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BRIEF COMMUNICATION

Evaluation of light-harvesting complex proteins as senescence-related protein markers in detached rice leaves

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Abstract

Ten light-harvesting complex (Lhc) proteins were investigated to determine which was the most appropriate protein marker of senescence in detached rice leaves. The levels of Lhc proteins were monitored by immunoblot analysis, which was conducted using commercially available antibodies raised against each Lhc protein. Among the Lhc proteins evaluated in this study, Lhca1, Lhcb1, Lhcb2, Lhcb3, and Lhcb5 were not appropriate to be used as senescence markers while others can be used after optimization of the procedure.

Additional key words: abscisic acid; *Oryza sativa*; pigment-binding proteins; senescence-associated genes; zeatin.

Senescence is a well-known developmental process that involves the disassembly of cellular components in the senescing tissue and the mobilization of essential nutrients to developing parts of the plant. During senescence, a large number of genes are differentially expressed. In general, genes involved in anabolic processes are downregulated, whereas genes essential for catabolic processes are upregulated during senescence. In a study conducted by Buchanan-Wollaston *et al.* (2005), microarray analysis revealed that changes in the expression of 800 genes were induced upon leaf senescence in *Arabidopsis*. Accordingly, these genes were collectively designated as senescence-associated genes (SAGs). Many representative SAGs have since been employed as senescence-related molecular markers in an attempt to demonstrate the severity of senescence in plants. In *Arabidopsis*, these genes include a series of SAGs, of which SAG12 and SAG13 encode cysteine proteinase and alcohol dehydrogenase, respectively (Mishina *et al.* 2006). In rice, twelve SAGs have been reported, including aspartic proteinase (Osl295) and aminotransferase (Osl2) (Lee *et al.* 2001). In contrast to SAGs, senescence downregulates many genes that were primarily involved in photosynthesis, such as ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and Rubisco activase (Weng *et al.* 2005). Although the transcriptional induction of SAGs coincides with the degradation of chlorophyll (Chl) or phenotypic senescence symptoms, this measurement is somewhat time-consuming and requires laborious experimental procedures such as Northern blot analysis. Therefore, protein levels have also been employed to evaluate the sequential symptoms of senescence in plants (Parrott *et al.* 2005). Pigment-binding proteins of the light harvesting complex (Lhc) of photosystem I and II, such as Lhca and Lhcb, are associated with Chl *a*, Chl *b*, and xanthophylls; therefore, the levels of these proteins are in parallel with the Chl levels. Due to their specific binding to Chl *a*, Chl *b*, and xanthophylls molecules, these antenna proteins (Lhc family proteins) may play a role in maintaining the balance between light harvesting and the utilization of the photosynthetic apparatus upon leaf senescence which is characterized by Chl degradation (Huner *et al.* 1998). In addition, antibodies for Lhca and Lhcb that exhibit a broad range of reactivity in dicotyledonous and monocotyledonous plants are readily available. As a result,

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Abbreviations: ABA – abscisic acid; Chl – chlorophyll; EDTA – ethylenediaminetetraacetate; Lhc – light-harvesting complex; Lhca – light-harvesting complex protein of photosystem I; Lhcb – light-harvesting complex protein of photosystem II; SAGs – senescence-associated genes.
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Lhca and Lhcb are employed as senescence-related protein markers (Lucinski *et al.* 2006, Jung *et al.* 2008). However, the expression levels of Lhc proteins and the efficacy of Lhc antibodies vary among plant species and among treatment methods used within a species (Rosiak-Figielek and Jackowski 2000, Jackowski and Zelisko 2001). Therefore, in this study, we evaluated changes in the Lhc proteins upon senescence of the detached leaves of rice to identify the Lhc that can best be used as a senescence-associated protein marker. To the best of our knowledge, no similar studies have been conducted to date. As shown in Table 1, the leaves of 4-week-old rice (*Oryza sativa* cv. Dongjin) plants that were grown in a plant growth room at 28°C and 70% humidity under a 16-h light/8-h dark cycle with a 150 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD), were employed. The apical 15 cm of the third leaf was used in all experiments. A group of five segments was transferred into a 50-cm^3 polypropylene conical tube containing 10 cm^3 of water and then incubated under the same growth conditions described above for the specified time periods. Chl contents were determined spectrophotometrically according to the method of Porra *et al*. (1989). Rice leaves (0.3 g) were extracted in 10 cm^3 of 80% (v/v) acetone. After centrifugation, absorbance was read at 646.6, 663.6, and 750 nm. Chl *a* and Chl *b* were calculated according to formulas described by Porra *et al*. (1989). Under these conditions, the Chl levels declined by 20% at 6 d and by 50% and 58% at 8 d and 10 d, respectively.

To evaluate the Lhc protein levels upon senescence of the detached rice leaves, we conducted immunoblot analysis of the rice leaves described in Table 1. The total protein extracts were prepared by homogenizing rice leaves in a mortar and pestle with 1 cm^3 of the homogenization buffer (80 mM Tris/HCl (pH 7.0), 20% (m/v) glycerol, 10 mM sodium metabissulfate, 10 mM sodium ascorbate, 1% (m/v) polyvinyl pyrrolidone, 5 mM β-mercapthoethanol, and 2 mM EDTA). The homogenates were then centrifuged for 10 min at 13,000 × *g*, after which the supernatants (30 μg protein equivalent) were subjected to 11% SDS-PAGE and then electroblotted onto PVDF membranes.

Table 1. Chlorophyll (Chl) contents [mg g^{-1}(fresh leaves)] upon the senescence of detached rice leaves. The means of five replicates ± SD. Within each column, values followed by the same letter are not significantly different at $P<0.05$ according to LSD multiple test.

Day	Chl *a*	Chl *b*	Total Chl
0	12.8 ± 0.6^{a}	22.4 ± 0.4^{a}	35.2 ± 1.0^{a}
4	12.6 ± 0.8^{a}	22.0 ± 0.7^{a}	34.6 ± 1.5^{a}
6	13.0 ± 0.6^{a}	14.9 ± 0.6^{b}	27.9 ± 1.2^{b}
8	10.8 ± 0.9^{b}	7.1 ± 0.5^{c}	17.9 ± 1.4^{c}
10	9.4 ± 0.7^{c}	5.4 ± 0.4^{d}	14.8 ± 1.1^{d}

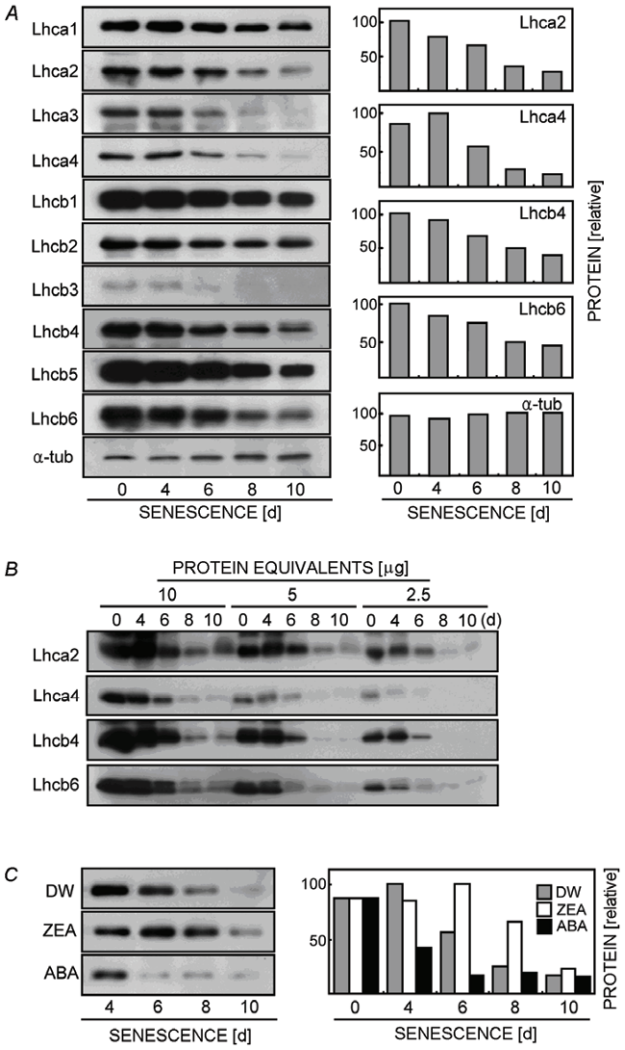

Fig. 1. Immunoblot analysis of Lhc proteins upon the senescence of detached rice leaves. *A*: immunoblot analysis of Lhca and Lhcb proteins (*left panel*) and relative protein abundance (*right panel*), *B*: protein dose response patterns of selected Lhc proteins, *C*: effects of hormonal treatments on the levels of Lhca4 protein (*left panel*) and relative protein abundance (*right panel*). Rice leaves were detached from 4-week-old seedlings and subjected to senescence by incubating rice leaves in water or in the presence of 5 μM zeatin or abscisic acid for 10 d. The expression of tubulin (*Santa Cruz Biotechnology*, Santa Cruz, CA, USA) was used to demonstrate equal loading of protein samples. Relative protein abundance was determined by *AlphaEase FC Imaging System* (*Alpha Innotech*, San Leandro, CA, USA).

Immunodetection was conducted according to standard procedures using polyclonal antibodies against Lhc proteins (*Agrisera*, Vännäs, Sweden).

Of the 14 Lhc proteins found in higher plants, ten Lhc proteins (Lhca1-Lhca4 and Lhcb1-Lhcb6) were monitored in the detached rice leaves that were challenged with senescence (Fig. 1*A*). The levels of Lhca1 remained unchanged throughout the experiment, whereas the levels

of Lhca2, Lhca3, and Lhca4 decreased rapidly as senescence proceeded. The clearest band pattern was produced by Lhca4. Even though the change in the pattern of Lhca3 was similar to that of Lhca4, it showed multiple Lhca3 bands which were supposed to be degraded proteins of Lhca3. When the Lhcb proteins were evaluated, Lhcb1 and Lhcb2 showed similar levels of proteins even after senescence, but the other Lhcb proteins decreased gradually upon senescence when compared to the level of Lhca4. For example, Lhcb5 was expressed in high levels not only in control rice leaves but also at 10 d after senescence. Conversely, the level of Lhcb3 was very low in rice leaves regardless of senescence. Among Lhcb proteins, Lhcb4 and Lhcb6 showed the best expression patterns which were positively correlated to the levels of Chl upon senescence in detached rice leaves. Furthermore, a series of dilution experiments were performed to judge whether the initial signal used for evaluation of senescence is in the region of the linear response of the antibody signal. As shown in Fig. 1*B*, protein concentrations ranging from 10 μg to 2.5 μg equivalents exhibited linear response to each Lhc antibodies. To further confirm the availability of Lhc protein as protein markers of senescence in detached rice leaves, the detached rice leaves were incubated in the presence of 5 μM of zeatin or abscisic acid, which are known to inhibit or accelerate senescence in plants, respectively. As shown in Fig. 1*C*, treatment of the detached rice leaves with zeatin resulted in a significant delay of the disappearance of Lhca4. However, the Lhca4 protein rapidly disappeared when rice leaves were treated with ABA. These results suggest that Lhca4 protein expression is closely coupled with the severity of senescence in detached rice leaves. In conclusion, the level of Lhca4 protein expression as well as other Lhc proteins such as Lhca2, Lhcb4 and Lhcb6 can be easily utilized as senescence-related protein markers in rice leaves. The rice plant is the most important food crop worldwide and a common monocotyledonous model plant for genetic analysis; therefore, it is expected that the use of Lhc proteins as the molecular protein markers of senescence will be useful in future studies of senescence in rice (Liu *et al.* 2008, Mishra *et al.* 2008).

References

Buchanan-Wollaston, V., Page, T., Harrison, E., Breeze, E., Lim, P.O., Nam, H.G., Lin, J.F., Wu, S.H., Swidzinski, J., Ishizaki, K., Leaver, C.J.: Comparative transcriptome analysis reveals significant difference in gene expression and signaling pathways between developmental and dark/starvation-induced senescence in *Arabidopsis*. – Plant J. **42**: 567-585, 2005.

Huner, N.P.A., Öquist, G., Sarhan, F.: Energy balance and acclimation to light and cold. – Trends Plant Sci. **98**: 358-364, 1998.

Jackowski, G., Żelisko, A.: Lhca polypeptides are maintained during dark-induced senescence of leaves. – Acta Physiol. Plant. **23**: 437-442, 2001.

Jung, S., Lee, H.J., Lee, Y., Kang, K., Kim, Y.S., Grimm, B., Back, K.: Toxic tetrapyrrole accumulation in protoporphyrinogen IX oxidase-overexpressing transgenic rice plants. – Plant Mol. Biol. **67**: 535-546, 2008.

Lee, R.H., Wang, C.H., Huang, L.T., Chen, S.C.G.: Leaf senescence in rice plants: cloning and characterization of senescence up-regulated genes. – J. Exp. Bot. **52**: 1117-1121, 2001.

Liu, L., Zhou, Y., Zhou, G., Ye, R.J., Zhao, L., Li, X.H., Lin, Y.J.: Identification of early senescence-associated genes in rice flag leaves. – Plant Mol. Biol. **67**: 37-55, 2008.

Lucinski, R., Schmid, V.H.R., Jansson, S., Klimmek, F.: Lhca5 interaction with plant photosystem I. – FEBS Lett. **580**: 6485-6488, 2006.

Mishina, T.E., Lamb, C., Zeier, J.: Expression of a nitric oxide degrading enzyme induces a senescence programme in *Arabidopsis*. – Plant Cell Environ. **30**: 39-52, 2006.

Mishra, S.K., Patro, L., Mohapatra, P.K., Biswal, B.: Response of senescing rice leaves to flooding stress. – Photosynthetica **46**: 315-317, 2008.

Parrott, D., Yang, L., Shama, L., Fischer, A.M.: Senescence is accelerated, and several proteases are induced by carbon "feast" conditions in barley (*Hordeum vulgare* L.) leaves. – Planta **222**: 989-1000, 2005.

Porra, R.J., Thompson, W.A., Kriedmann, P.E.: Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. – Biochim. Biophys. Acta **975**: 384-394, 1989.

Rosiak-Figielek, B., Jackowski, G.: The disappearance kinetics of Lhcb polypeptides during dark-induced senescence of leaves. – Aust. J. Plant Physiol. **27**: 245-251, 2000.

Weng, X.Y., Xu, H.X., Jiang, D.A.: Characteristics of gas exchange, chlorophyll fluorescence and expression of key enzymes in photosynthesis during leaf senescence in rice plants. – J. Integrative Plant Biol. **47**: 560-566, 2005.