sand and 2 mL of an extraction buffer. The extraction buffer (pH 7.9) was a modification of the buffer described by Kanbe *et al*. (2009). The buffer contained 50 mM Tris-HCl, 10 mM $MgCl_2$, 0.5 mM ethylenediaminetetraacetatic acid (EDTA)-NaOH, 5 mM dithiothreitol, 0.2% (w/v) polyvinylpolypyrrolindone and 0.1% (w/v) Triton X-100. The homogenate was filtered by one layer of Miracloth (Calbiochem Novabiochem, USA). An aliquot (0.2 mL) of the

homogenate was mixed with 0.8 mL of acetone to determinate the chlorophyll content as described by Arnon (1949). The remaining homogenate was centrifuged at 16 000 × g for 20 min at 4°C. The supernatant was moved to another tube on ice, and the pellet was re-extracted with 0.2 mL of extraction buffer. The supernatants were mixed and determined the concentration using BSA as a standard (Bradford, 1976). Soluble proteins (5 μg) were evaluated by SDS-PAGE in a 12% (w/v) gel containing 20% (w/v) SDS, and the gels were then stained with Coomassie brilliant blue R250. The Rubisco contents of the samples were determined and compared with that of commercial spinach (Sigma Co., Ltd., USA). The protein-band intensity of the large subunit of Rubisco was measured using ImageJ software (http://rsbweb.nih.gov/ij/).

2. 5. Statistical analysis

Statistical analyses were performed using R package (http://www.r-project.org/). Prior to the statistical analysis, all of the data were normalization using square root- or log-transformed values. Data are presented as the mean ± SD. The Tukey HSD *test* was used to compare parameters among the three rice genotypes and contrasting temperatures. The correlations were also analyzed using the R package. Terms were considered significant level at $P < 0.05$.

3. Results

3. 1. Effects of temperature on plant growth

Dry weight of 32R, 29S and Nb showed in Figs. 2.1-A1-2, B1-2, C1-2, D1-2, E1-2 and F1-2. Overall, total dry weight increased with a rising temperature. Total dry weight at 14/14°C showed the lowest in 32R and showed higher in 29S than in Nb, while total dry weight at 37/37°C showed the lowest in Nb and showed higher in 29S than in 32R. Total dry weight at 19/14, 25/20, 31/26 and 37/32°C showed the highest in 29S and showed higher in 32R than in Nb, however, in some cases total dry weight of 32R and Nb were not different. Similarly, the root dry weight also increased with increasing temperature. The root dry weight at 14/14°C regime showed the lowest in 32R and showed higher in 29S than in Nb, meanwhile root dry weight at higher temperature regimes showed higher in 29S than in both Nb and 32R and showed no difference between Nb and 32R. Root dry weight at 14/14°C, particularly at 15 dpe, showed a decrease of 2.5 and 1.9 times in 32R compared to in 29S and Nb, respectively. In addition, the leaf area of 32R, 29S and Nb are shown in Figs. 2. 1-A3, B3, C3, D3, E3 and F3. In general, leaf area increased with a rising temperature. The leaf area showed the highest in 29S and showed no difference between 32R and Nb at the six growth temperature regimes.

Figure 2.2 shows the results of RGR, NAR and LAR of 32R, 29S and Nb

under contrasting temperatures. RGR showed lower at 14/14°C regime than at others (Fig. 2. 2A). The 32R showed lower in RGR at 14/14°C than 29S and Nb, while 32R showed also lower in RGR at 19/14, 25/20, 31/26, 37/32 and 37/37°C than 29S but higher than Nb. RGR at 14/14°C decreased by 2.5 and 2.3 times in 32R compared to in 29S and in Nb, respectively. NAR also showed lower at 14/14°C regime than at others (Fig. 2. 2B). The 32R showed lower in NAR at 14/14°C than 29S and Nb, while 32R also showed lower in NAR at 19/14°C than 29S but higher than Nb. Both 32R and 29S showed higher in NAR at 25/20 and 31/26°C than Nb. NAR at 37/32 and 37/37°C were not different among genotypes. NAR at 14/14°C decreased by 2.8 and 2.4 times in 32R compared to in 29S and in Nb, respectively (Fig. 2. 2B); however, there were not significant difference in LAR in different genotypes and different growth temperature regimes (Fig. 2. 2C).

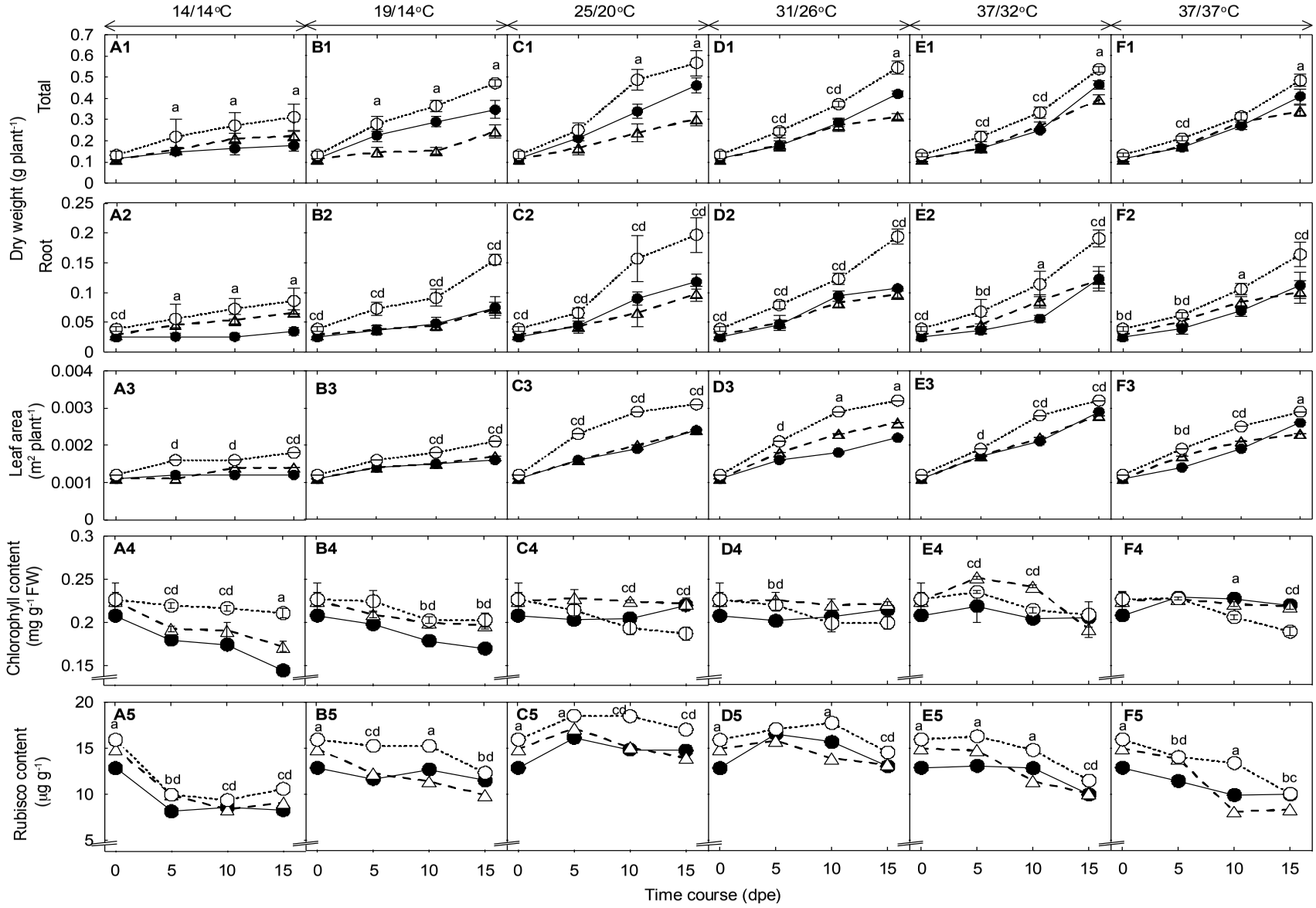

Fig. 2.1. Effects of temperature on dry weight, leaf area and the contents of chlorophyll and Rubisco of 32R (●), 29S (○) and Nb (△). The values are the mean ± SD (n = 6 for dry weight and leaf area, n = 3 for Rubisco contents and n = 30 for chlorophyll contents). The absence of a bar indicates that the SD was within the size of the symbol. Different letters indicate significance at $P < 0.05$ by Tukey's HSD *test* (where *a* is significant among 32R, Nb and 29S; *b* is significant between 32R and Nb; *c* is significant between 29S and Nb; and *d* is significant between 32R and 29S).

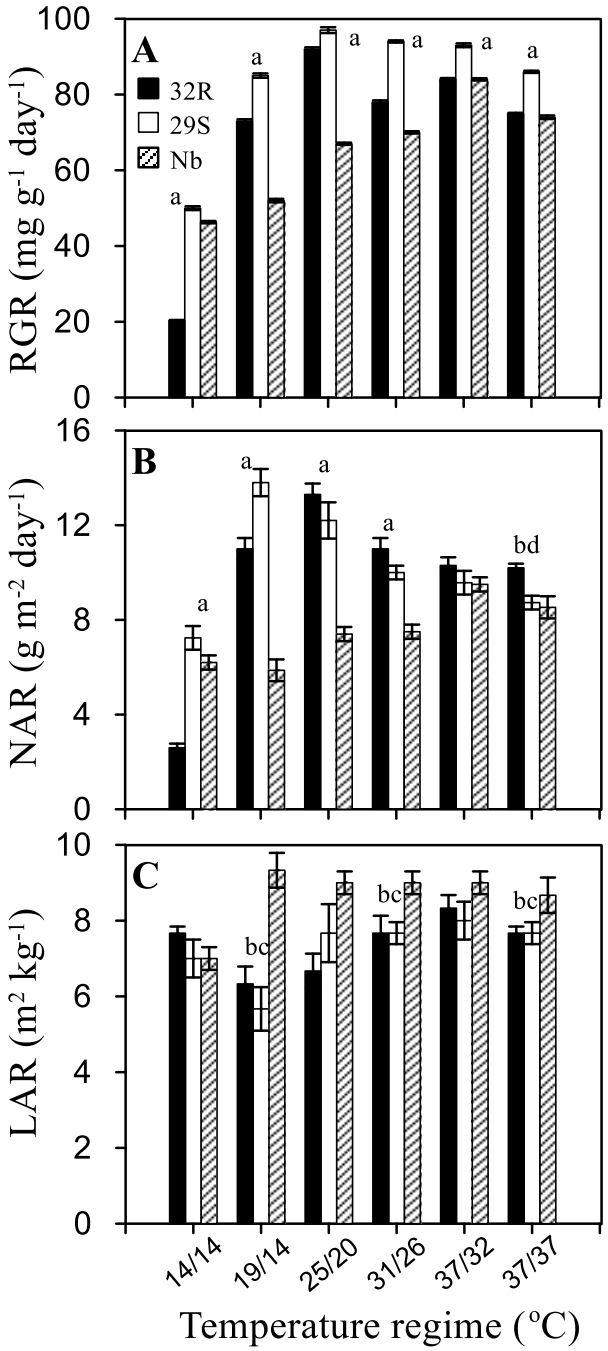

Fig. 2. 2. Effects of temperature on the relative growth rate (RGR), net assimilation rate (NAR), leaf area ratio (LAR) of 32R, 29S and Nb. Data are the mean ± SD (n = 18). The absence of a bar indicates that the SD was within the size of the symbol. Different letters show significant differences at $P < 0.05$ (where *a* is significant among 32R, Nb and 29S; *b* is significant between 32R and Nb; *c* is significant between 29S and Nb; and *d* is significant between 32R and 29S).

3. 2. Effects of contrasting temperature on leaf constituents

The chlorophyll contents of 32R, 29S and Nb were shown in Figs. 2. 1-A4, B4, C4, D4, E4 and F4. Overall, chlorophyll contents were rapidly decreased at a low temperature (Figs. 2. 1-A4 and 2. 1-B4). Chlorophyll contents showed lower in 32R than in 29S and Nb at 14/14 and 19/14°C regimes, but no difference at higher temperature regimes. In addition, the depression of chlorophyll content in the 32R occurred rapidly in comparison with that in the 29S and Nb.

The contents of Rubisco of 32R, 29S and Nb were shown in Figs. 2. 1-A5, B5, C5, D5, E5 and F5. Overall, Rubisco contents decreased at low and high temperatures (Figs. 2. 1A5 and 2. 1F5). Rubisco contents showed lower in 32R than in 29S, while showed no difference between 32R and Nb.

3. 3. Effects of temperatures on photosynthetic rates

3. 3. 1. Light response of photosynthesis

The photosynthetic rates of light response of 32R, 29S and Nb were estimated by using the rectangular hyperbola model as described by Ulqodry *et al*. (2014).

$$P - Li = \frac{I}{\alpha + \beta \times I} \quad (1)$$

where *P-Li* (μmol m^{-2} s^{-1}) is the photosynthetic rate of the individual leaf at a light intensity of *I* (μmol m^{-2} s^{-1}) and both α and β are coefficients to determine the convexity of the hyperbola.

$$P - Li = \frac{1}{\frac{\alpha}{I} + \beta} \quad (2)$$

When $I = \infty$, $P_{\max} - Li = \frac{1}{\beta}$ (3)

From (1) $\Rightarrow P' - Li = \frac{\alpha}{(\alpha + \beta \times I)^2}$ (4)

From (4) when $I = 0$, $P' - Li = \frac{1}{\alpha}$ (5)

The initial slope ($P' - Li = \frac{1}{\alpha}$) and maximum photosynthesis ($P_{\max} - Li = \frac{1}{\beta}$) of the photosynthetic light intensity response curves (*P-Li*) were estimated from (1). *P′-Li* based on absorbed light (quantum yield) describes the efficiency with which light is transformed into fixed carbon. *P′-Li* is determined at low light intensities when photosynthetic rate increases linearly with irradiance, with the light-driven electron transport rate (ETR) limiting photosynthesis (Lambers *et al*., 2008). P_{max}-*Li* is achieved when increases in light intensity at which photosynthesis is saturated (Kirk, 2011). *P′-Li* and P_{max}-*Li* of 32R, 29S and Nb

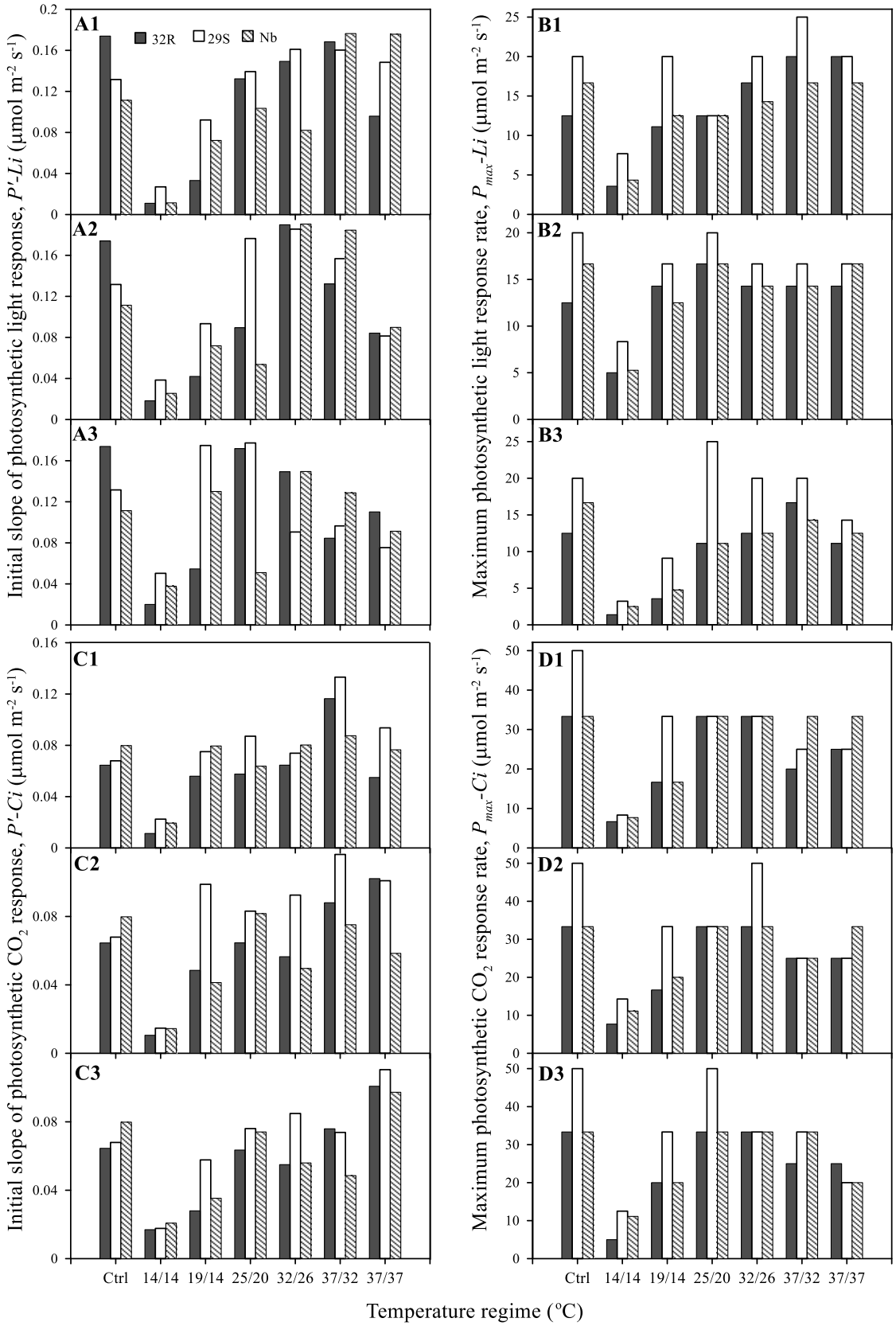

Fig. 2. 3. Effects of temperature on the rate of initial slope and maximum photosynthesis of 32R, 29S and Nb. The *P'-Li* (A1, A2 and A3) and P_{max}-*Li* (B1, B2 and B3) shows the photosynthetic light responses at 5, 10 and 15 dpe, respectively. The *P'-Ci* (C1, C2 and C3) and P_{max}-*Ci* (D1, D2 and D3) shows the photosynthetic CO_2 responses at 5, 10 and 15 dpe, respectively. Ctrl is the pre- treatment.

under contrasting temperatures were shown in Fig. 2. 3. The *P′-Li* and P_{max}-*Li* of the rice genotypes were decreased at 14/14℃ regime compared to other regimes. Before temperature treatment, the 32R showed higher in *P′-Li* than 29S and Nb, while the 32R showed lower in P_{max}-*Li* than 29S and Nb. After temperature treatment, especially at 14/14 and 19/14℃, the 32R showed lower in *P′-Li* and P_{max}-*Li* than 29S and Nb.

3. 3. 2. CO_2 response of photosynthesis

Like *P-Li*, the photosynthetic rates of CO_2 response of 32R, 29S and Nb were also estimated by using the rectangular hyperbola model.

$$P-Ci=\frac{C_i}{\alpha+\beta\times C_i} \quad (6)$$

where *P-Ci* (μmol m^{-2} s^{-1}) is the photosynthetic rate of the individual leaf at a CO_2 concentration (μmol mol^{-1}) and both α and β are coefficients to determine the convexity of the hyperbola.

$$P-Ci=\frac{1}{\frac{\alpha}{C_i}+\beta} \quad (7)$$

$$\text{When } C_i=\infty,\ P_{\max}-Ci=\frac{1}{\beta} \quad (8)$$

From (1) $\Rightarrow P'-Ci = \frac{\alpha}{(\alpha+\beta\times C_i)^2}$ (9)

From (4) when $C_i = 0$, $P'-Ci = \frac{1}{\alpha}$ (10)

The initial slope ($P'-Ci = \frac{1}{\alpha}$) and maximum photosynthesis ($P_{\max}-Ci = \frac{1}{\beta}$) of the photosynthetic CO_2 concentration response curves (*P-Ci*) were also estimated from (6). *P′-Ci* at light saturation generally reflects Rubisco capacity (Sage *et al*., 2011). *P′-Ci* is determined at low CO_2 concentration when photosynthetic rate increases linearly with the CO_2 concentration, calling Rubisco limitation. P_{max}-*Ci* achieved when increases in CO_2 concentration at which photosynthesis is saturated and normally occurs when the CO_2 concentration is high. P_{max}-*Ci* is predicted assuming that the rate of regeneration of RuBP is limiting, calling RuBP-limitation (Sharkey *et al*., 2007). *P′-Ci* and P_{max}-*Ci* of the 32R, 29S and Nb showed lower at 14/14℃ regime than at other regimes (Fig. 2. 3). The 32R showed lower in *P′-Ci* than 29S at six regimes. 32R showed lower in *P′-Ci* than Nb at pre-temperature treatment, 14/14, 19/14, 25/20 and 31/26℃, whereas showed higher in *P′-Ci* than Nb at 37/32 and 37/37℃. In additions, 32R showed lower in P_{max}-*Ci* than 29S at 14/14 and 19/14℃.

3. 3. 3. Temperature response of photosynthesis

Figure 2.4 shows the rate of photosynthetic leaf temperature response (*P-Ti*) of 32R, 29S and Nb under contrasting temperatures. Overall, the *P-Ti* decreased at a low and high leaf temperatures. It means that when leaf temperatures were ≤ 20 and > 30°C, the *P-Ti* decreased, whereas when the leaf temperatures were from 25 to 30°C, the *P-Ti* increased. *P-Ti* showed lower in 32R than in 29S, while showed no difference between 32R and Nb. In addition, *P-Ti* showed lower at 14/14°C regime compared to other regimes.

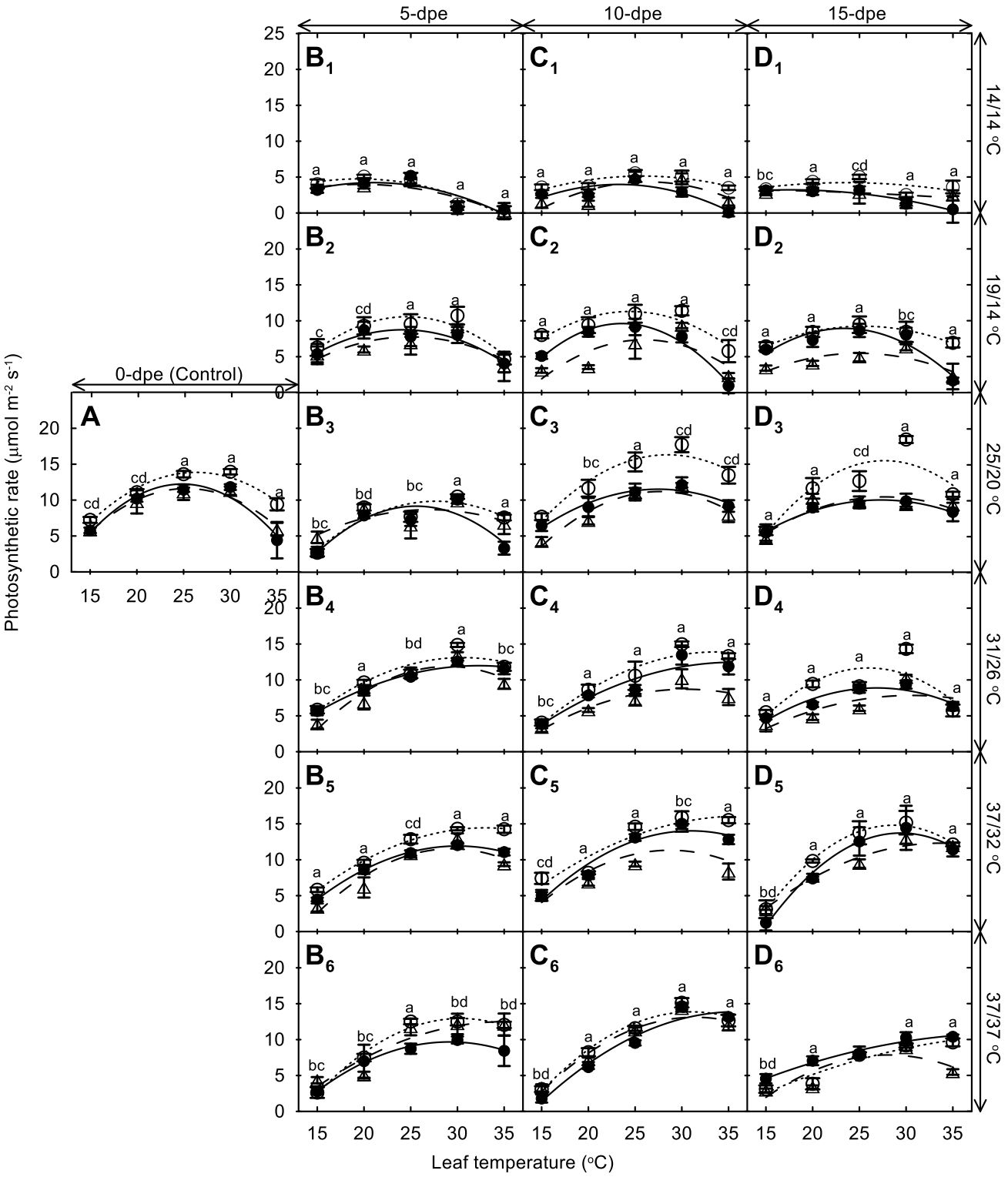

Fig. 2. 4. Effects of the growth temperature on the rate of the photosynthetic leaf temperature response of 32R (●), 29S (○) and Nb (△). The absence of a bar indicates that the SD was within the size of the symbol. Different letters indicate significant differences at $P < 0.05$ (where *a* is significant among 32R, 29S and Nb; *b* is significant between 32R and Nb; *c* is significant between 29S and Nb; and *d* is significant between 32R and 29S).

4. Discussion

The parameters of the plant growth and photosynthesis of both 32R and 29S were affected by the factors as follows: growth conditions, time courses and genotypes and by the interactions of these factors (Table 2. 1). In present study, effects of temperature on the plant responses of 32R were examined in comparison with those of the 29S and Nb. The seedlings at the 4th leaf stage of rice genotypes were exposed to a day/night temperature of 14/14, 19/14, 25/20, 31/26, 37/32 and 37/37°C for 5, 10 and 15 days. The present findings showed that the dry weight showed lower in 32R than in 29S and Nb at a low temperature, and total dry weight correlated strongly with root dry weight and leaf area. The RGR correlated strongly with the NAR. Rubisco, chlorophyll contents and the photosynthetic rates were limited at a low temperature, and showed lower in 32R than in 29S and Nb. The strong correlations between Rubisco and the rates of maximum photosynthesis and initial slope were found in 32R, but not found in 29S and Nb. In addition, RGR and NAR of 32R correlated positively with Rubisco. These results indicated that 32R contains traits of cold-sensitive genotypes that are related to limiting Rubisco at a low temperature, thus diminishing photosynthesis and limiting plant growth.

Table 2. 1. A two-way ANOVA of temperature, time course, genotype and their interactions effects on the characteristics of plant growth and photosynthesis of sheath blight resistant rice (32R), Nipponbare (Nb) and sheath blight susceptible rice (29S).

Parameter	F and (*P*-value)						
	Temperature (T)	Time Course (TC)	Genotype (G)	T*TC	TC*G	T*G	T*TC*G
Dw	41.75 (<0.001)	1005.03 (<0.001)	71.40 (<0.001)	42.58 (<0.001)	23.41 (<0.001)	0.0083 (0.93)	0.0014 (0.97)
LA	110.09 (<0.001)	731.19 (<0.001)	111.99 (<0.001)	55.88 (<0.001)	20.54 (<0.001)	1.71 (0.19)	0.97 (0.33)
Chlo	7.18 (0.007)	0.05 (0.82)	0.06 (0.79)	1.62 (0.20)	0.35 (0.56)	0.01 (0.92)	1.09 (0.29)
Rub	21.57 (<0.001)	0.86 (0.35)	1.01 (0.31)	0.68 (0.41)	0.02 (0.88)	47.55 (<0.001)	0.01 (0.91)
P-Ci	120.35 (<0.001)	80.94 (<0.001)	40.06 (<0.001)	25.36 (0.001)	0.6485 (0.42)	2.26 (0.13)	1.17 (0.28)
P-Li	98.42 (<0.001)	37.68 (<0.001)	56.23 (0.001)	29.83 (<0.001)	0.25 (0.62)	1.43 (0.23)	0.67 (0.41)
P-Ti	1166.56 (<0.001)	157 (<0.001)	231.62 (<0.001)	275.43 (<0.001)	1.57 (0.69)	10.68 (0.29)	11.79 (0.27)

Where T*TC is interaction between growth condition and time course, TC*G is interaction between time course and genotype, T*G is interaction between growth condition and genotype, and T*TC*G is interaction of three factors including growth condition, time course and genotype. Dw, LA, Chlo, P-Li, P-Ti, and P-Ci are total dry weight, leaf area, chlorophyll content, photosynthetic response of light intensity, leaf temperature and CO_2 concentration of 32R, Nb, and 29S, respectively.

The present findings agreed with previous studies that a low temperature causes a depression of the dry weight (Aghaee *et al*., 2011; Engels and Marschner, 1990; Nagai and Makino, 2009). In addition, the results in this study also showing total dry weight of genotypes were different at a low temperature. These differences related to root dry weight and leaf area. The relationship analysis indicated that total dry weight correlated strongly with root dry weight (Fig. 2. 5B1) and leaf area (Fig. 2. 5B2). Roots and shoots are different sensitivity to abiotic stress, because they have different functions: shoots essentially have a photosynthetic function, whereas roots take up water and nutrients. Thus, shoots and roots may vie and collaborate for the functions that a plant requirement. A decrease in roots growth often precedes the decrease of shoots growth (Gargallo-Garriga *et al*., 2014; Huang *et al*., 2012). This is reason to explain why total dry weight related strongly with root dry weight rather than with dry weight of leaves and stems of genotypes (data not shown).

The growth rate (GR) was appreciably faster in cold-resistant genotypes than in cold-susceptible genotypes. According to the results of this study, the 29S has both greater leaf area and more biomass compared with 32R and Nb. Poorter and Remkes (1990) reported that fast-growing species allocated more carbon to leaves than slow-growing species and that higher LAR in fast-growing species allows plants to fix more carbon per unit plant weight, with a positive correlation between LAR and RGR. However, the findings of this study indicated that there were strong correlations between RGR and NAR (Fig. 2. 5A3) rather than LAR

(Fig. 2. 5A4), root dry weight (Fig. 2. 5A1) and leaf area (Fig. 2. 5A2). The present findings are in agreement with the findings of Loveys *et al*. (2002) and Nagai and Makino (2009) that the variations of NAR play an important role in differences in RGR. Furthermore, Nagai and Makino (2009) indicated that although LAR was an important determinant for GR, no difference in the temperature responses of LAR was identified between rice and wheat. The explanation for the difference between species is different N-use efficiency for photosynthesis varying in growth temperatures. These suggest that differences in the temperature response of GR of genotypes are caused more by a difference in the temperature response of NAR. On the other hand, the optimal temperature for biomass production related to favorable temperature conditions of different genotypes. The present results indicated that the 32R showed the lowest total dry weight at a low temperature, while the Nb showed the lowest total dry weight at a high temperature. The Nb is a *japonica* and grows as a subtropical rice, thus the growth of Nb was limited at a high temperature. In contrast, growth of 32R was limited at a low growth temperature. According to Aghaee *et al*. (2011) the cold-susceptible rice genotypes showed more quickly decrease in dry weight than the cold-resistant rice genotypes at a low temperature. These suggest that the 32R may include the traits of cold-sensitive genotypes.

The present findings agreed with the findings of Aghaee *et al*. (2011) and Ulqodry *et al*. (2014) that the chlorophyll contents depressed in response to low temperatures. These results suggest that the photosynthetic pigments in 32R were

more susceptible to a low temperature than those in 29S and Nb. In addition, low and high temperatures cause the decrease of Rubisco contents in many crops such as rice, soybean and wheat (Makino and Sage, 2007; Sage *et al*., 2008; Vu *et al*., 2001; Yamasaki *et al*., 2002). In contrast, Nagai and Makino (2009) reported that the Rubisco contents in both wheat and rice increased at low (19/16 for rice and 13/10°C for wheat) and high (37/31°C) temperatures. These results suggest that there were different temperature effects on the leaf, thus inducing differences in the biochemical process and the rates of photosynthesis in plants.

The photosynthetic apparatus was destroyable and trended to be associated with damage to ligh absorption and electron transport components or deactivated Rubisco activase at low and high temperatures (Sage and Kubien, 2007). Ulqodry *et al*. (2014) reported that cold temperatures reduced the ETR and the maximal quantum efficiency of photosystem II (F_v/F_m) of *Rhizophora mucronata* leaves because of lowering of chlorophyll contents, causing decrease photosynthetic rates of *R. mucronata*. The present findings agreed with the findings of Ulqodry *et al*. (2014) that photosynthetic rates and chlorophyll contents were decreased at low temperatures. Furthermore, enzymatic reaction rates at low temperatures, mainly associated with the "dark reactions", are limited, whereas the oxygenating reaction of Rubisco at high temperatures increases more than the carboxylating one so that photorespiration becomes comparably more important (Lambers *et al*., 2008). The results of this study indicated that the rates of initial slope and maximum photosynthesis correlated strongly with Rubisco

(Fig. 2. 6). These suggest that limitations of the rates of initial slope and maximum photosynthesis related to limitations of the capacities of Rubisco. In addition, cold-resistant genotypes have ability to acclimatize high irradiance (Huner *et al*., 1998) and could alleviate the limitations in ETR and RuBP regeneration compared to cold-susceptible genotypes, thus photosynthetic rates of cold-tolerant genotypes are generally higher than those of cold-susceptible genotypes at low temperatures (Fracheboud and Leipner, 2003; Yamori *et al*., 2010). The results of this study indicated that the rates of initial slope and maximum photosynthesis limited in 32R in compared with in 29S and Nb at low temperatures, 14/14 and 19/14°C. In addition, the findings of this study also agreed with the findings of Kositsup *et al*. (2009) and Ulqodry *et al*. (2014) that *P-Ti* decreased at a low and high leaf temperatures, and showed lower at a low growth temperature regime compared with at higher growth temperature regimes. It means that 32R may contain the characteristics of cold-susceptible genotypes.

A previous study identified two genes of 32R which are located in the chromosome 7 that encode cytokinin-*O*-glucosyltransferase (Gaihre and Nose, 2011). Other research was done by Costanzo *et al*. (2011) also identified two genes that encode for cytokynin-*O*-glucosyltransferase in the progeny of the ShB-sensitive Cypress and ShB-moderately resistant Jasmine 85. Cytokinins are a major class of plant hormones that significantly influence plant growth and development, usually through interaction with other plant hormones, such as auxin, ethylene, gibberellin and abscisic acid (ABA), etc. (Li *et al*., 2015).

Glucosyltransferases are the most common enzymes in catalyzing the process of glycosylation, which could transfer sugar moieties from activated donor molecules to specific acceptor molecules such as phytohormone, plant secondary metabolites, amino acids, etc., and some of them were reported to participate in stress adaptation, such as involved in defending pathogen infections (von Saint Paul *et al*., 2011). The lowering of root dry weight of 32R may cause by the limitations of phytohormone during plants growth under low temperature. On the other hand, 32R and 29S were developed from the same parent (Tetep and CN_4-4-2) but they had different characteristics. The genetic relationship analysis indicated that 32R had 45% similar to Tetep, while 29S had 91% similar to Nb (Gaihre, 2015). The *indica* genotypes are adapted to tropical zones, while *japonica* genotypes are adapted to subtropical zones (Garris *et al*., 2005). The decreases in growth and photosynthesis during the seedling stage of 32R at low temperatures compared to both 29S and Nb might be due to 32R containing characteristics of the cold-sensitive *indica* genotype of Tetep. Although the 32R is cold-sensitive and low yield, it is ShB-resistant (Mutuku and Nose, 2012) and a high yield potential (Gaihre and Nose, 2013).

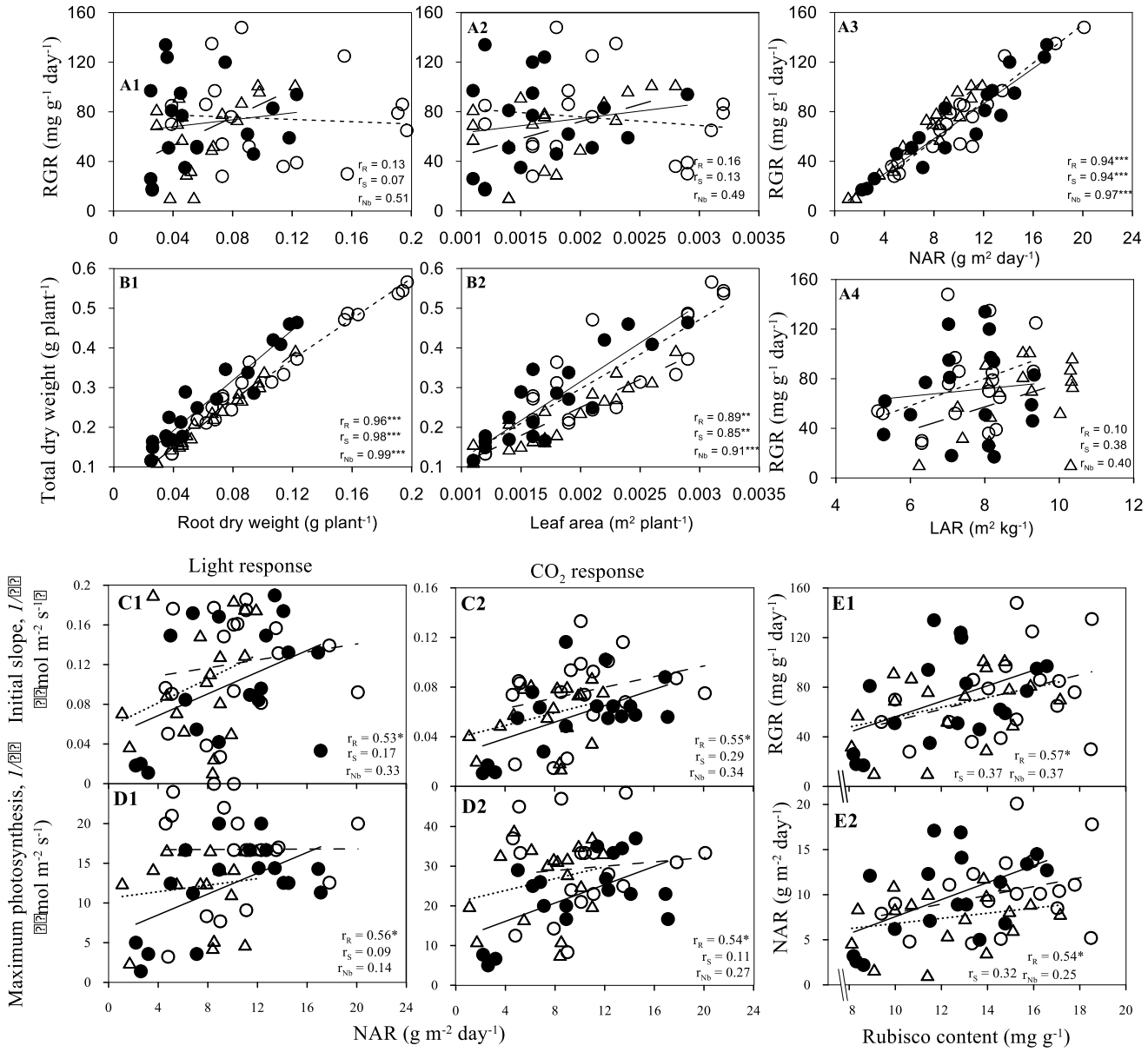

Fig. 2. 5. Correlation of parameters in the rate of growth and photosynthesis of 32R (●), 29S (○) and Nb (△). The r_R, r_S and r_{Nb} are r values for 32R, 29S and Nb, respectively. The symbols indicate the statistical significance at $***P < 0.001$, $**P < 0.01$ and $*P < 0.05$.

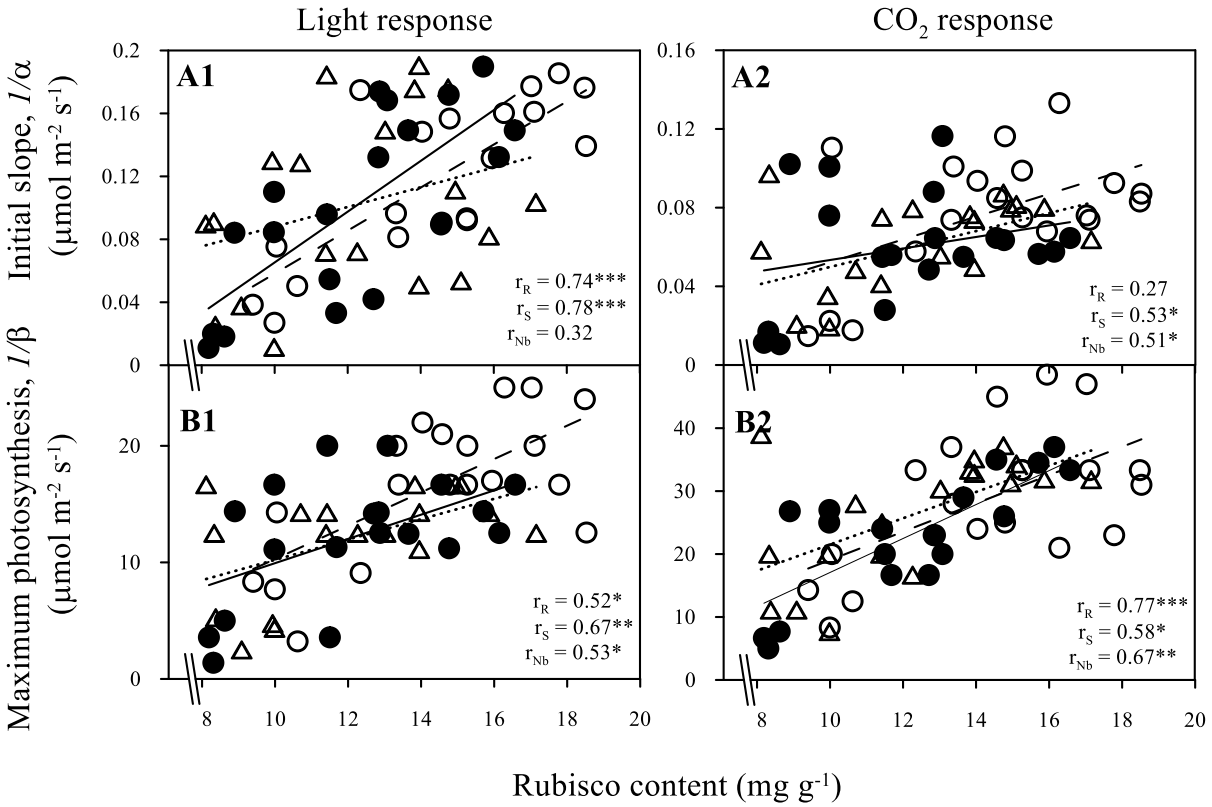

Fig. 2. 6. Correlations between the Rubisco content with the rate of initial slope (A1 and A2) and maximum photosynthesis (B1 and B2) of 32R (●), 29S (○) and Nb (△). The $r_{R,}$ r_S and r_{Nb} are r values for 32R, 29S and Nb, respectively. The symbols indicate statistical significance at ***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$.

In the present work, the rates of growth and photosynthesis of the ShB-resistant rice genotype were examined during juvenile seedling stage under contrasting growth temperatures. These results indicated that the limitations in total dry weight of 32R in compared to 29S and Nb were caused by limitations in its root dry weight, thus causing decrease RGR at low temperatures rather than at high temperatures. Furthermore, the variations in leaf constituents under contrasting temperatures caused the differences in photosynthesis. These results indicated that 32R is cold-sensitive genotype and poor growth. However, prominent genetic traits in 32R, high yield capacity and ShB resistance, may be useful for the development of a new ShB-resistant rice cultivar with a high yield through the use of QTL pyramiding.

5. Summary

The 32R rice genotype is resistant to ShB, with a high yield potential. The effects of temperature on the plant responses of 32R were examined in comparison with those of the ShB-susceptible rice genotype (29S) and Nb. The seedlings at the 4th leaf stage of rice genotypes were exposed to 14/14, 19/14, 25/20, 31/26, 37/32 and 37/37°C (day/night) for 5, 10 and 15 days. The dry weight, leaf area, photosynthesis, contents of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) and chlorophyll contents were examined. The dry weight showed lower in 32R than in 29S and Nb at a low temperature, and total dry weight correlated strongly with root dry weight and leaf area. The

relative growth rate (RGR) correlated strongly with the net assimilation rate (NAR). Rubisco, chlorophyll contents and the photosynthetic rates were limited at a low temperature and showed lower in 32R than in 29S and Nb. The strong correlations between Rubisco and the rates of maximum photosynthesis and initial slope were found in 32R, but not found in 29S and Nb. In addition, RGR and NAR of 32R correlated positively with Rubisco. These suggest that 32R contains traits of cold-sensitive genotypes that are related to limiting Rubisco at a low temperature, thus diminishing photosynthesis and limiting plant growth. Differences of growth among 32R, 29S and Nb were discussed in the relation of genotypes.

CHAPTER 3

Effect of cold stress on the root growth, accumulation of soluble proteins and free amino acids of the sheath blight-resistant rice genotype 32R

1. Introduction

Rice is one of the most important staple foods for more than half of the world population. However, rice is highly sensitive to biotic and abiotic stresses. Biotic stresses are caused by fungi, viruses, bacteria, pests and insects. Rice sheath blight disease (ShB) caused by *Rhizoctonia solani* Kuhn is one of the most serious fungal diseases reducing by more than 50% the rice yield, when plants are infected with *R. solani* (Lee and Rush, 1983). Since Kobayashi et al. (2006) reported that epidemics of ShB would increase in rice grown under elevated CO_2 concentrations in northern Japan, the increased risks of ShB to rice plants should be considered in the forecasting of the effects of climate change on rice yield. Rising temperature is one of the climate change factors that can provide better growth conditions for diseases in temperate zones (Case and

Tidwell, 2014). Furthermore, rice growth was easily impaired by low temperatures during the juvenile stages, leading to low productivity (Sipaseuth et al., 2007). In fact, recent results reported that seedling growth of a *R. solani*-resistant rice genotype, 32R dramatically decreased in comparison with that of the *R. solani*-susceptible rice genotype 29S at a low temperature (Chapter 2). In addition, 32R and 29S are near-isogenic rice lines that were developed from the crossing of Tetep (*Indica*, ShB resistance) and CN_4-4-2 (*Japonica*, ShB sensitivity), and continuously screened for ShB resistance and susceptibility for over 20 years (Wasano et al., 1985). The genetic relationship analysis indicated that 32R was 45% similar to Tetep, while 29S was 91% similar to Nb (Gaihre, 2015). Recently, Gaihre and Nose (2013) reported that 32R is ShB-resistant and shows a high yield potential. Therefore, 32R may be useful for the development of a new ShB-resistant rice cultivar in temperate areas with a high yield through the use of QTL pyramiding. However, before 32R can be cultivated in temperate zone its cold sensitivity should be examined.

Gaihre (2015) identified in 32R a gene located on the long arm of chromosome 7 that encodes cytokinin-*O*-glucosyltransferase which was assumed to interact with phytohormones and amino acids (Li et al., 2015; von Saint Paul et al., 2011). Cold stress is one of the serious environmental stresses affecting plant growth. Many studies indicated that plant amino acids (AAs), polyamines (PAs), organic acids and sugars display differential patterns in response to cold stress (Benina et al., 2013; Davey et al., 2009; Pathak et al., 2014; Zhao et al., 2013). The hypothesis of this study that roots would present a contrasting

metabolism in response to cold stress due to their different physiological functions in the acclimatization to stress. The seedlings of both 32R and 29S at the 4th leaf stage were subjected to a temperature of 14/14°C (day/night) for 5, 10 and 15 days post-exposure (dpe) in a growth chamber and were examined the contents of the total free amino acids to test this hypothesis.

2. Materials and Methods

2. 1. Plant materials and growth conditions

The seeds of 32R and 29S, as described in Chapter 2, were sterilized with a 0.1% solution of the insecticide Sumichion (Yashima Chemicals Industry Co., Ltd., Japan) and a 0.5% solution of the bactericide Tekurido C (Kumiai Chemicals Industry Co., Ltd., Japan) for one day and then soaked in tap water for two days at 25/20°C. Twelve seeds were sown in a plastic pot (20 × 26 × 10 cm) filled with a 1:1 ratio of peat moss to vermiculite. The pots were placed in growth chambers (KG-50 HLA, Koito Industries Co. Ltd., Japan) with a 14-h photoperiod and watered sufficiently to maintain wet but not saturated soil conditions until complete germination. The chamber temperatures were maintained at 25/20°C with a relative humidity of 70% and PPFD of 420 μmol m^{-2} s^{-1} at plant level. After the initial 14 days of growth, the plants were fertilized weekly with a half-strength concentration of the basal nutrient solution (Makino et al., 1988) with slight modifications. Half of the seedlings at the 4th leaf stage were transferred to a growth chamber with 14/14°C for 5, 10 and 15 dpe, and the

other conditions were similar to those at the initial growth stage. All the experiments were carried out with three replications.

2. 2. Measurements of root growth

The roots of the seedlings at 5, 10 and 15 dpe were washed with tap water and distilled water three times, respectively. The roots of six seedlings were oven-dried at 80°C for at least 48 h, and their dry weight was recorded. The other roots were immediately frozen with liquid nitrogen and stored at -80°C until use.

2. 3. Determinations of soluble protein and total free amino acid contents

Approximately 500 mg of frozen rice root material plus 2 mL of ice-cold Mg/Nondiet P-40 (NP-40) extraction buffer [0.5M Tris-HCl (pH 8.3), 2% (v/v) NP-40, 20mM $MgCl_2$, 2% β-mercaptoethanol, 1mM phenylmethylsulfonyl fluoride (PMSF), and 1% (w/v) polyvinylpyrrolidone] were homogenized using a mortar and pestle with 200 mg quartz sand. After filtration through one layer of sterile Miracloth (Calbiochem-Novabiochem, La Jolla, CA, USA), the homogenate was centrifuged at 12000×g (Tomy MX 105 refrigerated micro-centrifuge, Japan) at 4°C for 15 min. The supernatant was placed in a new micro-centrifuge tube and kept in an ice-box. The pellets were re-extracted with 1 mL of the same extraction buffer by vortex for about 2 min, then centrifuged at 12000×g at 4°C for 15 min. The supernatant was mixed with the supernatant in the first extraction. The protein concentration of the supernatant was assayed

according to the Bradford method (BioRad, Hercules, Ca, USA), using BSA as the standard.

Total FAA contents were determined using a ninhydrin assay, according to the method of Yemm et al. (1955) with slight modifications. Fine root powder (200 mg) was homogenized in 1 mL of 800 mL L^{-1} ethanol and centrifuged at 12 000 x g in 4°C for 15 min. The homogenate (0.1 mL) was mixed with 0.9 mL ninhydrin buffer [1% (w/v) ninhydrin in 0.5M citrate buffer (pH = 5.5), glycerol (870 mL L^{-1}) and 0.5M citrate buffer (pH = 5.5) in a ratio of 5:12:2]. The mixture was heated in a water bath for 10 min and then cooled at room temperature and the absorbance was determined at 570 nm in a UV-Spectrophotometer (UV-1800, Shimadza Co. Ltd., Japan). The blank was prepared by mixing 0.1 mL of 800 mL L^{-1} ethanol and 0.9 mL ninhydrin buffer with glycine as the standard.

Analyses of variance (ANOVA) were performed to determine the significance of the differences in genotypes, treatments, and time courses by using R packages (https://cran.r-project.org). The statistical significant level was $P < 0.05$.

3. Results

3. 1. Effects of cold stress on root growth

The root dry weight value showed lower under cold stress than under non-

cold stress conditions, and showed lower in 32R than in 29S by 53, 64 and 59% at 5, 10 and 15 dpe, respectively (Fig. 3. 1). Compared with non-cold stress condition, the root dry weight of 32R under cold stress decreased by 33, 62 and 69% at 5, 10 and 15 dpe, respectively, while the root dry weight of 32R under cold stress decreased by 15, 53 and 56% at 5, 10 and 15 dpe, respectively.

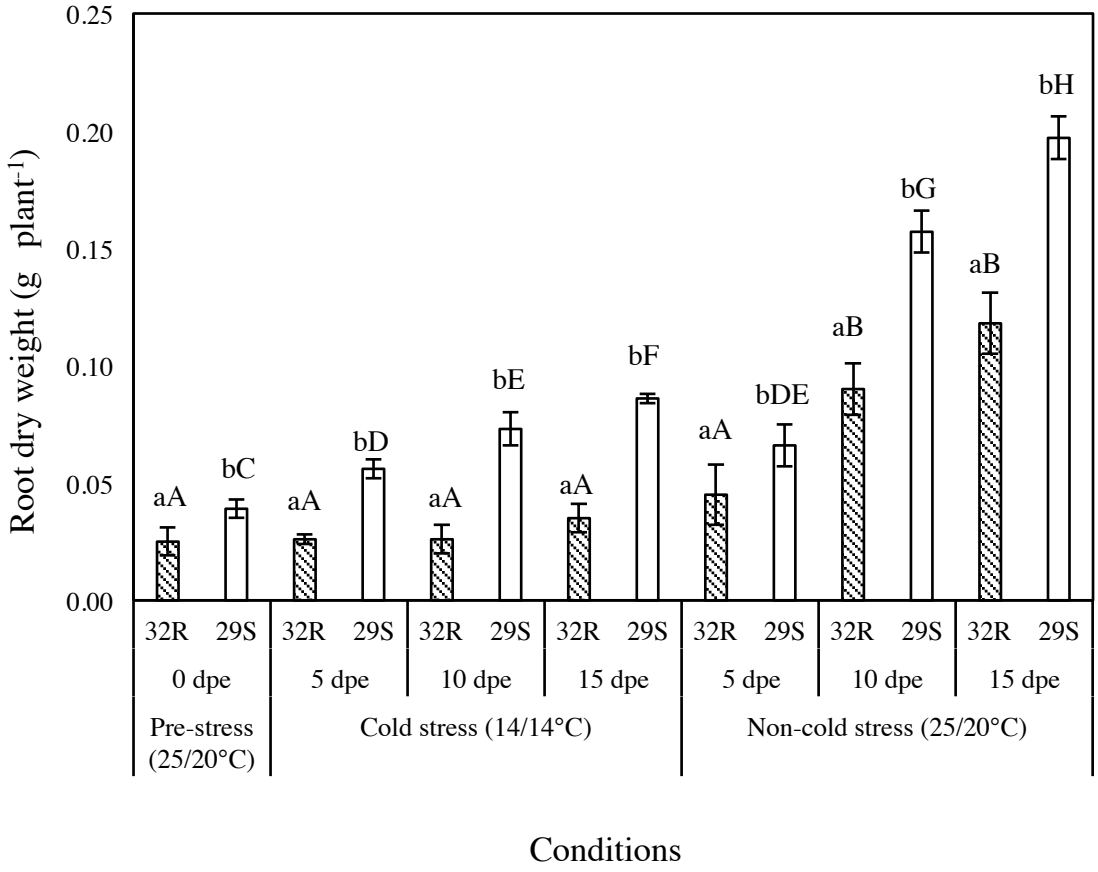

Fig. 3.1. Effects of cold stress on root dry weight (M ± SD, n = 6). Different letters indicate significant differences between 32R and 29S at $P < 0.05$ by Tukey's HSD test. Small letters indicate comparison between genotypes. Capital letters indicate comparison among conditions.

3. 2. Effects of cold stress on soluble protein contents

Soluble protein contents were lower under cold stress than under non-cold stress conditions (Fig. 3. 2). Soluble protein contents were lower in 32R than in 29S under pre-treatment and cold stress conditions, whereas they were higher in 32R than in 29S under non-cold stress conditions. Soluble protein contents of 32R decreased under cold stress in comparison with non-cold stress conditions by 13, 10 and 10% at 5, 10 and 15 dpe, respectively, whereas those of 29S decreased by 6, 10 and 5% at 5, 10 and 15 dpe, respectively under similar conditions (Fig. 3.2).

3. 3. Effects of cold stress on FAA contents

In contrast to the root dry biomass and soluble protein contents, the contents of FAAs were higher under cold stress than under non-cold stress conditions and were lower in 32R by 66 and 48% compared with 29S at 5 and 10 dpe, respectively, although no differences were detected at 15dpe (Fig. 3.3). Furthermore, the contents of FAAs of 32R increased by 122 and 225% at 10 and 15 dpe under cold stress conditions in comparison with under non-cold stress conditions, respectively (Fig. 3. 3).

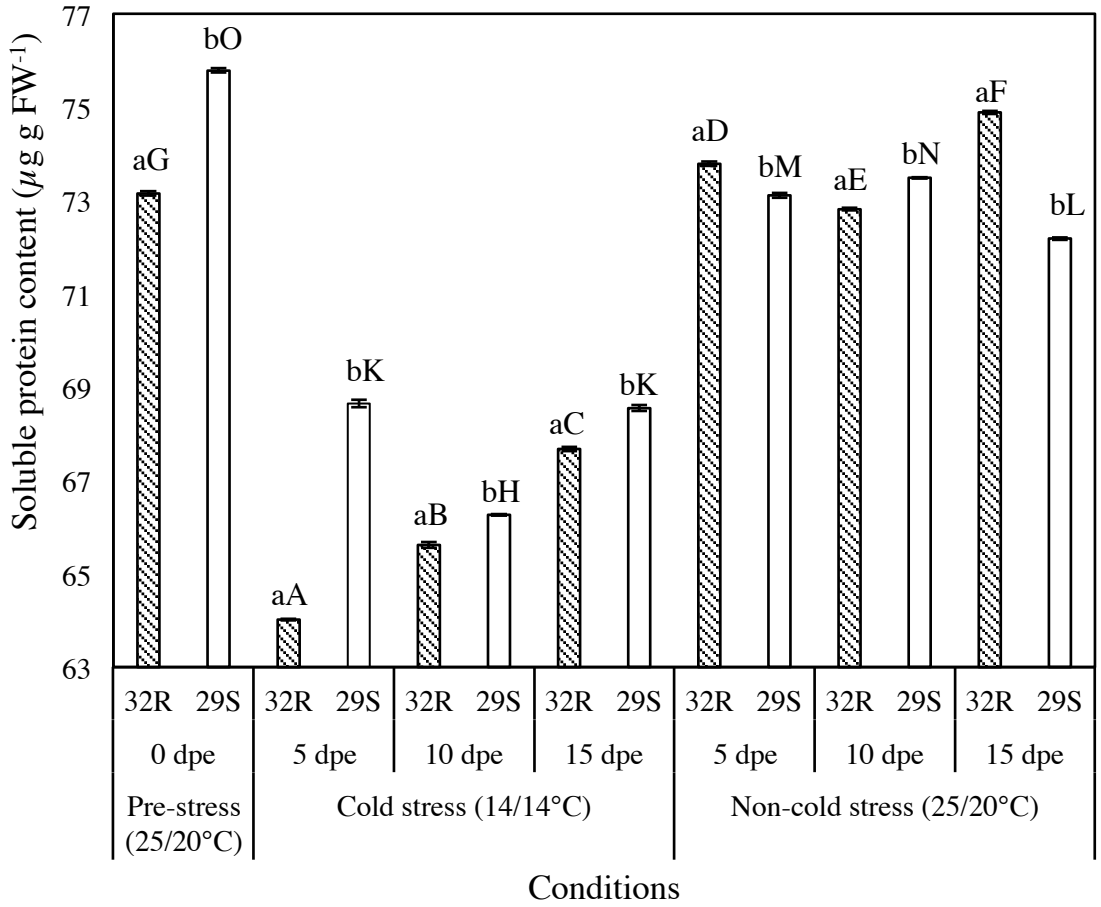

Fig. 3.2. Effects of cold stress on root soluble protein contents (M ± SD, n = 5). Symbols in the figures are similar to Fig.1. The absence of a bar indicates that the SD was within the bar of the chart.

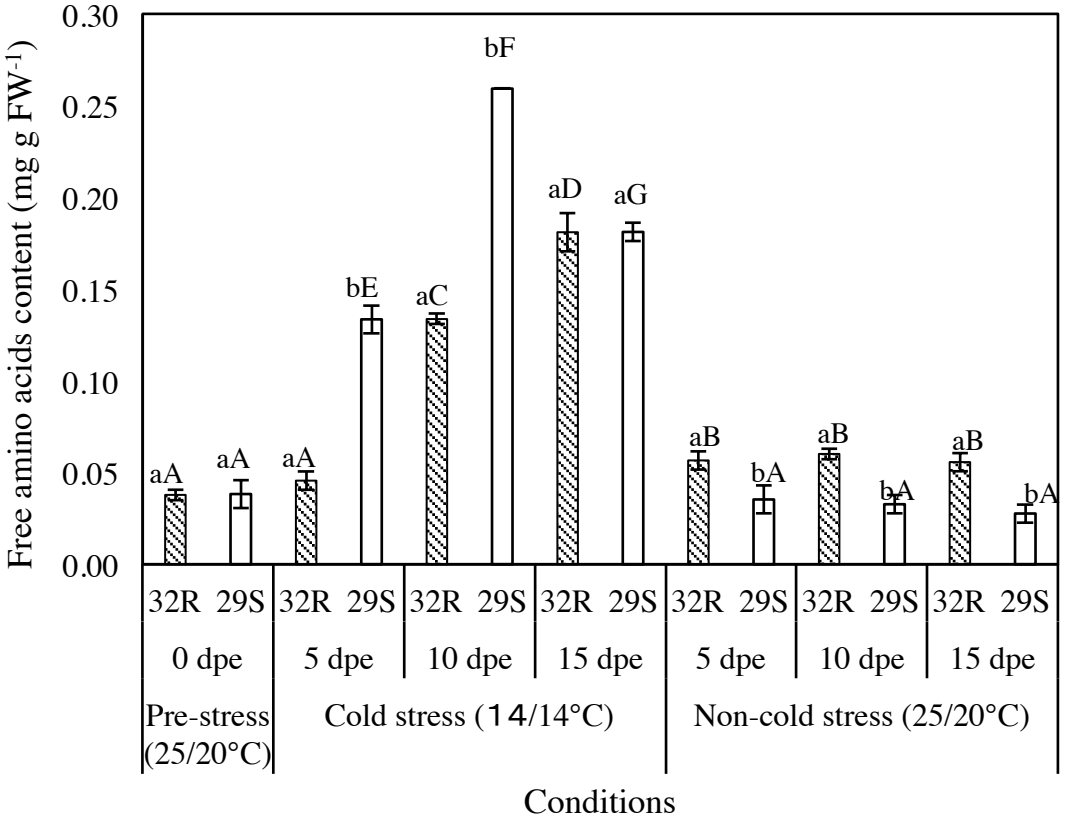

Fig. 3.3. Effects of cold stress on root free amino acids contents (M ± SD, n = 3). Symbols in the figures are similar to Fig.1.

4. Discussion

Cold stress limited the rates of photosynthesis, thus limiting the plant growth (Chapter 2). In the present study, the root growth of 32R and 29S were examined in response to cold stress with different time-points. The results of this study indicated that the root growth and soluble protein contents of both rice genotypes decreased in response to cold stress conditions (Figs 3.1 and 3.2), but the contents of FAAs in roots of both rice genotypes increased under similar conditions (Fig. 3.3). The results of two-way ANOVA indicated that root growth, the contents of soluble proteins and FAAs were affected by the factors as follow: growth conditions, time courses and genotypes and by the interactions of these factors (Table 3.1).

The root growth decreased in response to cold stress, and was lower in 32R than in 29S (Fig. 3.1). The 32R was known to be cold-sensitive rice genotype, whereas 29S is cold-tolerant rice genotype (Chapter 2), though 32R and 29S were developed from the same parent (Tetep × CN_4-4-2). The analysis of genetic relation also indicated that 32R was 45% similar to Tetep - *indica*, while 29S shows a 91% similar to Nb - *japonica* (Gaihre, 2015). The *indica* genotypes are adapted to tropical zones, while the *japonica* genotypes are adapted to subtropical zones (Garris et al., 2005). Thus it is possible that the lower value of the root dry weight of 32R was due to the incorporation of the cold-sensitive characteristics of the *indica* genotype of Tetep during crossing.

Table 3.1. A two-way ANOVA of temperature, time course, genotype and their interactions effects on root dry weight, concentration of free amino acids and soluble protein of 32R and 29S.

Parameter	F and (*P*-value)						
	Temperature (T)	Time Course (TC)	Genotype (G)	T*TC	TC*G	T*G	T*TC*G
Free amino acids	143.39 (<0.001)	14.76 (<0.001)	6.06 (0.02)	17.80 (<0.001)	4.33 (0.046)	27.27 (<0.001)	3.18 (0.08)
Soluble protein	394.81 (<0.001)	17.39 (<0.001)	0.57 (0.46)	0.46 (0.50)	9.04 (0.006)	0.63 (0.44)	3.64 (0.07)
Root dry weight	116.00 (<0.001)	45.30 (<0.001)	37.52 (<0.001)	17.84 (<0.001)	7.60 (0.01)	9.04 (0.006)	0.95 (0.33)

Where T*TC is interaction between growth condition and time course, TC*G is interaction between time course and genotype, T*G is interaction between growth condition and genotype, and T*TC*G is interaction of three factors including growth condition, time course and genotype.

Furthermore, soluble protein contents of roots in this study showed decrease in response to cold stress (Fig. 3.2). These findings are in contrast to those in studies showing that cold stress induced larger amounts of soluble proteins in many plants (Antikainen and Pihakaski, 1994; Cloutier, 1983; Karimzadeh et al., 2000; Terzioglu and Ekmekci, 2004). These differences could be related to degree of cold tolerance of the genotypes studied. Present study also indicated that soluble protein contents were lower in 32R than in 29S. As discussion above, 32R is cold-sensitive rice genotype, whereas 29S is cold-tolerant rice genotype. In addition, the findings of Terzioglu and Ekmekci (2004) who reported that soluble protein contents in the root of cold-tolerant wheat genotypes showed higher than those of cold-sensitive wheat genotypes. Thus, these findings suggest that synthesis and accumulation of these proteins may be related to the degree of cold tolerance of different genotypes.

In contrast to the root dry weight and protein contents, the contents of FAAs were higher in cold stress than non-cold stress conditions and were lower in 32R by 66 and 48% than in 29S at 5 and 10 dpe, respectively, but showed no difference at 15dpe (Fig. 3.3). Furthermore, the contents of FAAs of 32R increased by 122 and 225% at 10 and 15 dpe under cold stress in comparison with under non-cold stress conditions, respectively (Fig. 3.3). The results suggest that the differences in roots dry weight between 32R and 29S were clearly associated with the differential responses to cold stress. Zhao et al. (2013)

indicated that the chilling-sensitive *indica* rice variety IR29, which had less AAs than chilling-tolerant Lijiangxintuanhegu, a *japonica* rice variety. In addition, the contents of FAAs have been commonly reported to increase after cold treatment in many plants (Benina et al., 2013; Davey et al., 2009; Kaplan et al., 2004; Naidu et al., 1991; Zhao et al., 2013). However, it remains to be determined how the higher FAA contents contribute directly or indirectly to plant response to cold stress. The present findings also indicated that the FAAs were not different between 32R and 29S at 15 dpe under cold stress, whereas the soluble protein contents showed a significant difference at 15 dpe under cold stress. These results suggest that the changes in the FAA contents are not always directly associated with those of soluble protein contents, especially at later times following cold treatment.

The QTL analyses enabled to identify in 32R a gene located on the long arm of chromosome 7 that encodes cytokinin-*O*-glucosyltransferase (Gaihre, 2015). This enzyme which catalyzes the conjugation between cytokinin and *O*-glucoside in the process of glycosylation (Mok et al., 2005; Pineda Rodó et al., 2008), could transfer sugar moieties from activated donor molecules to specific acceptor molecules such as phytohormones and amino acids, and was reported to participate in stress adaptation such as defence against pathogen infections (von Saint Paul et al., 2011). As cytokinin is a phytohormone related to the nitrogen metabolism (Sakakibara et al., 2006), the increase of free amino acids under cold stress may be related to the role of cytokinin-*O*-glucosyltransferase. Based on the results, further studies should be carried out to elucidate the amino acid

metabolism of 32R, especially in response to cold stress.

In conclusion, root dry weight and protein contents of two rice genotypes decreased under cold stress conditions in comparison with non-cold stress conditions, and the cold-tolerant genotype, 29S showed higher values of root dry weight and protein contents than the cold-sensitive genotype, 32R. In contrast, synthesis and accumulation of free amino acids dramatically increased under cold stress in comparison with non-cold stress conditions, with a higher accumulation of free amino acids in 29S than in 32R. Although rice root growth decreased under cold stress conditions, the synthesis and accumulation of free amino acids in rice roots were related to the plant response to cold stress conditions, promoting the identification of genetic regulators for future rice breeding and metabolic engineering strategies.

5. Summary

The 32R is ShB-resistant with high yield potential, and is a cold-sensitive rice genotype. The accumulations of soluble proteins and free amino acids in the roots of 32R in response to cold stress were examined in comparison with 29S. The present findings showed the root dry weight value and protein contents of two rice genotypes decreased under cold stress conditions and were lower in 32R than in 29S. In contrast, the contents of FAAs dramatically increased under cold stress conditions and were lower in 32R than in 29S. These results indicated that existing the cold-sensitive characteristics and a QTL of ShB resistance encoding cytokinin-*O*-glucosyltransferase in 32R caused to limit the root growth and

contrasted the accumulations of soluble proteins and FAAs in root of both genotypes in response to cold stress.

CHAPTER 4

Metabolic profiling of cationic metabolites in root of the sheath blight-resistant rice genotype 32R under cold stress

1. Introduction

Cold stress is one of climate change factors affecting plant growth. Effects of temperature on the growth responses of ShB-resistant rice genotype 32R in comparison with those of ShB-susceptive rice genotype 29S have been investigated (Chapter 2). The results in Chapter 2 indicated that the dry weight at a low temperature was lower in 32R than in 29S and correlated strongly with root dry weight. The root dry weight was lower in 32R than in 29S (Chapter 3), whereas the leaf dry weight was not different between 32R and 29S. The limitations in roots under low temperature may cause to diminish the contents of the rubisco and chlorophyll relating to lower value of the photosynthetic rates, thus induce limiting plant growth of 32R in comparison with 29S (Chapter 2). In addition, many studies have been conducted on the genomic and proteomic levels

of plants in response to cold stress (Barah *et al*., 2013; Chinnusamy *et al*., 2007; Chinnusamy *et al*., 2010; Cui *et al*., 2005; Kreps *et al*., 2002), whereas few studies have been conducted on the metabolic levels of plants in response to cold stress. Metabolic levels of plants, such as determining the profiling of amino acids (AAs), organic acids (OAs) and sugars, display differentially in response to cold stress (Benina *et al*., 2013; Davey *et al*., 2009; Pathak *et al*., 2014; Zhao *et al*., 2013).

Roots and leaves are different sensitivity to abiotic stress, because they have different functions: leaves essentially have a photosynthetic function, whereas roots take up water and nutrients (Hibberd and Quick, 2002). Thus, leaves and roots may compete and collaborate for the functions that a plant achieves (Zerihun *et al*., 1998). Many metabolites that contribute to these functions, such as metabolites relating to the AA metabolism, were synthesized in leaves, allocated and used in large amounts in roots under abiotic stress (Huang *et al*., 2012). For example: the results of study on the common C_3 grasses *Alopecurus pratensis* and *Holcus lanatus* under drought stress by Gargallo-Garriga *et al*. (2014) who reported that the differences in metabolic responses between shoots and roots are because of their different functions: metabolites in shoots were deactivated during drought stress to diminish the intake of water and nutrients, while metabolites in roots were stimulated to boost the uptake of water and nutrients. The results in metabolic response of rice roots to cold stress also confirmed that root metabolites, FAA contents, were increased by ranging from

122 to 225% in comparison with non-cold stress (Chapter 3). In addition, a gene encoding cytokinin-*O*-glucosyltransferase located on chromosome 7 of 32R was identified as one of QTLs for ShB resistant (Gaihre and Nose, 2011 and Gaihre, 2015). This enzyme which was catalyzed the interaction between phytohormones and AAs (Li *et al*., 2015; von Saint Paul *et al*., 2011). Furthermore, as well known, the cytokinins are the phytohormones relating to the nitrogen metabolism (Sakakibara *et al*., 2006). However, no study has been conducted to investigate the response of AAs in root of 32R in response to cold stress.

The amino acid analysis, established by Spackman *et al*. (1958) using ion-exchange chromatography followed by post-column derivatization with ninhydrin, allowed for the first practical analysis of the AAs. Recently, the amino acid analysis is performed by using HPLC or GC-TOF/MS. However, both HPLC and GC-TOF/MS remain some challenges, such as larger amount of protein or peptide requirement in HPCL and difficult-comprehensive analysis of all AAs in GC-TOF/MS (Williams *et al*., 2007), and many AAs can not be detected by GC-TOF/MS (Noctor *et al*., 2007) in compared with calibrate electrophoresis-time of flight-mass spectrometry (CE-TOF/MS) (Williams *et al*., 2007). Thus, CE-TOF/MS is a novel strategy to analyze and differentially displays metabolic profiling of cationic and anionic metabolites. The CE-TOF/MS is unrequired derivatization prior to injection and used minimal sample preparation. More recently, many studies demonstrated the potential of CE-

TOF/MS method to identify the metabolites in plant (Maruyama *et al*., 2014; Ramautar *et al*., 2015; Yamakawa and Hakata, 2010).

The root growth decreased, but the contents of FAAs in roots increased under cold stress (Chapter 3). Genetic relationship analysis indicated that 32R is similar to Tetep-*indica*, while 29S is similar to Nb-*japonica*, though 32R and 29S were developed from the same parents (Tetep and CN_4-4-2) (Gaihre, 2015). This study complements and extends the hypothesis in the Chapter 3 that roots would present a contrasting metabolism in response to cold stress due to their different physiological functions in the cold stress. The seedlings of both 32R and 29S at the 4^{th} leaf stage were subjected to a temperature of 14/14°C (day/night) for 5, 10 and 15 dpe in a growth chamber and the utilities of CE-TOF/MS were used to analyze the metabolic profiling of cationic metabolites in the roots of 32R in comparison with 29S to test this hypothesis.

2. Materials and Methods

2. 1. Plant materials and growth conditions

Plant materials and growth conditions were done exactly as described in Chapter 3. Briefly, the rice seeds of 32R and 29S were sterilized by insecticide and bactericide. Twelve seeds were sown in a plastic pot (20 × 26 × 10 cm) filled with a 1:1 ratio of peat moss to vermiculite. Plants were grown under with

25/20°C, relative humidity of 70% and PPFD at plant level of 420 μmol m^{-2} s^{-1}. After the initial 14 days of growth, the plants were fertilized weekly with a half-strength concentration of the basal nutrient solution (Makino *et al*., 1988) with slight modifications. Half of the seedlings at the 4^{th} leaf stage were transferred to a growth chamber with a temperature of 14/14°C for 5, 10 and 15 dpe, and the other conditions were similar to those at the initial growth stage. All the experiments were conducted with three replications.

2. 2. Samples preparation for CE-TOF/MS

The fine powder of rice root (30 mg) was weighed and mixed with 0.5 mL ice-cold methanol containing internal standard-1 (H3304-1002; Human Metabolome Technologies, Tsuruoka, Japan) by being vortexed for 30 seconds. Internal standard-1 contains one positive ion (m/z = 182.048) and one negative ion (m/z = 231.070) for calibration of the quantification of mass spectrometry. The homogenate was mixed with 0.2 mL of ice-cold Milli-Q water and 0.5 mL of chloroform by being vortexed for 30 sec and were then centrifuged at 2300 × g at 4°C for 5 minutes. The separated methanol–water layer (0.4 mL) was ultrafiltrated (Ultrafree-MC-PLHCC, PLHCC (5K), UFC3LCCNB, Merck Millipore Ltd., Darmstadt, Germany) to remove proteins and macromolecules prior to capillary electrophoresis equipped with a time-of-flight-mass spectrometry (CE-TOF/MS) analysis by centrifuge at 7900 × g at 4°C for at least 3 hours. The filtrated solution was lyophilized, suspended in Milli-Q water

containing internal standards-3 (H3304-1004; Human Metabolome Technologies, Tsuruoka, Japan) and stored at –80°C until use. Internal standard-3 contains two positive ions (m/z = 87.0912 and m/z = 192.1383) and two negative ions (m/z = 209.0092 and m/z 150.9783) for calibration of the migration time for CE.

2. 3. Instrumentation

CE-TOF/MS was performed by using an Agilent G7100A Capillary Electrophoresis (CE) system equipped with an Agilent G6224A Time of Flight mass spectrometry, an Agilent 1100 isocratic high-performance liquid chromatography (HPLC) pump, an Agilent G1603A CE-MS adapter kit, and an Agilent G1607A CE-ESI-MS sprayer kit (Agilent Technologies, Waldbronn, Germany). The system was controlled by Agilent G2201AA ChemStation software version B.05.00 for CE (Agilent Technologies).

2. 4. CE-TOF/MS conditions

A fused silica capillary (H3305-2002, Human Metabolome Technologies-HMT), sheath liquid (H3301-1020, HMT) and cationic buffer solution (H3301-1001, HMT) were used in the analysis. The applied voltage was set at +27 kV, electrospray ionization was operated in the positive polarity. The drying gas temperature was monitored at 300°C with rate of 7 L/min. Stoptime was 30

minutes. Flow and maximum pressure of pump were 1 mL/min and 150 bar. Electrospray ionization-mass spectrometry (ESI-MS) was conducted in the positive ion mode. Mass range of TOF spectra was scanned from 50 to 1,000 *m/z* with a rate of 1.5 cycles/s.

2. 5. Data analysis of CE-TOF/MS results

Raw data acquired from CE-TOF/MS was analyzed using the MassHunter Quantitative analysis software (Agilent Technologies). Signal peaks matching to isotopomers, adduct ions, and other product ions of known metabolites were omitted. All the signal peaks possibly related to authentic compounds were extracted, and their migration times (MTs) were normalized by using those of the internal standards. The alignment of peaks was then performed by comparing the *m/z* values and normalized MT values of the internal standards, in which the difference of ± 10 ppm and ± 0.5 min were permitted, respectively. Finally, peak areas were normalized against those of the internal standards. The resultant peak area values were further standardized by sample weight.

2. 6. Statistical analyses

The up- and down-regulations of measured metabolites in roots of both genotypes under different growth conditions were classified by using the hierarchical cluster. Data of measured metabolites at 5, 10 and 15 dpe were

averaged for each genotype in each growth condition. The significance of the differences in genotypes and growth conditions were examined by the analyses of variance (ANOVA) with $P < 0.05$. Differentially changed metabolites were defined as those showing significant relative intensity increase or decrease relate to their respective controls at $P < 0.05$ in ANOVA. Volcano plot analyses were performed using the base 10 logarithm of *P*-value and the base 2 logarithm of fold change (FC) of the metabolic data of each genotype in the different growth conditions and of each growth condition between genotypes to understand the co-variance structure of metabolites and to reveal the overall variation pattern of the metabolic responses. Two-way ANOVA was also examined to understand the effects of growth temperature conditions, genotypes and time courses on the variation pattern of metabolites. All the analyses were conducted by using R packages (https://cran.r-project.org).

In addition, the map of these up- and down- regulated metabolites were conducted by mapping of measured metabolites in the present study and using the pathway maps of KEGG pathway (http://www.genome.jp/kegg/) as reference.

3. Results

Total 81 cationic metabolites from root samples of 32R and 29S were detected under cold stress and non-stress conditions by using the CE-TOF/MS (Table 4.1). The chemical compositions of identified metabolites in root of 32R

were similar to those in root of 29S. Base on the chemical taxonomy, these identified metabolites belong to many different subclasses, but major metabolites (48%) belong to the group of AAs, peptides and analogues (Fig. 4.1).

Figure 4.2 showed the hierarchical cluster analysis of the up- and down-regulations of 81 metabolites. The columns showed the growth conditions, while the raw displayed the names of metabolites that were detected in root of 32R and 29S. The dendrogram at the left provides the relationships of cationic metabolites of genotypes under different growth conditions. The dendrogram was cutoff at level dissimilarity 2.0 based on Euclidean distance equally with similarity above 65% at $P < 0.05$ as described by Suzuki and Shimodaira (2006). All metabolites were classified into four clusters. Cluster I showed the up-regulated metabolites of 29S under cold stress. Cluster II indicated the up-regulated metabolites of 32R under cold stress. Cluster III indicated the up-regulated metabolites of 29S under non-cold stress. Cluster IV showed the up-regulated metabolites of 32R under non-cold stress. Number of up-regulated metabolites by cold stress was much more than that by non-cold stress. The 41% (33/81) in 32R and 72% (58/81) in 29S of identified metabolites showed increase under cold stress, whereas 19% (15/81) in 32R and 24% (19/81) in 29S of identified metabolites showed increase under non-cold stress. 58% (47/81) identified metabolites of 32R were lower concentration than those of 29S.

Table 4.1. Cationic metabolites in root of 32R and 29S were detected by CE-TOF/MS.

ID	Abbreviation	Compound	MF	m/z	MT
1	Gly	Glycine	$C_2H_5NO_2$	76.04	7.65
2	Ser	Serine	C_3H_7NO3	106.05	9.09
3	Thr	Threonine	$C_4H_9NO_3$	120.06	9.54
4	Cys	Cysteine	$C_3H_7NO_2S$	122.03	10.19
5	Gln	Glutamine	$C_5H_{10}N_2O_3$	147.07	9.75
6	Glu	Glutamic acid	$C_5H_9NO_4$	148.06	9.91
7	Asp	Aspartic acid	$C_4H_7NO_4$	134.04	10.43
8	Arg	Arginine	$C_6H_{14}N_4O_2$	175.12	6.58
9	Pro	Proline	$C_5H_9NO_2$	116.07	9.77
10	Lys	Lysine	$C_6H_{14}N_2O_2$	147.11	6.37
11	Ala	Alanine	$C_3H_7NO_2$	90.06	8.27
12	Asn	Asparagine	$C_4H_8N_2O_3$	133.06	9.51
13	Val	Valine	$C_5H_{11}NO2$	118.09	9.10
14	Ile	Isoleucine	$C_6H_{13}NO_2$	132.10	9.27
15	Leu	Leucine	$C_6H_{13}NO_2$	132.10	9.37
16	Phe	Phenylalanine	$C_9H_{11}NO_2$	166.08	10.01
17	Tyr	Tyrosine	$C_9H_{11}NO_3$	182.08	10.26
18	Try	Tryptophan	$C_{11}H_{12}N_2O_2$	205.10	9.97
19	Met	Methionine	$C_5H_{11}NO_2S$	150.06	9.72
20	His	Histidine	$C_6H_9N_3O_2$	156.08	6.75
21	HyPro	Hydroxyproline	$C_5H_9NO_3$	132.06	10.80
22	Ornit	Ornithine	$C_5H_{12}N_2O_2$	133.10	6.31
23	Ava	5-Aminovaleric acid	$C_5H_{11}NO_2$	118.08	6.33
24	HoSer	Homoserine	$C_4H_9NO_3$	120.06	9.17
25	Betaine	Betaine	$C_5H_{11}NO_2$	118.09	10.16
26	Sarc	Sarcosine	$C_3H_7NO_2$	90.06	8.64
27	PCho	Phosphorylcholine	$C_5H_{15}NO_4P$	184.07	18.20
28	Citr	Citrulline	$C_6H_{13}N_3O_3$	176.10	10.00
29	β-Ala	β-Alanine	$C_3H_7NO_2$	90.06	6.801
30	Carno	Carnosine	$C_9H_{14}N_4O_3$	227.11	6.28
31	GABA	Gamma-aminobutyric acid	$C_4H_9NO_2$	104.07	7.11
32	Ala2	Ala-Ala	$C_6H_{12}N_2O_3$	161.09	8.64
33	β-Tyr	β-Tyrosine	$C_9H_{11}NO_3$	182.08	7.98
34	DPA	D- phenylalanine	$C_{12}H_{17}NO$	192.14	18.63

35	Apro	1-Aminocyclopropane-1-carboxylic acid	$C_4H_7NO_2$	102.06	5.91
36	Mefon	Methionine sulfone	$C_5H_{11}NO_4S$	182.05	10.77
37	Mefox	Methionine sulfoxide	$C_5H_{11}NO_3S$	166.05	10.92
38	GSSG	Glutathione disulfide	$C_{20}H_{32}N_6O_{12}S_2$	307.08	10.94
39	GSH	Glutathione	$C_{10}H_{17}N_3O_6S$	308.09	11.80
40	Aglu	N-Acetylglucosamine	$C_8H_{15}NO_6$	222.10	19.50
41	Apen	1-Aminocyclopentanecarboxylic acid	$C_6H_{11}NO_2$	130.08	9.51
42	MeSer	2-Methylserine	$C_4H_9NO_3$	120.06	9.82
43	Disul	Disulfiram	$C_{10}H_{20}N_2S_4$	297.07	19.61
44	TMLys	N6,N6,N6-Trimethyllysine	$C_9H_{20}N_2O_2$	189.16	6.74
45	AP3	Triapine	$C_4H_{10}N_2$	87.09	4.20
46	Put	Putrescine	$C_4H_{12}N_2$	89.11	4.45
47	Spr	Spermine	$C_{10}H_{26}N_4$	203.22	4.25
48	Spm	Spermidine	$C_7H_{19}N_3$	146.16	4.29
49	Amet	S-Adenosylmethionine	$C_{15}H_{22}N_6O_5S$	399.14	6.61
50	Adeni	Adenine	$C_5H_5N_5$	136.06	7.06
51	Hyxan	Hypoxanthine	$C_5H_4N_4O$	137.05	10.08
52	Guan	Guanine	$C_5H_5N_5O$	152.06	7.67
53	Guas	Guanosine	$C_{10}H_{13}N_5O_5$	284.10	11.26
54	Inos	Inosine	$C_{10}H_{12}N_4O_5$	269.09	16.77
55	Adeno	Adenosine	$C_{10}H_{13}N_5O_4$	268.10	9.01
56	DAde	2'-Deoxyadenosine	$C_{10}H_{13}N_5O_4$	252.11	9.19
57	Cyti	Cytidine	$C_9H_{13}N_3O_5$	244.09	8.85
58	Cyto	Cytosine	$C_4H_5N_3O$	112.05	6.72
59	Urac	Uracil	$C_4H_4N_2O_2$	113.03	18.73
60	Thym	Thymine	$C_5H_6N_2O_2$	127.05	18.73
61	Thymd	Thymidine	$C_{10}H_{14}N_2O_5$	243.10	18.73
62	Urid	Uridine	$C_9H_{12}N_2O_6$	245.07	18.75
63	Allan	Allantoin	$C_4H_6N_4O_3$	159.06	17.56
64	Chol	Choline	$C_5H_{14}NO$	104.11	6.37
65	Bald	Betaine aldehyde	$C_5H_{12}NO$	120.10	6.91
66	DMGly	N,N-Dimethylglycine	$C_4H_9NO_2$	104.07	9.88
67	Creati	Creatine	$C_4H_9N_3O_2$	132.07	8.11
68	Crnine	Creatinine	$C_4H_7N_3O$	114.07	6.77
69	AAA	2-Aminoadipic acid	$C_6H_{11}NO4$	162.07	9.19
70	GPCho	Glycerophosphocholine	$C_8H_{21}NO_6P$	258.11	19.15
71	Anth	Anthranilic acid	$C_7H_7NO_2$	138.05	9.70

72	Pheea	Phenylethanolamine	$C_8H_{11}NO$	138.09	6.81
73	Tyra	Tyramine	$C_8H_{11}NO$	138.09	7.64
74	DMTA	5'-Deoxy-5'-methylthioadenosine	$C_{11}H_{15}N_5O_3S$	298.09	8.48
75	PiP	Piperidine	$C_5H_{11}N$	86.10	6.15
76	Pyri	Pyridine	C_5H_5N	80.05	6.84
77	Sero	Serotonin	$C_{10}H_{12}N_2O$	177.10	8.21
78	Glusa	Glucosaminic acid	$C_6H_{13}NO_6$	196.09	9.67
79	Mmet	S-Methylmethionine	$C_6H_{15}NO_2S$	164.07	6.97
80	Octy	Octylamine	$C_8H_{19}N$	130.16	8.20
81	IbuA	Isobutylamine	$C_4H_{11}N$	74.10	5.69

MF: molecular formula, m/z: molecular weight, and MT: migration time.

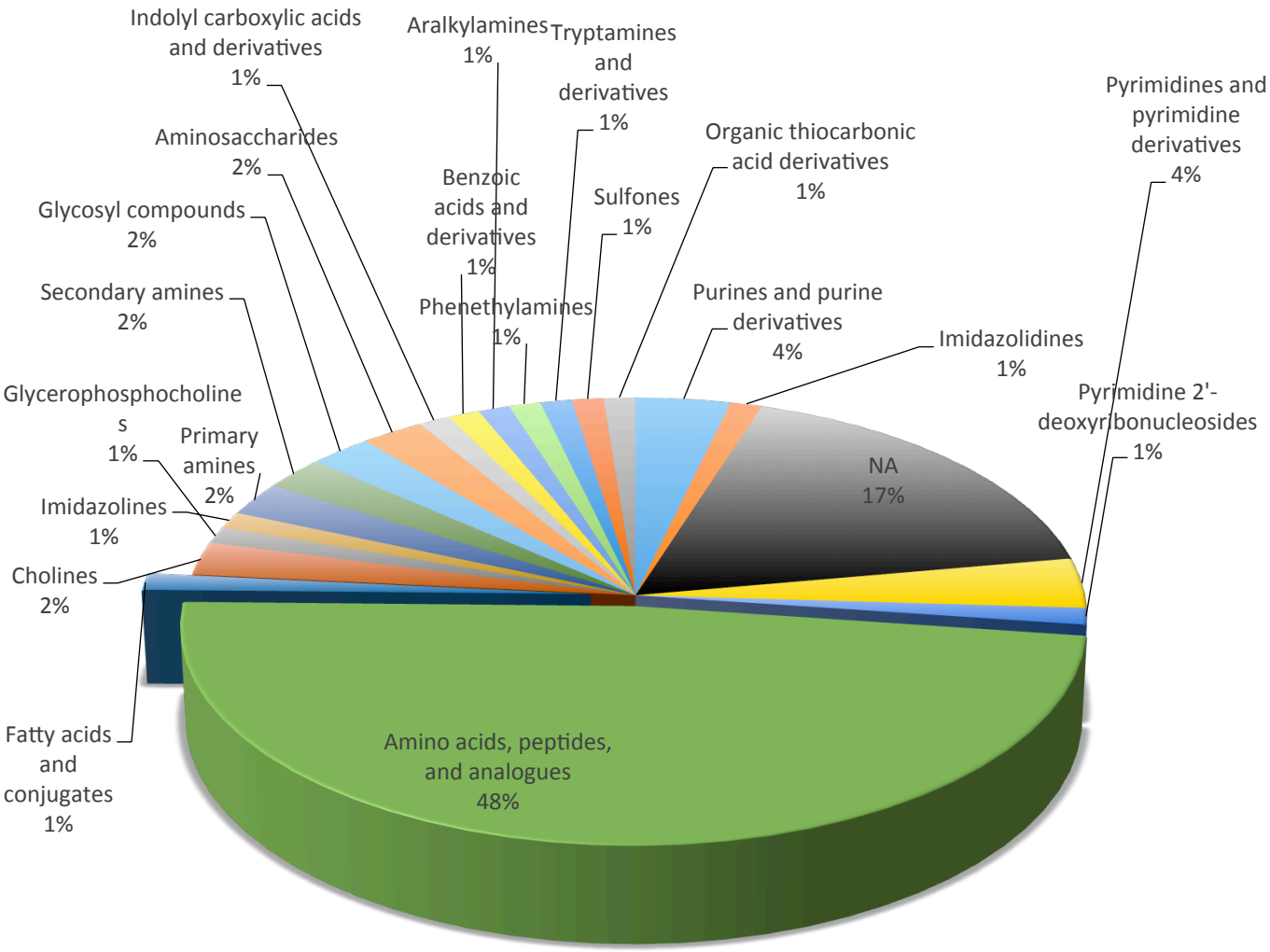

Fig. 4.1. Classification of detected metabolites of 32R and 29S based on chemical taxonomy of the human metabolome database.

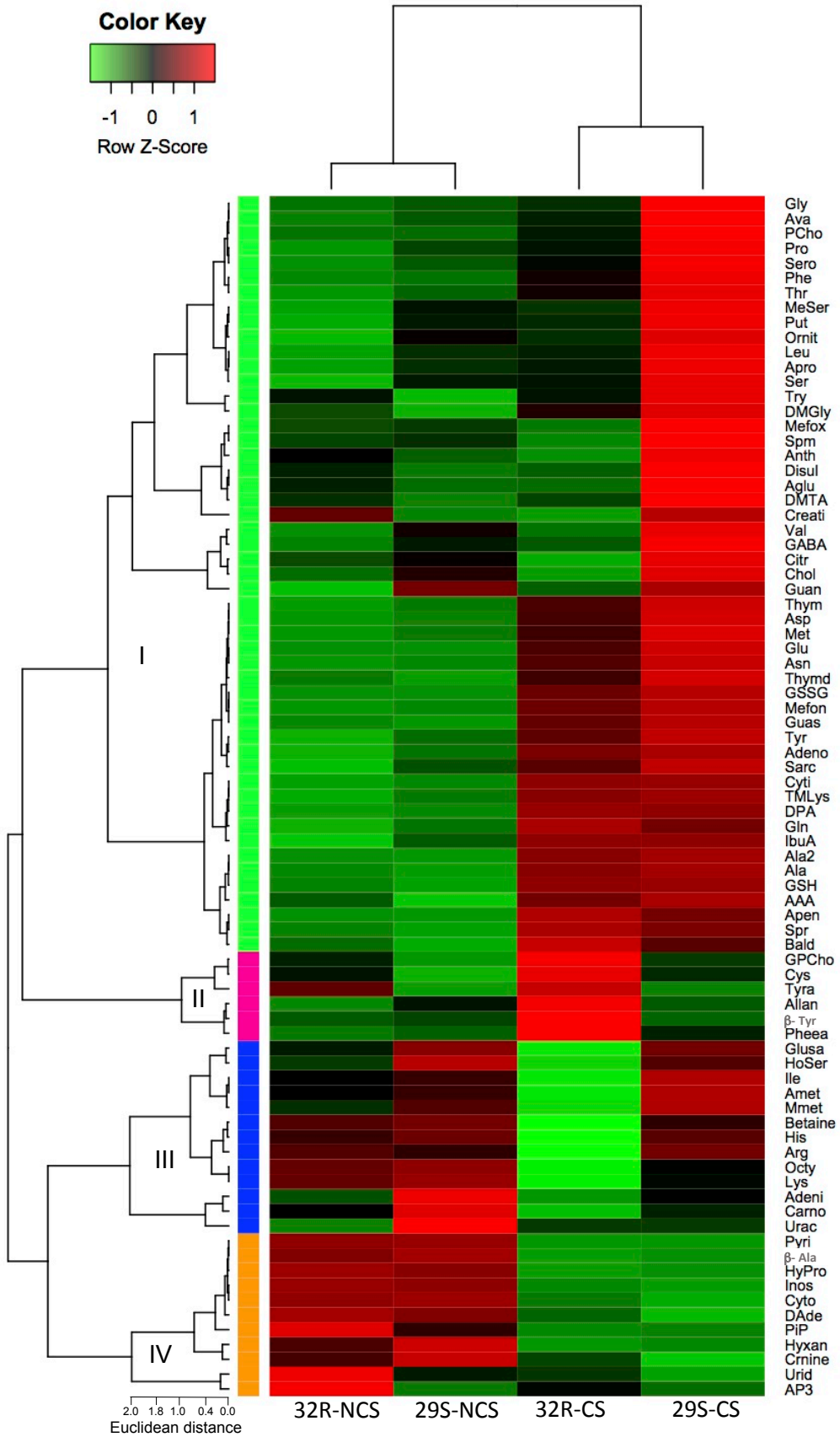

Fig. 4.2. Hierarchical cluster of detected cationic metabolites of 32R and 29S in cold stress (CS) and non-cold stress (NCS). Data is average of metabolites that obtained at 5 dpe, 10 dpe and 15 dpe. The dendrogram at the left provides a measure of the relationship of cationic metabolites in each sample and growth conditions. Each row represents a separate metabolite and each column a separate sample in each growth condtion. Different colors in dendrogram indicate relationship of metabolite group. Red and green colors indicate the up- and down-regulation of metabolites, respectively. The Roman numerals indicate the different cluster. Full name of the metabolites as defined in Table 4.1.

Figure 4.3 showed the volcano plots of differentially regulated metabolites of genotypes under different growth conditions. The fold change of metabolites in root of 32R and 29S under cold stress and non-cold stress conditions were different. Nine and six metabolites of 32R and 29S were changed under cold stress in comparison with non-cold stress conditions with FC > 2 and $P < 0.05$, respectively (Figs. 4.3-A, 4.3-B and Table 4.2). In 32R, there were seven up-regulated metabolites, serine (Ser), glutamine (Gln), glutamic acid (Glu), aspartic acid (Asp), asparagine (Asn), β-tyrosine (β-Tyr), and glutathione disulfide (GSSG) and two down-regulated metabolites, β-alanine (β-Ala) and S-methylmethionine (Mmet) under cold stress in comparison with non-cold stress. While in 29S, there were five up-regulated metabolites, Gln, Glu, Asp, Asn and GSSG and only β-Ala was down-regulated metabolite under cold stress in comparison with non-cold stress. Among of these differentially regulated metabolites under cold stress in comparison with non-cold stress, three metabolites, Mmet, β-Tyr and Ser were found in 32R, but not found in 29S (Fig. 4.3-A, 4.3-B and Table 4.2). In addition, compared 32R with 29S, only Asp was different under non-cold stress, whereas five metabolites, Ser, Mmet, arginine (Arg), β-Ala and β-Tyr were different under cold stress (Figs. 4.3-C and 4.3-D).

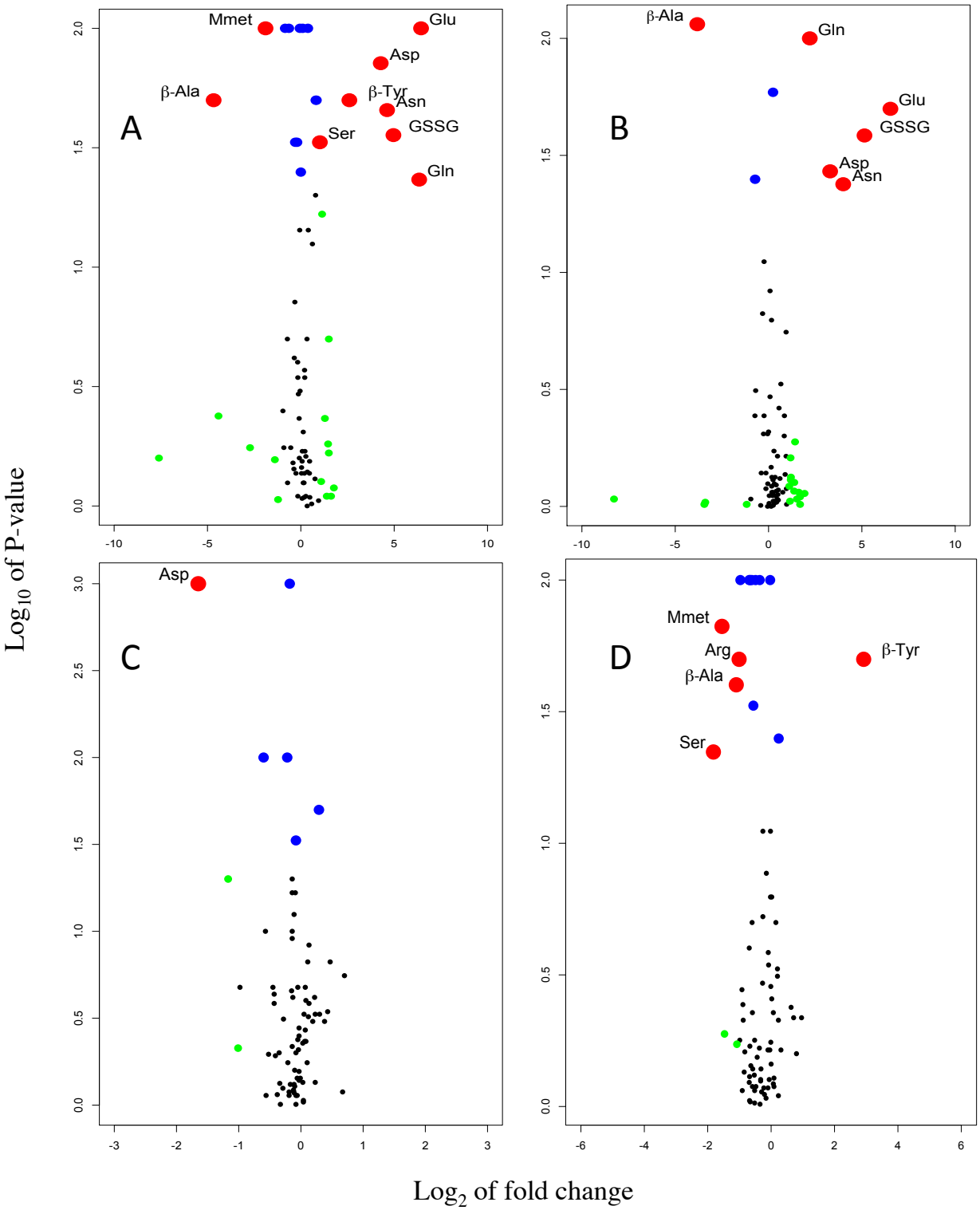

Fig. 4.3. Volcano plots of differentially regulated cationic metabolites. Comparison: A, between cold stress (CS) and non-cold stress (NCS) of 32R; B, betwwen CS and NCS of 29S; C, between 32R and 29S under NCS; D, between 32R and 29S under CS. The metabolites with $\log_2$ of fold change ($\log_2$FC) > 1 and $P < 0.05$ showed in red closed circles, with $P < 0.05$ showed in blue closed circles, with $\log_2$FC > 1 showed in green closed circles, and with $\log_2$FC < 1 and $P > 0.05$ showed in black closed circles. Full name of the metabolites as defined in Table 4.1.

Figure 4.4 showed the relationships of regulated metabolites in root of 32R and 29S under cold stress in comparison with non-cold stress. Four up-regulated AAs, Asn, Asp, Gln and Glu, related with Urea and TCA cycles. These AAs belong to cluster I (Fig. 4.2). Other three up-regulated AAs, GSSG, Ser and β-Tyr, related with glycolysis pathway. These AAs belong to clusters I and II (Fig. 4.2). While two down-regulated AAs, β-Ala and Mmet, related with PAs group in the Urea cycle. These AAs belong to clusters III and IV (Fig. 4.2).

Figure 4.5 showed the relationships of regulated metabolites in the root of 32R in comparison with that of 29S under each growth condition, cold stress and non-cold stress. Two AAs, Asp and Arg, related with Urea and TCA cycles were lower concentration in 32R than in 29S (Figs. 4.3, 4.6 and Table 4.2). These AAs belong to cluster I (Fig. 4.2). Other two AAs, β-Ala and Mmet, related with PAs group in the Urea cycle were lower concentration in 32R than in 29S (Figs. 4.3, 4.6 and Table 4.2). These AAs belong to clusters III and IV (Fig. 4.2). Two more AAs, Ser and β-Tyr, related with glycolysis pathway; the concentration of Ser was lower in 32R than in 29S, whereas concentration of β-Tyr was higher in 32R than in 29S (Figs. 4.3, 4.6 and Table 4.2). These AAs belong to clusters I and II (Fig. 4.2).

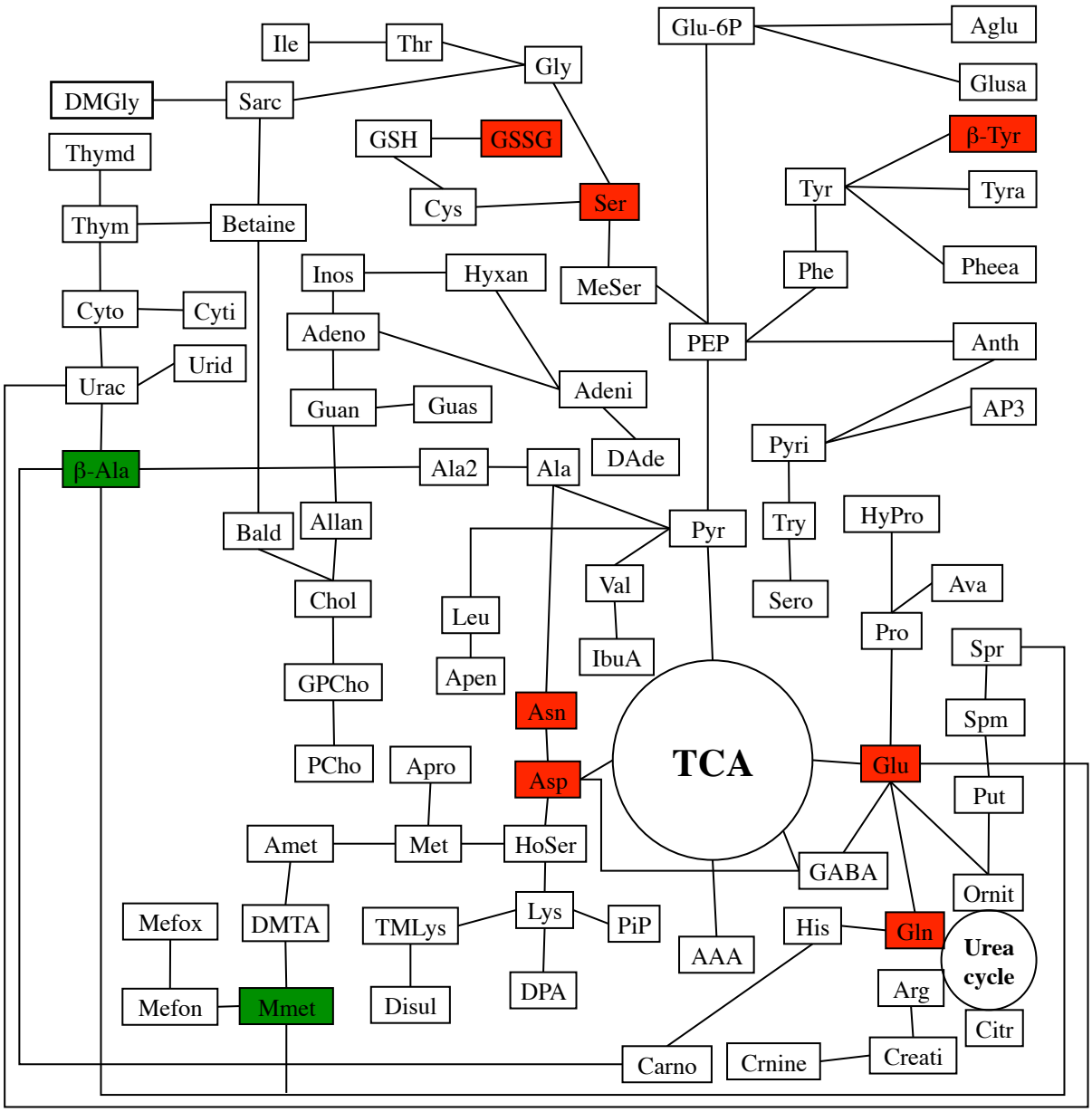

Fig. 4.4. Map of metabolic change under cold stress in comparison with non-cold stress condition derived from TCA and glycolysis pathway. Red and green show the up- and down-regulated metabolites. Full name of the metabolites as defined in Table 4.1.

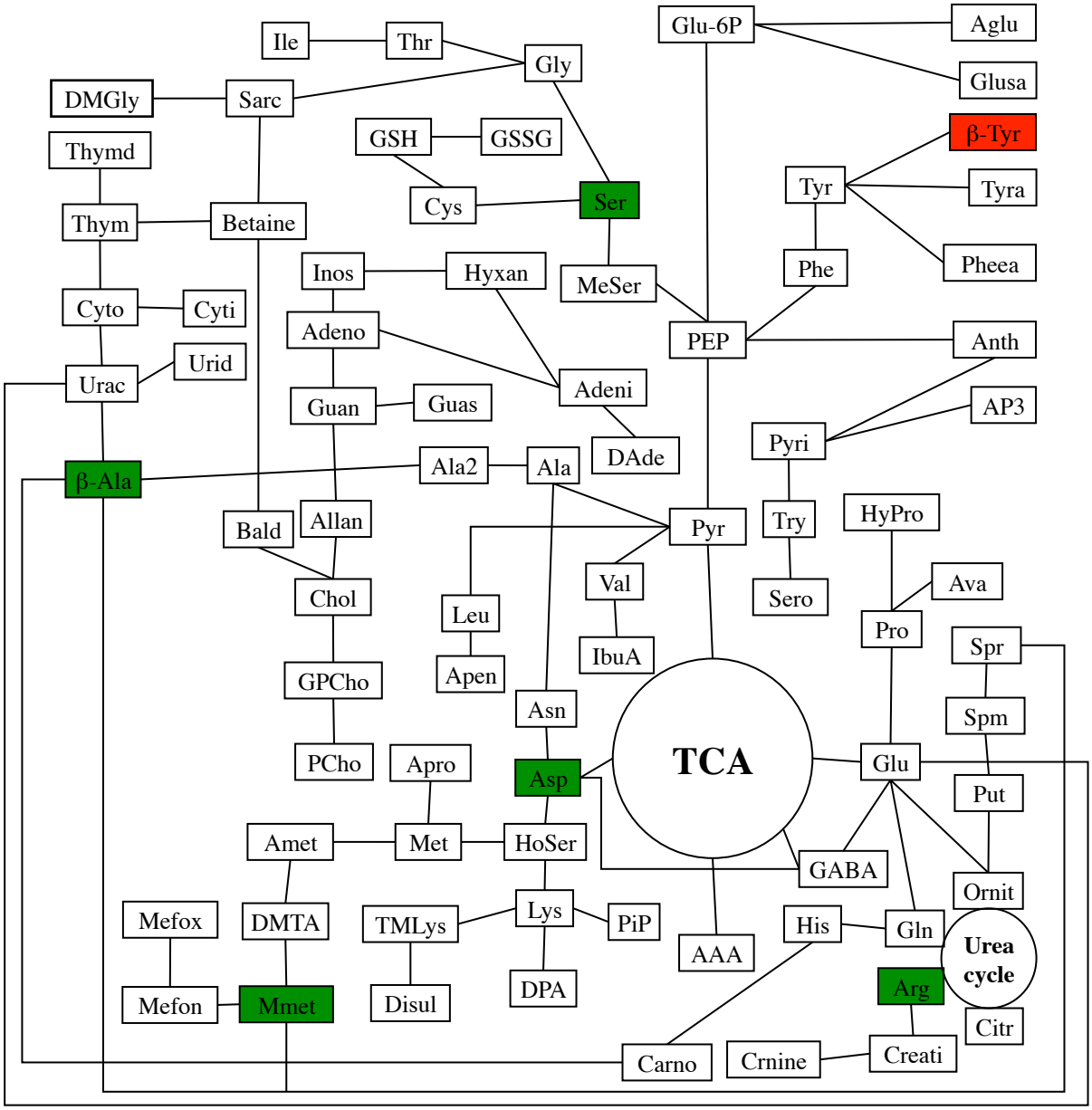

Fig. 4.5. Map of metabolic change between 32R in comparison with 29S under cold stress and non-cold stress condition derived from TCA and glycolysis pathway. Red and green show the up- and down-regulated metabolites. Full name of the metabolites as defined in Table 4.1.

Figure 4.6 showed the time course effects on the concentrations metabolites under cold stress and non-cold stress conditions. In comparison with non-cold stress, the contents of Ser, Asn, Gln, Glu, Asp and GSSG under cold stress increased in root of 32R by ranging from 1.2 to 205.2 times and in root of 29S by ranging from 1.1 to 122 times, whereas contents of β-Ala under cold stress decreased in root of 32R by ranging from 1.9 to 49.2 times and in root of 29S by 9.5 to 27.7 times (Figs. 4.6-D, 4.6-E, 4.6-F, 4.6-G, 4.6-H, 4.6-I and 4.6-A); contents of Mmet and Arg under cold stress decreased in root of 32R by ranging from 1.31 to 2.6 times, whereas increased in root of 29S by ranging from 1.1 to 1.5 times (Figs. 4.6-B and 4.6-J); and the content of β-Tyr increased in root of 32R by 7.8 times, whereas decreased in root of 29S by 4.3 times (Fig. 4.6-C). In addition, comparison among time courses, concentrations of β-Ala, Gln and Glu under cold stress increased in root of 32R by ranging from 1.04 to 1.07 times at 10 and 15 dpe, respectively, in comparison with 5 dpe and in root of 29S by ranging from 1.47 to 1.51 at 10 and 15 dpe, respectively, in comparison with 5 dpe (Figs. 4.6-A, 4.6-F and 4.6-G); concentrations of Ser, Asp and Arg under cold stress were higher in root of 32R at 10 dpe by ranging from 1.3 and 2.2 times than those at 5 and 15 dpe and in root of 29S at 10 dpe by 1.1 and 2.2 times than those at 5 and 15 dpe, whereas the concentrations of β-Tyr under cold stress were lower in root of 32R at 10 dpe by 17.1 and 20.8 times than those at 5 and 15 dpe, respectively and in root of 29S at 10 dpe by 1.3 and 2.8 times than those at 5 and 15 dpe, respectively (Figs. 4.6-H, 4.6-J and 4.6-D); in

the root of 32R under cold stress the concentrations of Mmet and Asn increased by 1.2 and 1.3 times at 10 and 15 dpe, respectively, in comparison with 5 dpe, whereas GSSG decreased by 1.2 and 1.4 times at 10 and 15 dpe, respectively, in comparison with 5 dpe (Figs. 4.6-B, 4.6-E and 4.6-I); and in the root of 29S the concentrations of Asn and GSSG increased at 10 dpe by ranging from 1.2 and 1.7 times in comparison with at 5 and 15 dpe, respectively, whereas the concentration of Mmet decreased at 10 dpe by 1.3 and 1.1 times in comparison with 5 and 15 dpe, respectively (Figs. 4.6-E, 4.6-I and 4.6-B).

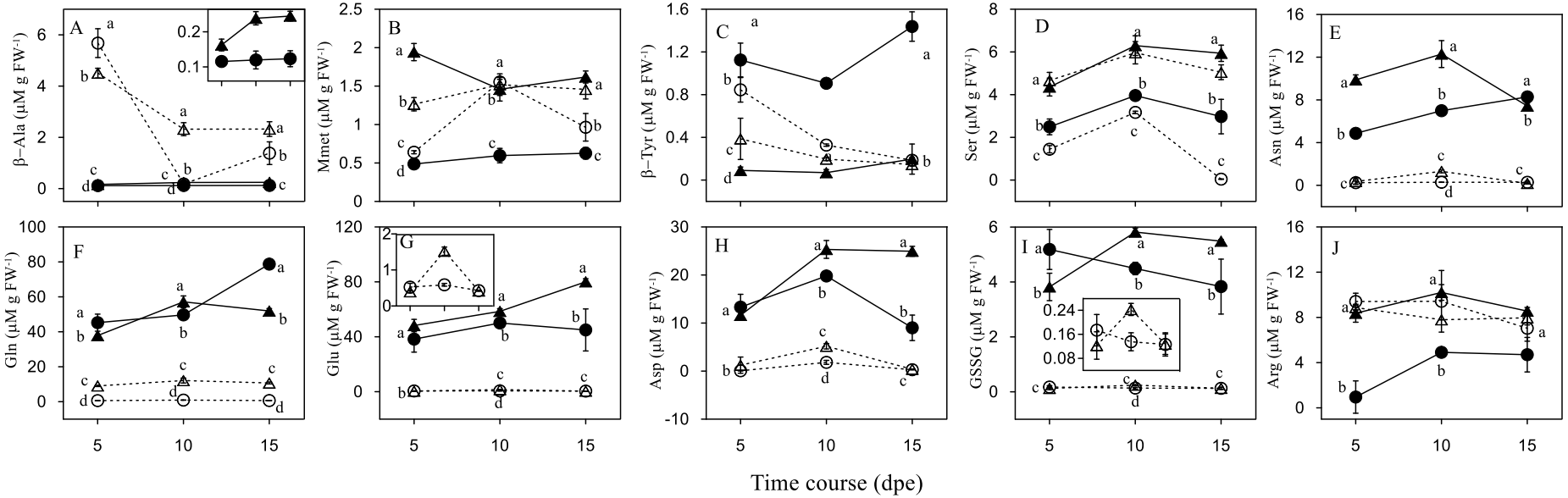

Fig. 4.6. Effects of time course on metabolites with significant changes based on volcano plot analysis in root of 32R and 29S under cold stress (●,▲) and non-cold stress condition (○,△). The values are the mean ± SD (n = 4). The absence of a bar indicates that the SD was within the size of the symbol. Different small letters indicate significantly at $P < 0.05$ by Tukey's test. Different capital letters indicate the different metabolites. Small graphs in the Figs. A, G and I described detail the difference between 32R and 29S for CS (A) and NCS (G and I) conditions. Full name of the metabolites as defined in Table 4.1.

4. Discussion

The two rice genotypes, 32R and 29S, are known to have contrasting cold tolerance at the phenotypic and physiological levels (Chapter 2) and accumulations of AAs in roots (Chapter 3). In this study, the CE-TOF/MS method was used to detail the changes that occur in the metabolites of root in the response to cold stress. Especially, metabolic profiling of cationic metabolites was conducted in order to achieve a comprehensive understanding of the dynamic metabolite networks during adaptation to cold stress. The present findings showed that 81 cationic metabolites including 48% AAs, peptides and analogues were identified in 32R and 29S under different growth conditions (Table 4.1 and Fig. 4.1). The relationship and level of these metabolites showed difference between 32R and 29S in the same growth condition and showed difference between cold stress and non-cold stress conditions of each genotype as shown in Fig. 4.2. In comparison with non-cold stress, nine metabolites, Ser, Gln, Glu, Asp, Asn, β-Tyr, GSSG, β-Ala and Mmet, were significantly changed in root of 32R and 29S under cold stress (Figs. 4.3 and Table 4.2). In addition, in comparison with 29S, the concentration of Asp under non-cold stress was lower in 32R, while the concentrations of Ser, Arg, Mmet and β-Ala under cold stress were lower in 32R, and the concentration of β-Tyr under cold stress was higher in 32R (Figs. 4.3, 4.6 and Table 4.2). The results of two-way ANOVA indicated that these metabolites were generally affected by the factors as follows: growth

Table 4.2. The significant changes of cationic metabolites under different conditions based on the volcano plot analysis.

Condition	Compound	Molecular formula	Molecular weight (m/z)	Migration time (min)	FC (abs)	Log2FC	*P*-value	Regulation
Pair CS and NCS of 32R (A)	β-Ala	$C_3H_7NO_2$	90.06	6.801	-25.528	-4.674	0.020	Down
	Mmet	$C_6H_{15}NO_2S$	164.07	6.970	-3.706	-1.89	0.010	Down
	β-Tyr	$C_9H_{11}NO_3$	182.08	7.980	6.063	2.6	0.020	Up
	Ser	$C_3H_7NO_3$	106.05	9.090	2.030	1.02	0.030	Up
	Asn	$C_4H_8N_2O_3$	133.06	9.510	24.590	4.62	0.020	Up
	Gln	$C_5H_{10}N_2O_3$	147.07	9.750	81.008	6.34	0.040	Up
	Glu	$C_5H_9NO_4$	148.06	9.910	86.823	6.44	0.010	Up
	Asp	$C_4H_7NO_4$	134.04	10.430	19.430	4.28	0.010	Up
	GSSG	$C_{20}H_{32}N_6O_{12}S_2$	307.08	10.940	31.125	4.96	0.030	Up
Pair CS and NCS of 29S (B)	β-Ala	$C_3H_7NO_2$	90.06	6.801	-14.123	-3.82	0.010	Down
	Gln	$C_5H_{10}N_2O_3$	147.07	9.750	4.627	2.21	0.010	Up
	Glu	$C_5H_9NO_4$	148.06	9.910	92.410	6.53	0.020	Up
	Asn	$C_4H_8N_2O_3$	133.06	9.510	16.000	4.00	0.040	Up
	Asp	$C_4H_7NO_4$	134.04	10.430	9.849	3.30	0.040	Up
	GSSG	$C_{20}H_{32}N_6O_{12}S_2$	307.08	10.940	35.261	5.14	0.030	Up
Pair 32R and 29S in NCS (C)	Asp	$C_4H_7NO_4$	134.04	10.430	-3.138	-1.65	0.001	Down
Pair 32R and 29S in CS (D)	β-Tyr	$C_9H_{11}NO_3$	182.08	7.980	7.568	2.92	0.020	Up
	Mmet	$C_6H_{15}NO_2S$	164.07	6.970	-2.928	-1.55	0.015	Down
	Arg	$C_6H_{14}N_4O_2$	175.12	6.580	-2.014	-1.01	0.020	Down
	β-Ala	$C_3H_7NO_2$	90.06	6.801	-2.143	-1.10	0.025	Down
	Ser	$C_3H_7NO_3$	106.05	9.090	-3.531	-1.82	0.045	Down

A is comparative metabolites of 32R between cold stress (CS) and non-cold stress (NCS), B is comparative metabolites of 29S between CS and NCS, C is comparative metabolites under NCS between 32R and 29S, and D is comparative metabolites under CS between 32R and 29S. Negative charge (-) indicated the down-regulation. Full name of metabolites as defied in Table 4.1. FC: fold change, abs: absolute value, CS: cold stress, and NCS: non-cold stress

Table 4.3. Two-way ANOVA and interactions effects on the significant changes of metabolites based on the volcano plot analysis.

Parameter	F and (*P*-value)						
	Temperature (T)	Time Course (TC)	Genotype (G)	T*TC	TC*G	T*G	T*TC*G
β-Ala	90.05 (<0.001)	23.25 (<0.001)	1.88 (0.18)	24.43 (<0.001)	2.74 (0.11)	0.96 (0.33)	2.34 (0.13)
Mmet	70.84 (0.018)	0.68 (0.41)	79.02 (< 0.001)	3.04 (0.09)	2.2 (0.15)	20.32 (<0.001)	0.68 (0.41)
β-Tyr	60.23 (0.006)	6.54 (0.014)	27.29 (<0.001)	14.09 (<0.001)	4.46 (0.041)	1.23 (0.27)	6.78 (0.013)
Ser	24.89 (<0.001)	13.93 (0.045)	22.93 (<0.001)	16.43 (<0.001)	1.12 (0.30)	1.18 (0.28)	4.68 (0.037)
Asn	431.18 (<0.001)	0.19 (0.66)	21.41 (<0.001)	0.36 (0.55)	10.81 (0.002)	13.84 (<0.001)	9.29 (0.004)
Gln	402.63 (<0.001)	17.88 (<0.001)	0.04 (0.83)	15.47 (<0.001)	2.33 (0.13)	15.60 (<0.001)	3.30 (0.08)
Glu	993.60 (<0.001)	22.08 (<0.001)	29.26 (<0.001)	22.26 (<0.001)	9.53 (0.004)	27.57 (<0.001)	9.36 (0.004)
Asp	263.23 (<0.001)	3.08 (0.09)	17.33 (<0.001)	4.08 (0.05)	12.00 (0.001)	6.68 (0.014)	15.31 (<0.001)
Arg	22.95 (<0.001)	1.79 (0.19)	36.14 (<0.001)	6.48 (0.015)	0.24 (0.63)	52.08 (<0.001)	1.56 (0.22)
GSSG	1106.13 (<0.001)	0.16 (0.69)	4.04 (0.05)	0.27 (0.60)	20.39 (<0.001)	3.50 (0.07)	19.04 (<0.001)

Where T*TC is interaction between growth condition and time course, TC*G is interaction between time course and genotype, T*G is interaction between growth condition and genotype, and T*TC*G is interaction of three factors including growth condition, time course and genotype. Full name of metabolites as defied in Table 4.1.

conditions, time courses and genotypes and by the interactions of these factors (Table 4.3).

Cold stress induced to increase the concentration of AAs in many plants, such as in herbaceous perennial plants (Sagisaka, 1987), *Arabidopsis thaliana* (Kaplan *et al*., 2004), *Arabidopsis lyrata* spp. petraea (Davey *et al*., 2009) and rice (Zhao *et al*., 2013). The present study indicated that contents of metabolites caused by cold stress were higher than those by non-cold stress (Fig. 4.2). The contents of 58 % (47/81) identified metabolites under cold stress were lower in 32R than those in 29S (Fig. 4.2). In previous study, Zhao *et al*. (2013) reported that metabolites of chilling-tolerant rice variety, Lijiangxintuanhegu, were higher concentrations than those of chilling-sensitive rice variety, IR29. In addition, the changes of metabolites showed difference in time courses under cold treatment (Fig. 4.6). For example, contents of Ser, Asp, GSSG and Arg showed increase with ranging from 4 to 11% at 10 dpe in comparison with 5 and 15 dpe, whereas Gln and Glu showed increase with ranging from 21 to 29 % at 15 dpe in comparison with 5 and 10 dpe. The present results are in agreement with the previous results as shown in Chapter 3 that concentrations of FAAs (FAAs belong to the group of AAs, peptides and analogues) in cold stress were higher than those in non-cold stress conditions and were lower in 32R than in 29S at 5 and 10 dpe (Fig. 3.3). The changes in the contents of FAAs under cold stress are

not always associated directly with those in contents of soluble proteins (Chapter 3). Though it remains to be understood how these increases in the contents of AAs contribute directly or indirectly to plant in response to cold stress, these were proposed to be involved in the stress responses through their interactions with the negatively charged macromolecules, such as DNA, RNA and organic acids, resulting changes in the physical and chemical properties of plant (Alcázar *et al*., 2006). In addition, previous study detected in 32R a gene located on the chromosome 7 encodes the cytokinin-*O*-glucosyltransferase (Gaihre and Nose, 2011). This enzyme which catalyzed the interaction between cytokinins and AAs (Li *et al*., 2015; von Saint Paul *et al*., 2011). Recent studies in the seedlings of *Arabidopsis* and 2-week-old rice seedlings reported that the expression of AtNRT genes (encoding nitrate transporters) as well as some ammonium and amino acid transporters were reduced after cytokinin supplement (Hirose *et al*., 2007; Kiba *et al*., 2011), thus cytokinin was proposed to play a negative role in the regulation of N uptake-related genes (Kiba *et al*., 2011). The results in Chapter 3 and Chapter 4 indicated that concentrations of AAs were lower in 32R than in 29S, these results can be explained because of existing a gene encoding cytokinin-*O*-glucosyltransferase in 32R that caused to inhibit the expression of genes that encode for amino acid transporters and accumulation of AAs, thus limiting root growth leading to poor plant growth in 32R in comparison with 29S.

In present study, the contents of many metabolites, such as Ser, Asp, Gln, Glu, Arg and Asn, were significant changes under cold stress in comparison with non-cold stress conditions. These metabolites have been known to be essential AAs in process of nitrogen assimilation, storage and transport in plant and were up-regulated during cold stress (Davey *et al*., 2009; Lam *et al*., 1995). The concentrations of Asn, Glu, Gln, Arg and Asp were lower in 32R than in 29S (Figs. 4.6-E, 4.6-F, 4.6-G, 4.6-H and 4.6-J). The findings of Hu *et al*. (2014) in metabolites of rice seed reported that *japonica* was higher levels of nitrogen containing compounds, such as γ-aminobutyrate, Ser, alanine, Glu, glycine, Glu, and agmatine and polyamines (putrescine and spermidine) than *indica*. Other studies reported that *japonica* and *indica* display different nitrogen uptake efficiency during the vegetative growth stage (Britto and Kronzucker, 2004; Fan *et al*., 2007). Previous results indicated that 32R contained the traits as *indica*, while 29S contained the traits as *japonica* (Chapters 2 and 3). These results suggest that existing a gene encoding cytokinin-*O*-glucosyltransferase in 32R has caused to inhibit the expression of genes that encode for amino acid transporters in the nitrogen metabolism.

The present study also indicated that the contents of GSSG of 32R and 29S were 31 and 35 times higher in cold stress than those in non-cold stress (Fig. 4.3 and Table 4.2). As well known, the GSSG derived from glutathione and Ser (Figs. 4.4 and 4.5) by catalyst of glutathione reductase (GR). GR and GSSG play key roles in determining the tolerance of plant under abiotic stress, and the rising

of GR caused increase of GSSG (Yousuf *et al*., 2012). Many studies reported that the GR increased in many plants under abiotic stress conditions, such as *Cicer arietinum* under salt stress (Eyidogan and Öz, 2007), *A. thaliana, Vigna mungo, Triticum aestivum, Capsicum annuum,* and *Brassica juncea* under cadmium treatment (Yousuf *et al*., 2012), and rice under drought condition (Sharma and Dubey, 2005). Furthermore, concentrations of GSSG under cold stress showed lower in 32R than in 29S. These results suggest that lower contents of GSSG in 32R in comparison with 29S under cold stress induced to reduce the cold-tolerant characterization in 32R, therefore caused limiting plant growth (Chapter 2) and root growth (Chapter 3) under cold stress.

The β-Ala is the precursor for β-Ala betaine, an tremendous osmoprotectant (Hanson *et al*., 1991; Rao *et al*., 2006; Rathinasabapathi *et al*., 2001). Results indicated that the contents of β-Ala were down-regulated in 32R and 29S under cold stress in comparison with non-cold stress conditions (Figs. 4.4-A and 4.4-B) and were lower in 32R than in 29S (Fig. 4.5-A). These results suggest that lower contents of β-Ala under cold stress are leading to lower capacity of osmoprotectant in 32R in comparison with 29S. In addition, Mmet is an important transitional compound in the sulphur metabolism. The abilities of Mmet to preserve cell membrane integrity or reduce the degree of membrane damage in both the leaves and roots of peas, maize, soybeans and eight winter wheat varieties during cold stress (Rácz *et al*., 2008). Furthermore, Páldi *et al*.

(2014) also reported that the Mmet participated to protect the photosynthetic apparatus under cold stress. The present findings indicated that contents of Mmet under cold stress were lower in 32R than in 29S (Fig. 4.6-B). As mention above, many metabolites were synthesized in leaf, but allocated and used in large amounts in root under abiotic stress (Huang *et al*., 2012). Therefore, these results can be proposed that Mmet may be synthesized and used directly in leaf to protect the photosynthetic apparatus prior moved to root or after synthezing in leaf moved immediately to root and then opposite-controlled mechanism from root to protect the photosynthetic apparatus in leaf. These findings can explain for lower rates of the photosynthesis in 32R than those in 29S as shown in Chapter 2, because the photosynthetic apparatus of 32R may be damaged by cold stress in comparison with that of 29S. These results suggest that the roles of the osmo- and cell membrane-protection were limited in root of 32R due to lowering of the concentration of β-Ala and Mmet.

In addition, β-Tyr has been demonstrated most comprehensively as a component of bacterial polyketide antibiotics and anticancer drugs that have potential applications in human medicine (Lohman and Shen, 2012). β-Tyr was unknown as a plant metabolite but rather it inhibited the growth of *Pseudomonas syringae* as well as the growth of seedlings of dicotyledonous plants, whereas β-Tyr in tobacco leaves has unlimited the attractive aphids, lepidopterans and sugarcane borers (Huang *et al*., 2011). Furthermore, β-Tyr have been known to

inhibit root growth of *Arabidopsis thaliana* and other dicots (Mach, 2015). Recently, a study in 119 rice cultivars indicated that β-Tyr has been determined in the seeds, leaves, roots, and root exudates of *japonica*, but absence in those of *indica* (Yan *et al*., 2015). They also reported the discovery of a rice tyrosine aminomutase, encoding by the gene TAM1 on chromosome 12, is an enzyme that converts α-tyrosine into β-Tyr. The present results indicated that the contents of β-Tyr were higher in 32R than in 29S (Fig. 4.6-C). These results suggest that the high contents of β-Tyr caused to inhibit the growth of root (Chapter 3) leading to inhibition in whole plant growth in 32R in comparison with 29S (Chapter 2).

5. Summary

The rice genotype 32R is ShB-resistant with high yield potential and poor root growth in response to cold stress. To clarify the root metabolic responses when the seedlings at 4th leaf stage were exposed to cold stress, profiling of cationic metabolites in root of 32R was examined by using CE-TOF/MS in comparison with 29S (a ShB-sensitive and cold tolerant rice genotype). Total 81 cationic metabolites were detected, and 48% (39/81) metabolite belongs to the group of AAs, peptides and anlogues. These metabolites related to TCA cycle and glycolysis pathway. Two-way ANOVA indicated that these metabolites were affected by the factors of growth conditions, time courses and genotypes, and by

the interactions of these factors. Contents of metabolites caused by cold stress were higher than those by non-cold stress. 58 % (47/81) identified metabolites under cold stress were lower concentration in 32R than that in 29S. The changes of metabolites showed difference in time courses under cold treatments. Many metabolites relating to the process of nitrogen assimilation, storage and transport, the cold-tolerant characterization, the osmo- and cell membrane-protection and inhibit root growth were lower contents in 32R than in 29S. These results suggest that existing a gene encoding cytokinin-*O*-glucosyltransferase in 32R has caused to inhibit the expression of genes that encode for amino acid transporters in the nitrogen metabolism leading to change the function of root in many metabolic processes under cold stress, thus causing poor root growth of 32R in comparison with that of 29S.

CHAPTER 5

General Discussion

The ShB-resistant rice genotype 32R was developed from Tetep (*indica,* ShB resistance) and CN_4-4-2 (*japonica,* ShB sensitivity) along with the ShB-susceptible rice genotype 29S (Wasano *et al*., 1985) and both rice genotypes were continuously screened for ShB resistance for over 20 years. Wasano and Hirota (1986) indicated that 32R showed more resistance to ShB than the parent - Tetep and 29S showed more susceptibility than Nb. Recently, many aspects relating to ShB resistance of 32R, such as metabolic pathways (Mutuku and Nose, 2012), proteomics analysis (Miyagi *et al*., 2006) and QTL analysis (Gaihre and Nose, 2011), have been studied. The rice genotype 32R is ShB-resistant, but its yield is poor, 7.9 MT/ha and is lower than that of Nb (9.6 MT/ha) and 29S (8.8 MT/ha) (Gaihre and Nose, 2013). However, when 32R was used to cross with NB, the yield of the F_1 generation (12.5 MT/ha) is higher than the parents (Gaihre and Nose, 2013). Although 32R is ShB-resistant and high yield potential, the observations identified that the seedlings growth of 32R during cold season

during cold season showed lower than those of 29S. Therefore, the aim of present study is to investigate how the genotype 32R in the seedling stage responses to different temperature conditions in the physiological and metabolic levers.

In Chapter 2, effects of temperature on the growth and photosynthesis in the juvenile seedling stage of 32R were studied and compared with those of 29S and Nb. The results indicated that the plant growth and photosynthesis of both 32R and 29S were affected by the factors of growth conditions, time courses and genotypes and by the interactions of these factors (Table 2. 1). The dry weight value at a low temperature showed lower in 32R than in 29S and Nb (Figs. 2.1-A1 and 2.1-A2), and total dry weight correlated strongly with root dry weight (Fig. 2.5-B1). Previous findings indicated that low temperature caused a depression of the dry weight (Aghaee *et al*., 2011; Engels and Marschner, 1990; Nagai and Makino, 2009). In addition, the RGR correlated strongly with the NAR (Fig. 2.5-A3). The results also indicated that the value of RGR at a low temperature showed smaller 2.6 times in 32R than in 29S (Fig. 2.2-A). Previous findings of Loveys *et al*. (2002) and Nagai and Makino (2009) also indicated that the variations of NAR play an important role in differences in RGR. Furthermore, rubisco, chlorophyll contents and the photosynthetic rates decreased at a low temperature and were lower in 32R than in 29S and Nb (Figs. 2.1, 2.3 and 2.4). The strong correlations between contents of Rubisco and the rates of maximum photosynthesis and initial slope of photosynthesis were found

in 32R, but not found in 29S and Nb (Fig. 2.6). RGR and NAR of 32R correlated positively with the contents of Rubisco (Figs. 2.5-E1 and 2.5-E2). Low and high temperatures also caused the decrease of Rubisco contents in many crops such as rice, soybean and wheat (Makino and Sage, 2007; Sage *et al*., 2008; Vu *et al*., 2001; Yamasaki *et al*., 2002). These results suggest that the ShB-resistant rice genotype 32R exists some traits of cold-sensitive genotypes that induced to limit the functions of root leading to limitation leaf biochemical components of 32R, thus diminishing photosynthesis and limiting plant growth.

To further investigate how low temperature (cold stress) inhibits the root functions of 32R, the accumulation of the biochemical components, soluble protein and FAAs, in root of 32R under cold stress has been examined in comparison with 29S (Chapter 3). The results of two-way ANOVA indicated that root growth, contents of soluble proteins and FAAs were affected by the factors of growth conditions, time courses and genotypes and by the interactions of these factors (Table 3.1). Root dry weight values of two rice genotypes were limited by cold stress conditions in comparison with non-cold stress conditions, and cold-tolerant genotype 29S showed higher in root dry weight than cold-sensitive genotype 32R (Fig. 3.1). Recent results in genetic relation indicated that 32R was 45% similar to Tetep - *indica*, while 29S was 91% similar to Nb - *japonica* (Gaihre, 2015). The *indica* genotypes adapted to tropical zones, while *japonica* genotypes adapted to subtropical zones (Garris *et al*., 2005). Thus, lower root dry weight of 32R might be due to 32R existing some cold-sensitive characteristics

of Tetep. Furthermore, the soluble protein contents also decreased in response to cold stress and showed lower contents in 32R than in 29S at all time courses (Fig. 3.2). These results are contrasted to many previous results that cold stress induced higher amounts of soluble proteins in many plants (Antikainen and Pihakaski, 1994; Cloutier, 1983; Karimzadeh *et al*., 2000; Terzioglu and Ekmekci, 2004). In addition, the results of this study indicated that contents of FAAs dramatically increased under cold stress and were lower in 32R by 66 and 48% than in 29S at 5 and 10 dpe, respectively, but were not different at 15dpe (Fig. 3.3). These results suggest that the changes in the contents of FAAs are not always associated directly with those in soluble protein contents under cold stress, thus the different roots dry weight of 32R and 29S were clearly associated with their different responses to cold stress.

Previous study detected in 32R a gene encoding cytokinin-*O*-glucosyltransferase located on chromosome 7 (Gaihre, 2015). This enzyme catalyzed the conjugation between cytokinin and *O*-glucoside in the process of glycosylation (Mok *et al*., 2005; Pineda Rodó *et al*., 2008) and could transfer sugar moieties from activated donor molecules to specific acceptor molecules such as cytokinins and amino acids. This enzyme was also reported to participate in abiotic stress adaptation, such as drought, heat and cold stress (Li *et al*., 2000; Novakova *et al*., 2007; von Saint Paul *et al*., 2011; Wu *et al*., 2015) and to induce short-shoot phenotypes and reduction crown root number in rice (Kudo *et al*., 2012). In addition, recent studies in the seedlings of *Arabidopsis* and 2-week-old

rice seedlings indicated that the expression of AtNRT genes (encoding nitrate transporters) as well as some ammonium and amino acid transporters were reduced after cytokinin supplement (Hirose *et al*., 2007; Kiba *et al*., 2011), thus cytokinin was proposed to play a negative role in the regulation of N uptake-related genes (Kiba *et al*., 2011). These increases of FAAs under cold stress are possibly related to the role of cytokinin-*O*-glucosyltransferase, therefore AAs metabolism in root of 32R is necessary further study to understand the mechanism of plant in response to cold stress.

Besides to investigate the role of metabolites in the AAs metabolism of rice roots under cold stress, the metabolic profiling of cationic metabolites (as well known that almost amino acids belong to cationic metabolites group) in two genotypes 32R and 29S were examined by using CE-TOF/MS as shown in Chapter 4. The two rice genotypes, 32R and 29S, have been known to have contrasting cold tolerance at the phenotypic and physiological levels (Chapter 2) and accumulation of AAs in roots (Chapter 3). The present study detected 81 cationic metabolites in root of 32R and 29S under different growth conditions. The group of AAs, peptides and analogues was major with 48% (39/81) of identified metabolite (Table 4.1 and Fig. 4.1). The contents of metabolites caused by cold stress were higher than those by non-cold stress, and 58 % (47/81) identified metabolites under cold stress were lower concentration in 32R than that in 29S (Fig. 4.2). Zhao *et al*. (2013) also reported that metabolites of chilling tolerant rice variety, Lijiangxintuanhegu, were higher concentration than those of

chilling sensitive rice variety, IR29. In comparison with non-cold stress condition, nine metabolites, Ser, Gln, Glu, Asp, Asn, β-Tyr, GSSG, β-Ala and Mmet, were significantly changed in root of 32R and 29S under cold stress (Figs. 4.3 and Table 4.2). In addition, in comparison with 29S, the concentration of Asp under non-cold stress was lower in 32R, while the concentrations of Ser, Arg, Mmet and β-Ala under cold stress were lower in 32R, and the concentration of β-Tyr under cold stress was higher in 32R (Figs. 4.3, 4.6 and Table 4.2). The results of two-way ANOVA indicated that these metabolites were affected by the factors of growth conditions, time courses and genotypes and by the interactions of these factors (Table 4.3). Previous studies also reported that levels of AAs in many plants increased in response to cold stress, such as in herbaceous perennial plants (Sagisaka, 1987), *Arabidopsis thaliana* (Kaplan *et al*., 2004), *Arabidopsis lyrata* spp. *petraea* (Davey *et al*., 2009) and rice (Zhao *et al*., 2013).

Present study indicated that these metabolites, such as Ser, Asp, Gln, Glu, Arg and Asn, showed up-regulation during cold stress, and previous studies reported that these metabolites have been known to be essential AAs in nitrogen assimilation, storage and transport in plant (Davey *et al*., 2009; Lam *et al*., 1995). The concentrations of Asn, Glu, Gln, Arg and Asp were lower in 32R than in 29S (Figs. 4.6-E, 4.6-F, 4.6-G, 4.6-H and 4.6-J). Low concentration of these metabolites under cold stress in 32R may be due to existing a QTL of ShB resistance encoding cytokinin-*O*-glucosyltransferase that has caused to inhibit the

expression of genes that encode for amino acid transporters in the nitrogen metabolism. Furthermore, the GSSG derived from glutathione and Ser (Figs. 4.4 and 4.5) by catalyst of glutathione reductase (GR). GR and GSSG play key roles in determining the tolerance of plant under abiotic stress, and the rising of GR caused increase of GSSG (Yousuf *et al*., 2012). The GSSG up-regulated in both genotypes during cold stress and showed lower in 32R than in 29S (Fig. 4.6-I). Lower content of GSSG in 32R under cold stress induced to reduce the capacity of cold tolerance in 32R and to diminish the process of nitrogen assimilation, storage and transport in plant, therefore caused limiting root growth (Chapter 3) and plant growth (Chapter 2) under cold stress. In addition, the β-Ala is the precursor for β-Ala betaine, an tremendous osmoprotectant (Hanson *et al*., 1991; Rao *et al*., 2006; Rathinasabapathi *et al*., 2001) and was lower concentration in 32R than in 29S (Fig. 4.6-A). The Mmet is an important transitional compound in the sulphur metabolism. The abilities of Mmet to preserve cell membrane integrity or reduce the degree of membrane damage in the time course of cold stress have been studied by Rácz *et al*. (2008). Mmet under cold stress was lower in 32R than in 29S (Fig. 4.6-B). Due to lowering of the concentration of β-Ala and Mmet that caused to limit root function, osmo- and cell membrane-protection leading to limiting the nitrogen assimilation, storage and transport in root of 32R in comparison with 29S. Moreover, β-Tyr have been known to inhibit root growth of *Arabidopsis thaliana* and other dicots (Mach, 2015). Recently, a study in 119 rice cultivars indicated that β-Tyr has been determined in the seeds,

leaves, roots, and root exudates of *japonica*, but absence in those of *indica* (Yan *et al*., 2015). However, the present study indicated that the β-Tyr showed higher in 32R than in 29S (Fig. 4.6-C). The high content of β-Tyr in 32R is the evidence for poor root growth (Chapter 3) leading to inhibition in whole plant growth in 32R in comparison with 29S (Chapter 2).

In summary, cold stress induced changes of many metabolites that related to many processes, such as nitrogen assimilation, storage and transport, the cold-tolerant characterization, the osmo- and cell membrane-protection and inhibit root growth in 32R and 29S (Fig. 5.1). The existing a gene encoding cytokinin-*O*-glucosyltransferase in 32R has caused to inhibit the expression of genes that encode for amino acid transporters in the nitrogen metabolism leading to limit the rubisco synthesis, reduce the rates of photosynthesis and NAR and finally reduce plant growth (Chapter 2). In other way, the effects of cold stress on the limitation of plant growth suggested that root growth, cold tolerance, osmoprotectant, cell membrane-protection affect to the growth seedlings of 32R under cold conditions (Chapters 3 and 4). Cold stress is a complex process involving coordinated activation of many metabolic pathways (Fernie *et al*., 2011; Guy *et al*., 2008; Usadel *et al*., 2008). Therefore, metabolic profiling studies should be conducted the multiplicity of the compatible solute-like network not only cationic metabolites but also anionic metabolites to complement comprehensively metabolic changes and the metabolic pathways

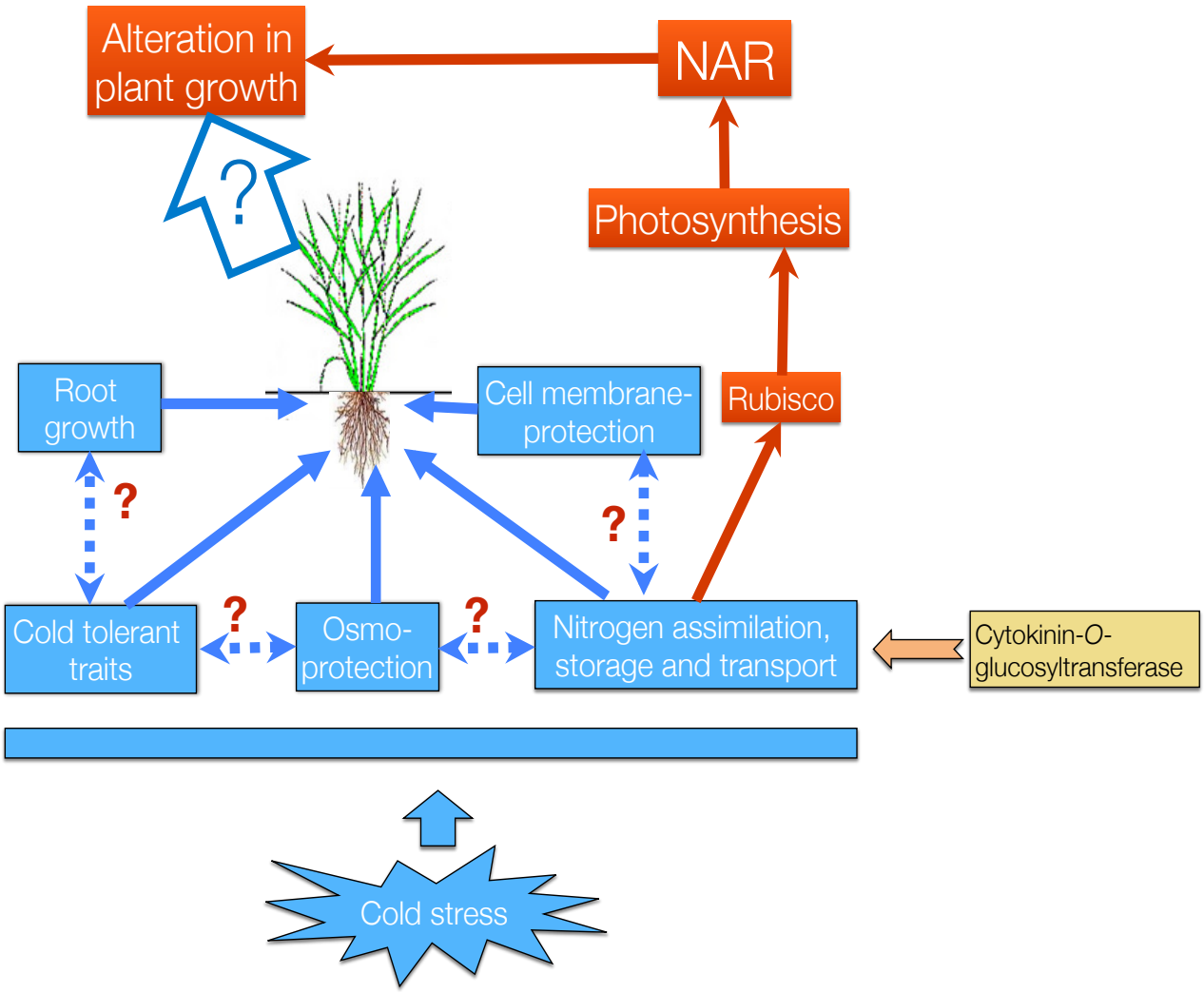

Fig. 5.1. Mechanism of cold stress effects on the rice seedling growth in the juvenile stage.

involved (Zhao *et al*., 2013). The identification of genes that are involved in plant metabolism and further functional characterization of those genes using the molecular biology methods to examine the genetic loci that control the biosynthesis of metabolites by using of mass spectrometry based metabolic profiling to identify metabolic qualitative trait loci (mQTL) should be further considered to apply for future breeding not only in rice but other crops.

Future studies

Effects of temperature on the physiological and metabolic characteristics of ShB-resistant rice genotype 32R have been conducted. These studies investigated the different plant performances and metabolic changes under temperature effects, but temperature effects on rice are complex processes. Therefore, to apply for rice breeding, the relationship metabolomic profiling and genetic regulation are necessary to consider in the future.

Cold stress causes biochemical changes in the rice roots as shown in Chapters 3 and 4. Both 32R and 29S were developed from the same parents (Tetep × CN_4-4-2), but they had different characteristics. Genetic relationship analysis indicated that 32R and 29S are similar to *indica* and *japonica*, respectively. However, it is well established that cold stress leading to improvement the cold tolerance is a complex process involving coordinated activation of many biochemical pathways (Guy et al., 2008; Usadel et al., 2008; Fernie et al., 2011). Therefore, metabolic profiling studies need to conduct the multiplicity of the compatible solute-like network and the metabolic pathways involved (Zhao et al., 2013). As shown in Chapter 4, the effect of cold stress on the variation of cationic metabolites was investigated, however to complement comprehensively metabolic changes not only cationic metabolites but also anionic metabolites, the variation of anionic metabolites in

root of 32R and 29S in response to cold stress given their different physiological and growth response should be clarified.

As showed in the Chapter 4, many cationic metabolites in 32R and 29S were identified the changes in the response to cold stress, however the identification of genes that are involved in plant metabolism, and further functional characterization of those genes were not conducted here. Therefore, application of different molecular biology techniques examine the genetic loci that control the biosynthesis of metabolites by using of mass spectrometry based metabolic profiling to identify metabolic qualitative trait loci (mQTL) should be investigated to apply for breeding a new rice variety in the future. These can also apply not only in rice breeding but any other future crop breeding.

Summary

Rice sheath blight (ShB), caused by *Rhizoctonia solani* Kuhn, is one of the most serious fungal diseases in diminishing rice production. Along with a progress of the climate change, the ShB is expanding in temperate region. The 32R is resistant to ShB and high yield potential, but the juvenile growth of 32R is poor under cold temperature. Using QTL pyramiding to develop new rice genotype with ShB resistance, high yield and cold tolerance is expecting for temperate zone under climate change, 32R is a valuable candidate for future rice breeding.

This study aimed to investigate the effects of temperature on juvenile growth and the metabolic characteristics during juvenile stage of 32R in comparison with ShB-susceptible rice genotypes 29S and Nipponbare (Nb), a standard *Japonica* variety. The 32R and 29S were developed from the crossing of Tetep (*Indica*, ShB resistance) and CN_4-4-2 (progeny of Chugoku 45 and Nb, *Japonica* and ShB sensitivity), but they showed different responses under temperature effects. First, effects of temperature on plant growth and photosynthesis in seedling stage were studied. The results showed that growth of 32R is lower than that of 29S and Nb, because 32R was limited in dry weight, leaf area, RGR, and NAR at low temperature. Furthermore, photosynthetic rate of 32R was lower than 29S and Nb, because its rubisco and chlorophyll content were inhibited at low temperature. These findings indicated

that 32R contains some traits of cold-sensitive rice genotypes, thus diminishing photosynthesis causes growth limitation. Second, the changes in root growth, soluble protein and free amino acids (FAAs) of genotypes under cold stress were studied. The results showed that root dry weight and soluble protein were decreased, whereas FAAs increased under cold stress. They were lower in 32R than in 29S. However, the changes in FAAs were not always associated directly with those in soluble protein under cold stress. Third, to further understand the responses of root metabolites to cold stress, metabolic profiles of cationic metabolites were studied by using CE-TOF/MS. The results showed that 81 metabolites including 55.6% amino acids (AAs), 4.9% polyamines, 16.0% nucleotides (Nus) and 23.5% other small molecular compounds were identified. These metabolites participate in many metabolic pathways. Of 81 measured metabolites, several metabolites participate in nitrogen assimilation, osmo-protection and cell membrane-protection were changed under cold stress, and they were lower in 32R than in 29S. These results indicated that cold stress caused variations in many metabolites of root, and the changes were contrasted between 32R and 29S.

These results of this study led to conclusion that 32R contains some traits of cold-sensitive. The contrasting changes in root metabolites caused changes in the physiological functions of roots, thus limiting seedling growth. In addition, these results provided useful information for reconstructing

metabolic networks, and expediting the identification of genetic regulators and metabolic engineering strategies in the ShB-resistance for future rice breeding.

Abstract in Japanese

イネ紋枯病（ShB）は、イネ生産を阻害する重要病害のひとつで、気候変動に伴う気温の上昇とともに温帯域における罹病地域の拡大が続いてい。また、抵抗性主導遺伝子の存在がないとされる ShB に対する抵抗性品種開発において、QTL 解析を用いた新たな品種開発に系統 32R は、我国における有用な育種素材と期待されている。イネ系統 32R は ShB 抵抗性を示す高収量の可能性を有した系統であるが、その初期生育は低温によって抑制されることが観察されてきた。

本研究では、系統 32R の初期生育に対する温度の影響を ShB 感受性系統の 29S 及び日本晴との比較検討を行った。32R と 29S は、インディカ型で ShB 抵抗性品種である Tepep とジャポニカ型で ShB 感受性系統の CN_4-4-2（日本晴と中国 45 号の交配後代）との交配から選抜育成された系統である。

まず、初期生育に対する温度の影響を検討し、32R が 29S 及び日本晴に比べ、低温下での生育が抑制されることを、乾物重、葉面積、RGR、NAR で明らかにした。また、光合成速度の抑制は Rubisco 及びクロロフィル含量に起因するものであった。次に、低温下で根の生長及び可溶性タンパクと全アミノ酸含量が 32R で低下することを明らかにした。また、全アミノ酸含量と可溶性タンパク含量の関係は温度によって異なることも観察された。次に、

低温が根の生理的特性に及ぼす影響について、カチオン性代謝産物の動態について解析し、81 種類の代謝産物（アミノ酸 55.6%、核酸 16%、ポリアミン 4.9%、その他小分子物質 23.5%）を同定した。特に低温下において窒素同化、浸透圧調節タンパク、細胞膜構成タンパクの合成に係る代謝経路が 29S に比べ 32R で抑制されていることが明らかになった。つまり、低温条件下において根の多くの代謝経路が影響を受け、その影響の仕方は 29S と 32R で異なることが確認された。

以上のことから、イネ系統 32R は低温感受性で、特に根部の窒素代謝に起因する生育の抑制が生じることが明らかになった。また、本研究で明らかになった低温によって影響を受ける代謝ネットワークの特性は、今後 32R を活用した ShB 抵抗性イネ品種の開発に有用な成果として利用することが期待される。

References

Aghaee A., Moradi F., Zare-Maivan H., Zarinkamar F., Irandoost H.P. and Sharifi P. 2011. Physiological responses of two rice (*Oryza sativa* L.) genotypes to chilling stress at seedling stage. African Journal of Biotechnology 10: 7617-7621.

Alcázar R., Marco F., Cuevas J., Patron M., Ferrando A., Carrasco P., Tiburcio A. and Altabella, T. 2006. Involvement of polyamines in plant response to abiotic stress. Biotechnololy Letters 28: 1867-1876.

Alexandratos N. and Bruinsma J. 2012. World agriculture towards 2030/2050: the 2012 revision. ESA Working Paper No. 12-03. Rome, FAO.

Antikainen M. and Pihakaski S. 1994. Early developments in RNA, protein, and sugar levels during cold stress in winter rye (*Secale cereale*) leaves. Annals of Botany 74: 335-341.

Arnon D.I. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiology 24: 1-15.

Atkin O.K., Scheurwater I. and Pons T.L. 2006. High thermal acclimation potential of both photosynthesis and respiration in two lowland Plantago species in contrast to an alpine congeneric. Global Change Biololgy 12: 500-515.

Baker J.M., Hawkins N.D., Ward J.L., Lovegrove A., Napier J.A., Shewry P.R. and Beale M.H. 2006. A metabolomic study of substantial equivalence of field-grown genetically modified wheat. Plant Biotechnology Journal 4: 381-392.

Barah P., Jayavelu N.D., Rasmussen S., Nielsen H.B., Mundy J. and Bones A.M. 2013. Genome-scale cold stress response regulatory networks in ten *Arabidopsis thaliana* ecotypes. BMC Genomics 14: 1-16.

Benina M., Obata T., Mehterov N., Ivanov I., Petrov V., Toneva V., Fernie A. and Gechev T.S. 2013. Comparative metabolic profiling of *Haberlea rhodopensis*, *Thellungiella halophyla*, and *Arabidopsis thaliana* exposed to low temperature. Frontiers in Plant Science 4: 1-11.

Bradford M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248-254.

Britto D.T. and Kronzucker H.J. 2004. Bioengineering nitrogen acquisition in rice: can novel initiatives in rice genomics and physiology contribute to global food security? BioEssays 26: 683-692.

Bruhn D.A.N., Leverenz J.W. and Saxe H. 2000. Effects of tree size and temperature on relative growth rate and its components of *Fagus sylvatica* seedlings exposed to two partial pressures of atmospheric [CO_2]. New Phytologist 146: 415-425.

Case M. and Tidwell A. 2014. Climate impacts threatening Japan today and tomorrow, Climate Change Programme, WWF Japan, pp. 1-14. Available at: https://www.wwf.or.jp/activities/lib/pdf_climate/environment/WWF_NipponChanges_lores.pdf.

Chinnusamy V., Zhu J. and Zhu J.-K. 2007. Cold stress regulation of gene expression in plants. Trends in Plant Science 12: 444-451.

Chinnusamy V., Zhu J.-K. and Sunkar R. 2010. Gene regulation during cold stress acclimation in plants. Methods in Molecular Biology 639: 39-55.

Cloutier Y. 1983. Changes in the electrophoretic patterns of the soluble proteins of winter wheat and rye following cold acclimation and desiccation stress. Plant Physiology 71: 400-403.

Cook D., Fowler S., Fiehn O. and Thomashow M.F. 2004. A prominent role for the CBF cold response pathway in configuring the low-temperature metabolome of Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America 101: 15243-15248.

Costanzo S., Jackson A. and Brooks S. 2011. High-resolution mapping of *Rsn1*, a locus controlling sensitivity of rice to a necrosis-inducing phytotoxin from *Rhizoctonia solani* AG1-IA. Theoretical and Applied Genetics 123: 33-41.

Cruz R.V., Harasawa H., Lal M., Wu S., Anokhin Y., Punsalmaa B., Honda Y., Jafari M., Li C. and Ninh N.H. 2007. Asia. Climate change 2007: Impacts, adaptation and vulnerability. Contribution of working group II to the fourth assessment report of the intergovernmental panel on climate change. In Parry M.L., Canziani O.F., Palutikof J.P., Linden P.J.v.d. and Hanson C.E. eds., Cambridge University Press, Cambridge, UK. pp. 469-506.

Cui S., Huang F., Wang J., Ma X., Cheng Y. and Liu J. 2005. A proteomic analysis of cold stress responses in rice seedlings. Proteomics 5: 3162-3172.

Davey M., Ian Woodward F. and Paul Quick W. 2009. Intraspecfic variation in cold-temperature metabolic phenotypes of *Arabidopsis lyrata* ssp. petraea. Metabolomics 5: 138-149.

de Souza A.P., Cocuron J.-C., Garcia A.C., Alonso A.P. and Buckeridge M.S. 2015. Changes in whole-plant metabolism during grain-filling stage in *Sorghum*

bicolor L. (Moench) grown under elevated CO_2 and drought. Plant Physiology 169: 1755-1765.

Engels C.H. and Marschner H. 1990. Effect of sub-optimal root zone temperatures at varied nutrient supply and shoot meristem temperature on growth and nutrient concentrations in maize seedlings (*Zea mays* L.). Plant and Soil 126: 215-225.

Evans G.C. 1972. The quantitative anaylysis of plant growth. Oxford: Blackwell Scientific. pp. 734.

Eyidogan F. and Öz M. 2007. Effect of salinity on antioxidant responses of chickpea seedlings. Acta Physiologiae Plantarum 29: 485-493.

Fan X., Jia L., Li Y., Smith S.J., Miller A.J. and Shen Q. 2007. Comparing nitrate storage and remobilization in two rice cultivars that differ in their nitrogen use efficiency. Journal of Experimental Botany 58: 1729-1740.

Fernie A.R., Aharoni A., Willmitzer L., Stitt M., Tohge T., Kopka J., Carroll A.J., Saito K., Fraser P.D. and DeLuca V. 2011. Recommendations for reporting metabolite data. Plant Cell Online 23: 2477-2482.

Fracheboud Y. and Leipner J. 2003. The application of chlorophyll fluorescence to study light, temperature, and drought stress. In DeEll J. and Toivonen P.A. eds., Practical applications of chlorophyll fluorescence in plant biology. Springer US. pp. 125-150.

Fuhrer J. 2003. Agroecosystem responses to combinations of elevated CO_2, ozone, and global climate change. Agriculture Ecosystems & Environment 97: 1-20.

Gaihre Y.R. 2015. Study on sheath blight resistance and yielding capacity by phenotype and QTL analysis in rice line 32R. PhD Thesis. Kagoshima University, Japan. pp. 43-57.

Gaihre Y.R. and Nose A. 2011. QTL analysis of sheath blight disease resistance in the rice line 32R derived from Tetep. Japanese Journal of Crop Science 80 (Extra issue 2): 172-173.

Gaihre Y.R. and Nose A. 2013. High yielding capabilities and genetic variation in crossing of sheath blight disease resistant rice line. Field Crops Research 149: 133-140.

Gargallo-Garriga A., Sardans J., Perez-Trujillo M., Rivas-Ubach A., Oravec M., Vecerova K., Urban O., Jentsch A., Kreyling J., Beierkuhnlein C., Parella T. and Penuelas J. 2014. Opposite metabolic responses of shoots and roots to drought. Scientific Reports 4. 1-7.

Garris A.J., Tai T.H., Coburn J., Kresovich S. and McCouch S. 2005. Genetic structure and diversity in *Oryza sativa* L. Genetics 169: 1631-1638.

Ghini R., Hamada E. and Bettiol W. 2008. Climate change and plant diseases. Scientia Agricola 65: 98-107.

Gnanamanickam S.S. 2009. Biological control of rice diseases. CRC Press. pp. 108.

Grime J.P. and Hunt R. 1975. Relative growth rate: Its range and adaptive significance in a local llora. Journal of Ecology 63: 393-422.

Groth D.E. and Nowick E.M. 1992. Selection for resistance to rice sheath blight through number of infection cushions and lesion type. Plant Disease 76: 721-723.

Guy C., Kaplan F., Kopka J., Selbig J. and Hincha D.K. 2008. Metabolomics of temperature stress. Physiologia Plantarum 132: 20-235.

Guy C., Kopka J. and Moritz T. 2008. Plant metabolomics coming of age. Physiologia Plantarum 132: 113-116.

Hanson A.D., Rathinasabapathi B., Chamberlin B. and Gage D.A. 1991. Comparative physiological evidence that β-Alanine betaine and choline-O-sulfate act as compatible osmolytes in *Halophytic limonium* species. Plant Physiology 97: 1199-1205.

Hardjawinata S. 1980. Macroclimatic aspects of rice production in Southeast Asia. In Cowell R.L. ed., Proceding agrometerology of the rice crop. IRRI, Phillippines. pp. 57-68.

Hatfield J.L. and Prueger J.H. 2015. Temperature extremes: Effect on plant growth and development. Weather and Climate Extremes 10: 4-10.

Hibberd J.M. and Quick W.P. 2002. Characteristics of C_4 photosynthesis in stems and petioles of C_3 flowering plants. Nature 415: 451-454.

Hirose N., Makita N., Kojima M., Kamada-Nobusada T. and Sakakibara H. 2007. Overexpression of a type-a response regulator alters rice morphology and cytokinin metabolism. Plant and Cell Physiology 48: 523-539.

Hu C., Shi J., Quan S., Cui B., Kleessen S., Nikoloski Z., Tohge T., Alexander D., Guo L., Lin H., Wang J., Cui X., Rao J., Luo Q., Zhao X., Fernie A.R. and Zhang D. 2014. Metabolic variation between japonica and indica rice cultivars as revealed by non-targeted metabolomics. Scientific Reports 4: 1-10.

Huang B., Rachmilevitch S. and Xu J. 2012. Root carbon and protein metabolism associated with heat tolerance. Journal of Experimental Botany 63: 3455-3465.

Huang T., Jander G. and de Vos M. 2011. Non-protein amino acids in plant defense against insect herbivores: Representative cases and opportunities for further functional analysis. Phytochemistry 72: 1531-1537.

Huner N.P.A., Öquist G. and Sarhan F. 1998. Energy balance and acclimation to light and cold. Trends in Plant Science 3: 224-230.

Hunt W.F. and Halligan G. 1981. Growth and developmental responses of perennial ryegrass grown at constant temperature. I. Influence of light and

temperature on growth and net assimilation. Functional Plant Biology 8: 181-190.

Jiao J., Leonardos E.D. and Grodzinski B. 2005. Approaches to measuring plant bioproductivity and growth. In Pessarakli M. ed. Handbook of Photosynthesis, Second Edition. CRC Press. pp. 699-716.

Kanbe T., Sasaki H., Aoki N., Yamagishi T. and Ohsugi R. 2009. The QTL analysis of rubisco in flag leaves and non-structural carbohydrates in leaf sheaths of rice using chromosome segment substitution sines and backcross progeny F_2 populations. Plant Production Science 12: 224-232.

Kaplan F., Kopka J., Haskell D.W., Zhao W., Schiller K.C., Gatzke N., Sung D.Y. and Guy C.L. 2004. Exploring the temperature-stress metabolome of Arabidopsis. Plant Physiology 136: 4159-4168.

Karimzadeh G., Francis D. and Davies M.S. 2000. Low temperature-induced accumulation of protein is sustained both in root meristems and in callus in winter wheat but not in spring wheat. Annals of Botany 85: 769-777.

Kiba T., Kudo T., Kojima M. and Sakakibara H. 2011. Hormonal control of nitrogen acquisition: roles of auxin, abscisic acid, and cytokinin. Journal of Experimental Botany 62: 1399-1409.

Kirk J.T.O. 2011. Light and photosynthesis in aquatic ecosystems. Cambridge University Press. pp. 330-387.

Kobayashi T., Ishiguro K., Nakajima T., Kim H.Y., Okada M. and Kobayashi K. 2006. Effects of elevated atmospheric CO_2 concentration on the infection of rice blast and sheath blight. Phytopathology 96: 425-431.

Kositsup B., Montpied P., Kasemsap P., Thaler P., Améglio T. and Dreyer E. 2009. Photosynthetic capacity and temperature responses of photosynthesis of rubber trees (*Hevea brasiliensis* Müll. Arg.) acclimate to changes in ambient temperatures. Trees - Structure and Function 23: 357-365.

Kreps J.A., Wu Y., Chang H.S., Zhu T., Wang X. and Harper J.F. 2002. Transcriptome changes for Arabidopsis in response to salt, osmotic, and cold stress. Plant Physiology 130: 2129-2141.

Kudo T., Makita N., Kojima M., Tokunaga H. and Sakakibara H. 2012. Cytokinin activity of cis-zeatin and phenotypic alterations induced by overexpression of putative cis-zeatin-*O*-glucosyltransferase in rice. Plant Physiology 160: 319-331.

Lam H.M., Coschigano K., Schultz C., Melo-Oliveira R., Tjaden G., Oliveira I., Ngai N., Hsieh M.H. and Coruzzi G. 1995. Use of Arabidopsis mutants and genes to study amide amino acid biosynthesis. The Plant Cell 7: 887-898.

Lambers H., Chapin F.S., III and Pons T. 2008. Photosynthesis. In Lambers H. ed. Plant physiological ecology. Springer New York. pp. 11-99.

Lambers H. and Poorter H. 2004. Inherent variation in growth rate between higher plants: A search for physiological causes and ecological consequences. advances in ecological research. Academic Press. pp. 283-362.

Lee F.N. and Rush M.C. 1983. Rice sheath blight: a major rice disease. Plant Diseases 67: 829-832.

Li R., Sosa J.L. and Zavala M.E. 2000. Accumulation of zeatin-*O*-glycosyltransferase in *Phaseolus vulgaris* and *Zea mays* following cold stress. Plant Growth Regulation 32: 295-305.

Li X., Qian Q., Fu Z., Wang Y., Xiong G., Zeng D., Wang X., Liu X., Teng S., Hiroshi F., Yuan M., Luo D., Han B. and Li J. 2003. Control of tillering in rice. Nature 422: 618-621.

Li Y.-j., Wang B., Dong R.-r. and Hou B.-k. 2015. AtUGT76C2, an *Arabidopsis* cytokinin glycosyltransferase is involved in drought stress adaptation. Plant Science 236: 157-167.

Lohman J.R. and Shen B. 2012. 4-Methylideneimidazole-5-one-containing aminomutases in enediyne biosynthesis. In David A.H. ed. Methods in Enzymology. Academic Press. pp. 299-319.

Loveys B.R., Scheurwater I., Pons T.L., Fitter A.H. and Atkin O.K. 2002. Growth temperature influences the underlying components of relative growth rate:

An investigation using inherently fast- and slow-growing plant species. Plant, Cell & Environment 25: 975-988.

Luck J., Spackman M., Freeman A., Tre¸bicki P., Griffiths W., Finlay K. and Chakraborty S. 2011. Climate change and diseases of food crops. Plant Pathology 60: 113-121.

Mach J. 2015. Production of the non-protein amino acid β-tyrosine in rice. The Plant Cell 27: 949-949.

Makino A., Mae T. and Ohira K. 1988. Differences between wheat and rice in the enzymic properties of ribulose-1,5-bisphosphate carboxylase/oxygenase and the relationship to photosynthetic gas exchange. Planta 174: 30-38.

Makino A. and Sage R.F. 2007. Temperature response of photosynthesis in transgenic rice transformed with 'Sense' or 'Antisense' *rbcS*. Plant and Cell Physiology 48: 1472-1483.

Maruyama K., Urano K., Yoshiwara K., Morishita Y., Sakurai N., Suzuki H., Kojima, M., Sakakibara H., Shibata D., Saito K., Shinozaki K. and Yamaguchi-Shinozaki K. 2014. Integrated analysis of the effects of cold and dehydration on rice metabolites, phytohormones, and gene transcripts. Plant Physiology 164: 1759-1771.

Miyagi S., Agarie S., Ohno Y., Ishida Y.-i., Morinaga H. and Nose A. 2006. Proteomic analysis of defense responses in rice resistant line against

infection of sheath blight fungus *Rhizoctonia solani*. The 4th International Symposium on Rice Functional Genomics, October, 2006, Montpellier, France. pp. 9-11.

Mok M.C., Martin R.C., Dobrev P.I., Vanková R., Ho P.S., Yonekura-Sakakibara K., Sakakibara H. and Mok D.W.S. 2005. Topolins and hydroxylated thidiazuron derivatives are substrates of cytokinin-*O*-glucosyltransferase with position specificity related to receptor recognition. Plant Physiology 137: 1057-1066.

Mutuku J.M. and Nose A. 2010. *Rhizoctonia solani* infection in two rice lines increases mRNA expression of metabolic enzyme genes in glycolytic, oxidative pentose phosphate pathway and secondary metabolism. Tropical Agrriculture and Development 54: 119-131.

Mutuku J.M. and Nose A. 2012. Changes in the contents of metabolites and enzyme activities in rice plants responding to *Rhizoctonia solani* Kuhn infection: activation of glycolysis and connection to phenylpropanoid pathway. Plant and Cell Physiology 53: 1017-1032.

Nagai T. and Makino A. 2009. Differences between rice and wheat in temperature responses of photosynthesis and plant growth. Plant and Cell Physiology 50: 744-755.

Naidu B.P., Paleg L.G., Aspinall D., Jennings A.C. and Jones G.P. 1991. Amino acid and glycine betaine accumulation in cold-stressed wheat seedlings. Phytochemistry 30: 407-409.

Noctor G., Bergot G., Mauve C., Thominet D., Lelarge-Trouverie C. and Prioul J.-L. 2007. A comparative study of amino acid measurement in leaf extracts by gas chromatography-time of flight-mass spectrometry and high performance liquid chromatography with fluorescence detection. Metabolomics 3: 161-174.

Novakova M., Dobrev P., Motyka V., Gaudinova A., Malbeck J., Pospisilova J., Haisel D., Storchova H., Dobra J., Mok M.C., Mok D.W.S., Martin R. and Vankova R. 2007. Cytokinin function in drought stress response and subsequent recovery. In Xu Z., Li J., Xue Y. and Yang W. eds., Biotechnology and Sustainable Agriculture 2006 and Beyond. Springer Netherlands. pp. 171-174.

Oikawa A., Matsuda F., Kusano M., Okazaki Y. and Saito K. 2008. Rice metabolomics. Rice 1: 63-71.

Páldi K., Rácz I., Szigeti Z. and Rudnóy S. 2014. S-methylmethionine alleviates the cold stress by protection of the photosynthetic apparatus and stimulation of the phenylpropanoid pathway. Biologia Plantarum 58: 189-194.

Pathak M.R., Teixeira da Silva J.A. and Wani S.H. 2014. Polyamines in response to abiotic stress tolerance through transgenic approaches. GM Crops & Food 5: 87-96.

Pineda Rodó A., Brugière N., Vankova R., Malbeck J., Olson J.M., Haines S.C., Martin R.C., Habben J.E., Mok D.W.S. and Mok M.C. 2008. Over-expression of a zeatin-*O*-glucosylation gene in maize leads to growth retardation and tasselseed formation. Journal of Experimental Botany 59: 2673-2686.

Poorter H. and Remkes C. 1990. Leaf area ratio and net assimilation rate of 24 wild species differing in relative growth rate. Oecologia 83: 553-559.

Poorter H. and Werf A.V.d. 1998. Is inherent variation in RGR determined by LAR at low irradiance and by NAR at high irradiance? A review of herbaceous species. In Lambers H., Poorter H. and Vuuren M.M.I.V. eds., Inherent variation in plant growth. Physiological mechanisms and ecological consequences. Backhuys Publishers. pp. 309-336.

Quero J.L., Villar R., Marañón T., Zamora R., Vega D. and Sack L. 2008. Relating leaf photosynthetic rate to whole-plant growth: drought and shade effects on seedlings of four *Quercus* species. Functional Plant Biology 35: 725-737.

Rácz I., Páldi E., Szalai G., Janda T., Pál M. and Lásztity D. 2008. S-methylmethionine reduces cell membrane damage in higher plants exposed to low-temperature stress. Journal of Plant Physiology 165: 1483-1490.

Ramautar R., Somsen G.W. and de Jong G.J. 2015. CE-MS for metabolomics: Developments and applications in the period 2012–2014. Electrophoresis 36: 212-224.

Rao K.V.M., Raghavendra A.S. and Reddy K.J. 2006. Physiology and molecular biology of stress tolerance in plants. Springer Netherlands. pp. 345.

Rathinasabapathi B., Fouad W.M. and Sigua C.A. 2001. β-alanine betaine synthesis in the Plumbaginaceae. purification and characterization of a trifunctional, S-adenosyl-l-methionine-dependent N-methyltransferase from *Limonium latifolium* leaves. Plant Physiology 126: 1241-1249.

Sage R., Kocacinar F. and Kubien D. 2011. C_4 photosynthesis and temperature. In Raghavendra A.S. and Sage R.F. eds., C_4 photosynthesis and related CO_2 concentrating mechanisms. Springer Netherlands. pp. 161-195.

Sage R.F. and Kubien D.S. 2007. The temperature response of C_3 and C_4 photosynthesis. Plant, Cell & Environment 30: 1086-1106.

Sage R.F., Way D.A. and Kubien D.S. 2008. Rubisco, rubisco activase, and global climate change. Journal of Experimental Botany 59: 1581-1595.

Sagisaka S. 1987. Amino acid pools in herbaceous plants at the wintering stage and at the beginning of growth. Plant and Cell Physiology 28: 171-178.

Sakakibara H., Takei K. and Hirose N. 2006. Interactions between nitrogen and cytokinin in the regulation of metabolism and development. Trends in Plant Science 11: 440-448.

Sato H., Ideta O., Ando I., Kunihiro Y., Hirabayashi H., Iwano M., Miyasaka A., Nemoto H. and Imbe T. 2004. Mapping QTLs for sheath blight resistance in the rice line WSS2. Breeding Science 54: 265-271.

Sharkey T.D., Bernacchi C.J., Farquhar G.D. and Singsaas E.L. 2007. Fitting photosynthetic carbon dioxide response curves for C_3 leaves. Plant, Cell & Environment 30: 1035-1040.

Sharma P. and Dubey R.S. 2005. Modulation of nitrate reductase activity in rice seedlings under aluminium toxicity and water stress: role of osmolytes as enzyme protectant. Journal of Plant Physiology 162: 854-864.

Shulaev V., Cortes D., Miller G. and Mittler R. 2008. Metabolomics for plant stress response. Physiologia Plantarum 132: 199-208.

Sipaseuth B.J., Fukai S., Farrell T.C., Senthonghae M., Sengkeo P.S., Linquist B. and Chanphengsay M. 2007. Opportunities to increasing dry season rice productivity in low temperature affected areas. Field Crops Research 102: 87-97.

Spackman D.H., Stein W.H. and Moore S. 1958. Automatic recording apparatus for use in chromatography of amino acids. Analytica Chimica Acta 30: 1190-1206.

Stirling C.M., Heddell-Cowie M., Jones M.L., Ashenden T.W. and Sparks T.H. 1998. Effects of elevated CO_2 and temperature on growth and allometry of five native fast-growing annual species. New Phytologist 140: 343-354.

Suzuki R. and Shimodaira H. 2006. Pvclust: an R package for assessing the uncertainty in hierarchical clustering. Bioinformatics 22: 1540-1542.

Terzioglu S. and Ekmekci Y. 2004. Variation of total soluble seminal root proteins of tetraploid wild and cultivated wheat induced at cold acclimation and freezing. Acta Physiologia Plantarum 26: 443-450.

Ulqodry T., Matsumoto F., Okimoto Y., Nose A. and Zheng S.-H. 2014. Study on photosynthetic responses and chlorophyll fluorescence in *Rhizophora mucronata* seedlings under shade regimes. Acta Physiologia Plantarum 36: 1903-1917.

Usadel B., BlÄSing O.E., Gibon Y., Poree F., HÖHne M., GÜNter M., Trethewey R., Kamlage B., Poorter H. and Stitt M. 2008. Multilevel genomic analysis of the response of transcripts, enzyme activities and metabolites in *Arabidopsis rosettes* to a progressive decrease of temperature in the non-freezing range. Plant, Cell & Environment 31: 518-547.

Villar R., Marañón T., Quero J.L., Panadero P., Arenas F. and Lambers H. 2005. Variation in relative growth rate of 20 *Aegilops* species (Poaceae) in the field: The importance of net assimilation rate or specific leaf area depends on the time scale. Plant and Soil 272: 11-27.

von Saint Paul V., Zhang W., Kanawati B., Geist B., Faus-Keßler T., Schmitt-Kopplin P. and Schäffner A.R. 2011. The *Arabidopsis* glucosyltransferase UGT76B1 conjugates isoleucic acid and modulates plant defense and senescence. The Plant Cell 23: 4124-4145.

Vu J.C.V., Gesch R.W., Pennanen A.H., Allen L.H.J., Boote K.J. and Bowes G. 2001. Soybean photosynthesis, rubisco, and carbohydrate enzymes function at supraoptimal temperatures in elevated CO_2. Journal of Plant Physiology 158: 295-307.

Wasano K. 1988. The resistance to the rice diseases of bacterial leaf blight (*Xanthomonas campestris* pv. oryzae) and sheath blight (*Rhizoctonia solani*), and some problems in breeding of disease resistance of crops. *In*: Japanese Society of Breeding (eds.). Recent Advances in Research on Plant Breeding 30: 103–121. (In Japanse).

Wasano K. and Hirota Y. 1986. Varietal resistance of rice to sheath blight disease caused by *Rhizoctonia Solani* Kuhn by the syringe inoculation method. Bulletin of the Faculty of Agriculture Saga Univeristy 60: 49-59. (In Japanse with English summay).

Wasano K., Hirota Y. and Kido Y. 1985. Varietal differences in the resistance to sheath blight of rice, *Rhizoctonia solani* Kuhn, and effectiveness of selection for the resistance from the cross, Tetep×CN_4-4-2. Report of the Kyushu Branch of the Crop Science Society of Japan 52: 16-22. (In Japanse with English summay).

Wasano K., Oro S. and Kido Y. 1983. The Syringe Inoculation Method for Selecting Rice Plants Resistant to Sheath Blight, *Rhizoctonia solani* Kunh. Japanese Journal of Tropical Agriculture 27: 131-139 (In Japanese with English summary).

Williams B.J., Cameron C.J., Workman R., Broeckling C.D., Sumner L.W. and Smith J.T. 2007. Amino acid profiling in plant cell cultures: An inter-laboratory comparison of CE-MS and GC-MS. Electrophoresis 28: 1371-1379.

Wu L., Hu X., Wang S., Tian L., Pang Y., Han Z., Wu L. and Chen Y. 2015. Quantitative analysis of changes in the phosphoproteome of maize induced by the plant hormone salicylic acid. Scientific Reports 5: 1-16.

Yamakawa H. and Hakata M. 2010. Atlas of rice grain filling-related metabolism under high temperature: Joint analysis of metabolome and transcriptome demonstrated inhibition of starch accumulation and induction of amino acid accumulation. Plant and Cell Physiology 51: 795-809.

Yamasaki T., Yamakawa T., Yamane Y., Koike H., Satoh K. and Katoh S. 2002. Temperature acclimation of photosynthesis and related changes in photosystem II electron transport in winter wheat. Plant Physiology 128: 1087-1097.

Yamori W., Hikosaka K. and Way D. 2014. Temperature response of photosynthesis in C_3, C_4, and CAM plants: Temperature acclimation and temperature adaptation. Photosynthesis Research 119: 101-117.

Yamori W., Noguchi K., Hikosaka K. and Terashima I. 2010. Phenotypic plasticity in photosynthetic temperature acclimation among crop species with different cold tolerances. Plant Physiology 152: 388-399.

Yan J., Aboshi T., Teraishi M., Strickler S.R., Spindel J.E., Tung C.-W., Takata R., Matsumoto F., Maesaka Y., McCouch S.R., Okumoto Y., Mori N. and Jander G. 2015. The tyrosine aminomutase TAM1 is required for β-tyrosine biosynthesis in rice. The Plant Cell 27: 1265-1278.

Yemm E.W., Cocking E.C. and Ricketts R.E. 1955. The determination of amino-acids with ninhydrin. Analyst 80: 209-214.

Yoshida S. 1981. Fundamentals of rice crop science. The IRRI, Philippines. pp. 1-64.

Yousuf P., Hakeem K., Chandna R. and Ahmad P. 2012. Role of glutathione reductase in plant abiotic stress. In Ahmad P. and Prasad M.N.V. eds., Abiotic stress responses in plants. Springer New York. pp. 149-158.

Zerihun A., McKenzie B.A. and Morton J.D. 1998. Photosynthate costs associated with the utilization of different nitrogen–forms: influence on the carbon balance of plants and shoot–root biomass partitioning. New Phytologist 138: 1-11.

Zhao X.-Q., Wang W.-S., Zhang F., Zhang T., Zhao W., Fu B.-Y. and Li Z.-K. 2013. Temporal profiling of primary metabolites under chilling stress and its association with seedling chilling tolerance of rice (*Oryza sativa* L.). Rice 6: 23.

Zheng A., Lin R., Zhang D., Qin P., Xu L., Ai P., Ding L., Wang Y., Chen Y., Liu Y., Sun Z., Feng H., Liang X., Fu R., Tang C., Li Q., Zhang J., Xie Z., Deng Q., Li S., Wang S., Zhu J., Wang L., Liu H. and Li P. 2013. The evolution and pathogenic mechanisms of the rice sheath blight pathogen. Nat Commun 4: 1-10.