Morphology changes of rice root nucleus under iron stress

A Polosoro*, W Enggarini, T Hadiarto, and N Ohmido

1Indonesian Center for Agricultural Biotechnology and Genetic Resource Research and Development (ICABIOGRD), Jl. Tentara Pelajar No. 3A, Cimanggu, Bogor, Indonesia.
2Graduate School of Human Development and Environment, Kobe University, 3-11 Tsurukabuto, Nada-ku, Kobe 657-8501 Japan.

*Corresponding author: aqwin@pertanian.go.id, +62-818-0890-9220

Abstract, Understanding the response of the Root Apical Meristem (RAM) region under environmental stress is a long-standing issue in plant biology. Our previous study successfully gave new evidence in the epigenetic level that there is a heterochromatinization, especially in the proximal meristem region in rice under environmental stresses (high iron and salinity). These changes are suspected to be responsible for the reduction of the growth rate of RAM. On this study, we used sensitive (Ciherang), moderate (Nipponbare) and tolerant (Mentong and Siam Arjuna) rice varieties to see the meristematic cell response under high iron content environment. For the methodology, we use the nucleus size as the indicator for cell phase. Normal meristematic cells are dominant in the interphase stage (spread to G0, S, and G1 phase). However, our observation and quantitative analysis showed that iron stress reduced the S, G0, and cell cycle phase and pushed the cell to form G0-like phase. There is no significant difference between tolerant and sensitive rice varieties response, this proves that this response is a general mechanism in abiotic stresses.

Keyword: rice, iron stress, interphase, RAM

1. Introduction
Rice (Oryza sativa) is an essential crop for most Asian countries, majority inbreeding species that successfully supply as primary nutrition over half of the world's population [1]. An understanding of the physiological, developmental, and morphological mechanisms in cultivated rice is crucial for developing new varieties, especially in the aspects of quality, reliability, and sustainability as the world's food supply. The rapid growth of the human population, remarkably in developing countries push the researchers to accelerate the breading process to get more option in stress-tolerant, resource-use efficient, and highly productive varieties.

One of the main problems in rice cultivation is heavy metal-contained soil by natural or artificial contamination. In high concentration, heavy metal can decrease rice production significantly. Most iron (Fe)-polluted land is found in developing countries including Malaysia, India, Myanmar, Thailand,
Laos, Cambodia, Vietnam, Bangladesh, the Philippines, Sri Lanka and Madagascar, which are rice-producing countries [2,3]. For example, approximately 60% of the paddy fields in Central Africa and West Africa suffer from Fe poisoning. However, Indonesia suffers worse, the large scale of iron toxicity spread in 1 million ha of intensified tidal and peatland areas in the provinces of Southern Kalimantan and Southern Sumatra [4].

Iron is a crucial precursor in several biochemical and cellular mechanisms. This metal also has the primary function as electron acceptors and donors, cofactors in the enzymatic process and necessary in biological pathways, such as photosynthesis in chlorophyll, DNA synthesis, nitrogen metabolism and hormone synthesis [5]. Typically, the soil contains two types of iron oxidation forms; the divalent (Fe$^{2+}$) and trivalent (Fe$^{3+}$) which is related to several environmental conditions such as pH, redox potential (Eh) and water status. Mostly iron will be stable in high oxidation stage in the aerobic environment, while the divalent state is stable in anaerobic condition.

The plant growth regulator abscisic acid (ABA) has a crucial role in plant growth, development, biotic, and abiotic adaptation. Its function not only promotes seed differentiation and dormancy but also contributes to organizing the immune system under abiotic stresses [6]. ABA signaling pathways have been elucidated rapidly in much detail just over a couple of years later. A significant example has been shown in progress at the discovery of ABA signaling complex in Arabidopsis, which regulates gene expression [7–9]. Our previous study also successfully gave new evidence in the global epigenetic level that there is a heterochromatinization, especially in the proximal meristem region in rice under environmental stresses (high iron and salinity) and also by exogenous ABA addition [10]. These changes are suspected to be responsible for the reduction of the growth rate of RAM and also cell transformation into a transient phase [11].

In the present study, the meristematic cell response was analyzed under high iron content environment. High iron content environment was set for treating germinated rice roots. Several rice cultivars, including sensitive, moderate, and tolerant varieties. The nucleus size population were counted based on size range and plotted on the curve for evaluating the meristematic cell response. The result provides the population changes of nucleus specimens in the proximal meristem region.

2. Materials and methods
2.1. Plant materials
The experiment used several rice varieties, which was characterized by high iron condition as sensitive Ciherang (ICABIOGRAAD-Indonesia), moderate Nipponbare (Kobe University-Japan), and tolerant Siam Arjuna and Mentong (ICABIOGRAAD-Indonesia).

2.2. Treatments
To treated plant samples, the seeds were germinated in the growth chamber (NK System Biotron, Japan) at 25–30 °C under a 12-h photoperiod. 3-5-day-old seedlings were treated by dipping in 150 ppm (mg/L) iron sulfate (FeSO$_4$) for 6 h.

2.3. Slide preparation
RAM tissues were cut and fixed for 10 min under vacuum condition in 4% PFA buffer (paraformaldehyde in MTGB buffer), followed by 20 min in room temperature. To remove the fixative agent, the roots were washed twice in sterile distilled water for 10 min. To remove the cell wall, the samples were treated by an enzyme mixture of 1% Cellulose Onozuka RS [Yakult Co., Japan] and 2% Pectolyase Y-23 [Kikkoman, Japan] for 1 h and washed twice for 10 min each with 1x PBS buffer. The proximal meristem tissues were separated from the elongation zone, squashed on the glass slide, and covered by a cover glass. The cover glass was detached after freezing in liquid nitrogen and immediately transferred to cold 1x PBS buffer for 10 min. For the nucleus imaging, the sample stained by 0.5 ng/μL
DAPI solution for 10 min in the darkroom and washed by distilled water for couple time. A cover glass was placed on the samples after the addition of a drop of Vectashield. The specimens were observed by a fluorescence microscope (BX60, Olympus-Japan), and the images were taken by a cooled CCD camera (SPOT RT3, Spot Imaging Solitons).

2.4 Data analysis
The nucleus size was converted to the actual wide by a pixel-size table and clustered by each 2 µm$^2$ range for counting the population. The histogram was projected to the line curve based on each population percentage and nucleus size.

3. Results
3.1 Nucleus size changes
Significant changes in the nucleus population of the four rice cultivars were observed during 24 h of treatment with 150 ppm iron (FeSO$_4$). Visual observation of rice root proximal meristem nucleus (fig. 1) shows the changes of global nucleus dimension in all rice varieties under iron treatment. In general, nucleus specimens were reduced in size after the treatment, and the nucleus population became more homogenous in the smaller size. Moreover, telophase specimens became challenging to find in the treatment sample. However, this result should be validated based on several statistical techniques to make more objective conclusions.

![Figure 1](image)

**Figure 1.** Visual observation of root proximal meristem nucleus of (a) Ciherang control, (b) Ciherang treatment, (c) Nipponbare control, (d) Nipponbare treatment, (e) Mentong control, (f) Mentong treatment, (g) Siam Arjuna control, and (h) Siam Arjuna treatment under high iron content medium. bar = 10 um

We used box and whisker plot and counting the mean to investigate the nucleus size data distribution and more objective observation. Nucleus size data were taken from the nuclei of 225 Ciherang, 174 Nipponbare, 221 Mentong and 160 Siam Arjuna in the proximal meristem region. All controls have nucleus size of approximately 9 µm$^2$, and they were reduced to 7 µm$^2$ after 6 hours of iron treatment. Moreover, the data distribution also dramatically changed after the treatment. All 4th quartile of the control nucleus specimens has a wide distribution, from 11 µm$^2$ to 18 µm$^2$, gone after iron exposure. The nucleus size distribution also spread to a narrower dimension within range 3-12 µm$^2$ from 3-18 µm$^2$ before the treatment. However, there are no significant changes in the minimal size nucleus, which is still distributed around 3-4 µm$^2$. We also found 50% of nucleus size population (in quartile 2nd and 3rd) spread on 5-8 µm$^2$, which was far smaller compared to the control which was about 7-11µm$^2$. This showed that the nucleus transformed into one size specimen.
Figure 2. Nucleus size data distribution compared between varieties and treatment. The lower bar indicates the extreme lower quartile; the upper bar, the extreme upper quartile; the lower line of the box, the lower quartile; the upper line of the box, the upper quartile; and the cross shows the median. (a) Ciherang control, (b) Ciherang treatment, (c) Nipponbare control, (d) Nipponbare treatment, (e) Mentong control, (f) Mentong treatment, (g) Siam Arjuna control, and (h) Siam Arjuna treatment.

3.2. Nucleus phase arrest

Figure 3. The proximal nucleus population based on the nucleus size on the rage 2 µm$^2$ in a). Ciherang, b). Nipponbare, c). Mentong and d). Siam Arjuna. circle = control and triangle = iron treatment.

We utilized a histogram plot from all nucleus size population data from the range of every 2 µm$^2$ size difference to analyze the population of nucleus specimens. The results (figure 3) successfully imitated
flow cytometry histogram results in the meristematic nucleus. Entirely untreated variety showed two peaks at 7-10 µm² and 13-15 µm² that were estimated as G1 and G2 checkpoints. However, 6 hours of iron exposure successfully erased the second peak and increased the population of G1-like checkpoint in all cultivars. The G1 checkpoint climbed from just only 25% to 35-45 % proximal nucleus population. The single peak curve demonstrated that the nucleus on the proximal region arrested on the G1 checkpoint.

4. Discussion

4.1. Effect of iron stress on rice meristematic activity

In this study, the high iron content environment not only successfully changes the average size of the rice nuclei but also changes the nucleus composition from heterogenous size to smaller homogenous size nuclei. Several studies have also been performed to investigate the effects of environmental stresses on the RAM activities, the stress can initiate early maturation of RAM [12], reduction of cell division [13–15], reduction of nucleus size [13,15], nucleus heterochromatinization [10], and transformation of nuclei into a transient state [11].

Using the box and whisker plot diagram, we can identify extinction of 25% of nucleus specimens (11 µm² to 18 µm² wide) during 6-hour treatment. The histogram analysis also detects the erasure of G2 phase and increment of the G1 population, but there are no significant differences between varieties. The G1 phase arrest dominates in the canonical cell cycle in several biological processes such as meristematic quiescence, dormancy, and terminal differentiation. Meristematic quiescence is in substance-related to non-cell-autonomous regulation of meristem cell identity, and especially by the effect of ubiquitin-dependent proteolysis, which also associated with reactive oxygen species (ROS), abscisic acid (ABA), and auxin [16]. All varieties also show domination of G1-like phase under iron stress, and it is also supported by our previous research that demonstrated the link between heterochromatinization of proximal meristem with an increment of ABA, ROS, and also IAA negative effect [10].

4.2. G1 arrest and nucleus heterochromatinization

The association between G1 arrest and heterochromatinization might be apparent since global heterochromatinization in the meristematic nucleus during stress condition will decrease. Histone acetylation is needed for the specification and activation of replication origins during the transition at the G1 and in S phase. In G2 phase, a deposition of CENH3 was discovered in the centromeric region, but the mechanism still unknown [17]. During mitosis, euchromatinization occurs globally, which is induced by histone modification, mainly phosphorylation. Finally, at the end of the cell cycle, heterochromatinization spread to avoid re-replication or endoreduplication (E) and re-entry into S phase [18]. Heterochromatinization will close DNA accessibility and affects gene transcription and DNA replication. As a result, protein synthesis and DNA replication will be interrupted, and it will reduce the S and G2 nucleus population and raises of G1 nucleus specimens.

4.3. G1 arrest as a transient phase (Meristematic quiescence)

In the case of the cell culture, growing the plant cell in the salt medium will give stress condition that leads to cellular reprogramming and development of a transient, dedifferentiated, stem cell-like state. This transition is related to chromatin decondensation or heterochromatinization that usually accompanied by DNA transposition and/or recombination [11]. This transient phase is categorized as meristematic quiescence, a transient state of the suppressed cell cycle in undifferentiated cells. It is also classified as embryonic quiescence, including organizing centre (OC) and the quiescent centre (QC) in the same state [16].

ROS and redox signaling pathways are proved as an essential player to maintain the balance of quiescence and proliferation in the RAM and SAM [19]. In the case of G1 arrest in QC cells, antioxidants ascorbate and glutathione have an important role which is dependent on the polar auxin maximum in the QC. Therefore, the ascorbate and glutathione are both highly oxidized in the QC, but on the contrary
in the proximal meristem [20]. Our previous experiment and this paper also proved the robust evidence about the connection between G1 arrest and ROS increment in proximal meristem tissues.

5. Conclusion
In conclusion, our experiment demonstrates the response of rice root nucleus to high iron exposure by suppressing the growth rates and arresting the nucleus in the G1 phase. The mitotic activity reduction probably is a result of global heterochromatinization and also a mechanism for defending meristematic cell under environmental stress. However, there is no significant difference between tolerant and sensitive rice varieties response, which indicates that this response is a general mechanism in abiotic stresses. Future research should evaluate the G1 arrest state actual status by using more integrated parameters such as cell physiology and epigenetics for discovering the real mechanism of G1 arrest under environmental stresses.

6. References
[1] Zhao K, Tung C, Eizenga G C, Wright M H, Ali M L, Price A H, Norton G J, Islam M R, Reynolds A, Mezey J, Mcclung A M, Bustamante C D and Mccouch S R 2011 a rich genetic architecture of complex traits in Oryza sativa Nat. Commun. 2 467
[2] Asch F, Becker M and Kpongor D S 2005 A quick and efficient screen for resistance to iron toxicity in lowland rice J. Plant Nutr. Soil Sci. 168 764–73
[3] Tam Aung 2006 Physiological mechanisms of iron toxicity tolerance in lowland rice Master of Science Tam Aung (Universität zu Bonn)
[4] Isumudji 1990 Alleviating iron toxicity in lowland rice Indon. Agric. Res. Dev. J. 12 67-72.
[5] Jeong J and Connolly E L 2009 Iron uptake mechanisms in plants: Functions of the FRO family of ferric reductases Plant Sci. 176 709–14
[6] Cutler S R, Rodriguez P L, Finkelstein R R and Abrams S R 2010 Abscisic Acid: Emergence of a Core Signaling Network vol 61
[7] Lee S C and Luan S 2012 ABA signal transduction at the crossroad of biotic and abiotic stress responses Plant, Cell Environ. 35 53–60
[8] Raghavendra A S, Gonugunta V K, Christmann A and Grill E 2010 ABA perception and signalling Trends Plant Sci. 15 395–401
[9] Yoshida T, Mogami J and Yamaguchi-Shinozaki K 2014 ABA-dependent and ABA-independent signaling in response to osmotic stress in plants. Curr. Opin. Plant Biol. 21 133–9
[10] Polosoro A, Enggarini W and Ohmido N 2019 Global epigenetic changes of histone modification under environmental stresses in rice root Chromosom. Res.
[11] Grafi G, Florentin A, Ransbotyn V and Morgenstern Y 2011 The Stem Cell State in Plant Development and in Response to Stress Front. Plant Sci. 2 1–10
[12] Ji H, Liu L, Li K, Xie Q, Wang Z, Zhao X and Li X 2014 PEG-mediated osmotic stress induces premature differentiation of the root apical meristem and outgrowth of lateral roots in wheat J. Exp. Bot. 65 4863–72
[13] Fernandez-Marcos M, Sanz L, Lewis D R, Muday G K and Lorenzo O 2011 Nitric oxide causes root apical meristem defects and growth inhibition while reducing PIN-FORMED 1 (PIN1)-dependent acropetal auxin transport Proc. Natl. Acad. Sci. 108 18506–11
[14] Sacks M M, Silk W K and Burman P 1997 Effect of Water Stress on Cortical Cell Division Rates within the Apical Meristem of Primary Roots of Maize. Plant Physiol. 114 519–27
[15] West G, Inzé D and Beemster G T S 2004 Cell Cycle Modulation in the Response of the Primary Root of Arabidopsis to Salt Stress Plant Physiol. 135 1050 LP – 1058
[16] Velappan Y, Signorelli S and Considine M J 2017 Cell cycle arrest in plants: what distinguishes quiescence, dormancy and differentiated G1 ? 495–509
[17] Lermontova I, Rutten T and Schubert I 2011 Deposition, turnover, and release of CENH3 at Arabidopsis centromeres Chromosoma 120 633–40
[18] Raynaud C, Mallory A C, Latrasse D, Jégou T, Bruggeman Q, Delarue M, Bergounioux C and Benhamed M 2014 Chromatin meets the cell cycle J. Exp. Bot. 65 2677–89
[19] Considine M J and Foyer C H 2013 Redox Regulation of Plant Development *Antioxid. Redox Signal.* 21 1305–26

[20] Jiang K, Meng Y L and Feldman L J 2003 Quiescent center formation in maize roots is associated with an auxin-regulated oxidizing environment *Development* 130 1429 LP – 1438