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**NOTE**

# Photosynthesis-related proteins of cup-shaped galls in *Litsea acuminata* leaves

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ABSTRACT: Insect-induced galls are an atypical growth and differentiation form of plant tissue. The objective of this research was to study the expression of photosynthesis-associated proteins in Cecidomyiidae galls derived from the leaves of *Litsea acuminata* using a Western blot analysis of antibodies against light-harvesting complex (LHC) proteins isolated from non-galled and galled leaves and gall tissues. These LHC proteins involved in RC-1a, RC-1b, LHCb4, LHCb5, CP47, and CP-1a showed different responses in galls and leaves and exhibited a remarkable potential modulation role in regulating gall development. All photosynthetic proteins were repressed in gall tissues, indicating that light reaction functioning was significantly repressed. Compared to non-galled and galled leaves, galls demonstrated significantly lower chlorophyll (Chl) content and photosystem II maximum quantum efficiency ($F_v/F_m$) values, suggesting that insect infestations reduced photosynthetic efficiency. In addition, there were significant and positive correlations between LHCb5, CP-1a, and CP47 vs. Fv/Fm values and Chl content in galls and leaves, indicating that gall infections induced physiological changes, and therefore, that the Fv/Fm value could be utilized as a tool to easily and quickly study the eco-physiology of galls.

KEY WORDS: *Chlorophyll fluorescence, gall, light-harvesting complex protein, photosynthesis, western blot.*

## INTRODUCTION

Gall tissues induce abnormal tissue formation on host plants by ovipositing or feeding. Insect galls are one of the adaptive strategies that plants use to keep insect larvae within a tumor-like tissue outgrowth that is apart from normal plant leaves, stems, and other organs (Stone and Schönrogge, 2003). Net photosynthesis rates in galls are usually much lower than in normal leaf tissues (Dorchin *et al.,* 2006). Galls cause multiple physiological changes in host plants, such as changes in nutrient composition, deficiencies in pigment-protein complexes, lower chlorophyll (Chl) content, and higher secondary metabolite content, all of which may impact the photosynthetic capacity of host leaves (Motta *et al*., 2005; Dima *et al.,* 2006). The physiological changes in plants in response to gall-inducing insects have been listed (Rohfritsch, 1992). Insect-induced galls cause infected leaves to synthesize fewer photosynthetic pigments and instead redirect that energy into producing more proteins and other compounds that protect the plant system against the insects in the gall (Nabity *et al.,* 2013). In photosynthesis, light reactions require the participation of protein complexes in thylakoid membranes and the mobile electron carriers plastoquinone, plastocyanin, and ferredoxin (Lu and Yao 2018). Light-harvesting chlorophyll a/b-binding proteins are the apoproteins of the light-harvesting complex (LHC) of photosystem (PS), and are normally complexed with Chl and xanthophylls and serve as the antenna complex (Jansson, 1999).

Previously, we found that herbivorous insects caused deficiencies in the pigment-protein complexes of PSI and PSII in an oval-pointed cecidomyiid gall of *Machilus thunbergii* Sieb. & Zucc. leaves at an early stage of development. Insect-induced galls contained low amounts of light-harvesting Chl, but still possessed normal grana stacking and thylakoid morphology (Yang *et al.,* 2003). The gall and infected leaves of *M. thunbergii* were deficient in the LHC2b protein, and leaf-derived cecidomyiid galls were sinks in *M. thunbergii* leaves (Yang *et al.,* 2007; Huang *et al.,* 2014). We also demonstrated that gall tissues in *Litsea acuminata* leaves lost their photosynthetic ability as well as experienced a reduction in Chl and other photopigments (Huang *et al.,* 2015), followed by examining the transcriptome of gall tissues and showed a significant down-regulation of photosynthesis-related genes (Shih *et al.,* 2018). Recently, we reported that leaf derived cecidomyiid galls induced by different insects can alter the distribution of chloroplasts, and there is a positive correlation between confocal images and transmittance spectra of chloroplast in cecidomyiid galls, and therefore, the transmittance spectra could be utilized as a tool to easily and quickly study the ecophysiology of galls (Huang *et al.*, 2019). Although the physiological and biochemical regulation of galls have been demonstrated, no work has been done on the photosynthesis-associated proteins of *L. acuminata* galls, and their relationships to the chlorophyll fluorescence (ChlF) of galls have not been

studied ever. Thus, the aim of this research was to detect the LHC proteins of PSI (RC-1a, RC-1b, and CP-1a) and PSII (LHCb4 and LHCb5, and CP47) in cup-shaped galls on *L. acuminata* leaves. In addition, the correlations among LHC proteins, Chl content, and ChlF (the maximum quantum efficiency of PSII, $F_v/F_m$) values of galls and leaves were also evaluated.

## MATERIALS AND METHODS

Gall tissues induced by a gall midge (genus *Bruggmanniella*, Cecidomyiidae, Diptera) on the leaves of *Litsea acuminata* (Lauraceae) were collected from the Erhtzupin of Yangmingshan National Park (25°11.08′N 121°31.365′E) in Taiwan in December, 2018. Mature cup-shaped gall tissues are shown in Fig. 1A. Details regarding a gall midge and its gall tissue were described in our previous paper (Huang *et al.,* 2015). Leaves and galls were frozen with liquid nitrogen for further analysis. The Chl contents of leaves and galls were determined according to Yang *et al.* (1998). ChlF was detected at dawn on dark-adapted galls and uninfected tissues of the same leaf at room temperature with a Pocket Plant Efficiency Analyzer (Hansatech, U.K.), and the procedures for $F_v/F_m$ analysis were described by Huang *et al.* (2015).

Leaf and gall tissue total protein were extracted by P-PER Plant Protein Extraction Kit (Thermo Fisher Scientific, USA). One hundred milligrams of each sample (fresh weight) were ground up in liquid nitrogen and proteins were extracted according to manufacturer instructions. Extracted proteins were stored at -80 ℃ for the following analysis. Ten micrograms of total protein in each sample were loaded for sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane and blocking was performed by using 3% bovine serum albumin (BSA, Sigma Aldrich, USA). After blocking, membranes were hybridized with antibodies against reaction center proteins (CP-1a, CP47, RC-1a, and RC-1b, developed against spinach CP-1a, CP47, RC-1a, and RC-1b, respectively) and the light harvesting complex (LHCb4 and LHCb5, Agrisera No. AS04045 and AS01009, respectively, Agrisera, Sweden) at 4 ℃ overnight. Membranes were incubated in HRP-conjugated secondary antibody (Goat-anti-rabbit IgG, Jackson ImmunoResearch, USA) at room temperature for 2 hr after washing. Chemiluminescence signals were visualized by imager (Biospectrum 810, UVP, Upland, CA, USA). The quantification of relative protein contents was determined with computing software (Image Quant v. 3.19.4, Molecular Dynamics, Sunnyvale, CA, USA). Band intensity was measured by VisionWorks LS (UVP) for comparing the 'relative' values of the protein contents among leaves and galls.

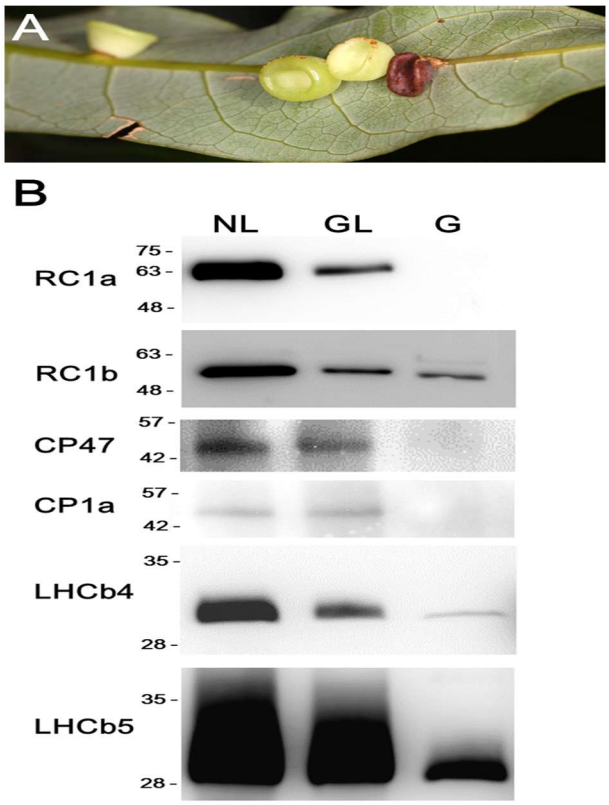


**Fig. 1.** Gall image and Western blot. **A.** Cup-shaped galls on a leaf of *Litsea acuminata*. **B.** Immunoblot of photosystem-related proteins (RC-1a, RC-1b, CP47, CP-1a, LHC-b4, and LHC-b5) against non-galled leaves (NL), galled leaves (GL) and gall tissues (G). Ten micrograms of total protein in each sample were loaded for sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Galls and leaves were sampled from three plants, with three replicates from each of the plants. Differences in $F_v/F_m$ values and Chl contents among tissues were examined using a completely randomized analysis of variance (ANOVA). For significant values, means were separated by the least significant difference test at $p \leq 0.05$. Relationships among photosystem-related protein content, $F_v/F_m$ value, and chlorophyll content were examined using simple linear regression model. The regression line was calculated from the pooled results for all protein contents of non-galled leaves, galled leaves, and gall tissues. All statistical analyses were conducted using JMP software, version 5.01 (SAS Institute, Cary, NC).

## RESLUTS AND DISCUSSION

Western blot analysis of the protein expressions of cup-shaped galls and host leaves were analyzed and shown to differentially express proteins in gall tissues (Fig. 1B). A clear band of RC-1a was detected in leaf tissues (63 kDa) as expected, but this protein was not detected in galls. Although their morphology is distinct from that of leaves, gall tissue had significantly reduced

levels of RC-1b (55 kDa), LHC-b4 (30 kDa), LHC-b5 (30 kDa), CP47 (45 kDa), and CP-1a (45 kDa), as did host leaves, having expressed proteins identified as those found in leaves. These photosystem-related protein quantities presented as ''relative'' values for comparisons of relative protein contents among leaves and galls are shown in Fig. S1. Non-galled leaves had significantly higher accumulations of all proteins than galled leaves and galls. Because of the deficiency in photosynthesis and photosystems, the observed decreased synthesis of RC-1a, RC-1b, LHC-b4, LHC-b5, CP47, and CP-1a and the accompanying low-regulation of the Chl-degradation function were reasonable because their substrate (Chl) was not synthesized in the gall (Huang *et al.*, 2015). These data are consistent with our previous transcriptomic findings, which showed the significant down-regulation of ~95% of genes related to either PSI or to PSII (Shih *et al.,* 2018). Moreover, the expression of genes associated with Chl-biosynthesis and degradation were also repressed (Shih *et al.,* 2018).

Nabity *et al.* (2013) showed that the expression of photosynthesis-related genes was reduced in galls compared to undamaged leaves, and the phylloxera-induced functioning of stomata in plants and insect transcriptionally reprograms galler-induced tissue beyond primary metabolism to include downstream secondary processes. The ability of this gall-forming insect to manipulate its host by inducing the formation of stomata, anatomical structures that balance proteins and nutrient transport, allows this parasite to reduce the negative effects of herbivory and increase the compatibility between this parasite and its host. Our results showed similar results in the comparison of gall tissue and host leaves, suggesting that host leaves remained as functional as undamaged leaves. Compared to the host leaf, RC-1a content in galls was undetectable and galled leaves lost their RC-1a photosynthetic ability. RC-1a related to photosynthesis was differentially expressed among tissues, and an up-regulation in RC-1a levels may also indicate an increase in the photorespiration rate.

Insect-induced galls are transformed from leaves infected by insects and contain an abnormal pigment-protein complex composition in PSI and PSII (Huang *et al.*, 2014). Galls have a lower content of Chl-protein complexes than leaves, which is related to deficiencies in the pigment-protein complexes CP1, A1, AB1, and AB2 (Yang *et al.,* 2007). The content of Chl-protein complexes is closely related to light absorption, and their deficiencies have been shown to have lower efficiencies of electron utilization (Gilmore and Ball, 2000). In our study, the amount of all examined proteins in galled leaves were decreased, suggesting that the development of cup-shaped galls might be regulated by these proteins derived externally, for example, from the insect larvae. The RC-1a protein of galls is not a remnant component of gall formation, probably because RC-1a protein is missing throughout the life of the gall. The incomplete organization of PSI may affect the gall photosynthetic functions of light-harvesting, energy transfer, and photochemical energy conversion performed in pigment protein complexes. All analyzed LHC proteins were repressed in the galls, and the translation responses in gall tissues revealed that the functions of the light reaction and Calvin cycle were significantly repressed. These results revealed the photosynthetic protein adaptive machinery of plant cells after gall induction, and also demonstrated the feasibility and reliability of uncovering functional gene groups via bioinformatics methods, especially via transcriptomic approaches to analyze whether the LHC proteins were up-regulated in the host leaf.

The photosynthesis-associated proteins shown in present study provides the correlation with our previous published photosynthesis-related dataset on chlorophyll fluorescence and Chl content (Huang *et al.,* 2015). Relationships among the $F_v/F_m$ value and Chl content vs. photosynthesis-related proteins in non-galled and galled leaves and cup-shaped galls of *L. acuminata* are presented in Fig. 2. Regression analysis showed that Fv/Fm values and Chl content were significantly and positively correlated with all photosynthetic proteins at determination coefficients ($R^2$) ranging from 0.4028 ~ 0.9499 in Fv/Fm value (Fig. 2 left panel) and 0.4807 ~ 0.9785 in Chl content (Fig. 2 right panel). In particular, the relationship of Chl content and Fv/Fm value in the LHC-b5, CP-1a, and CP47 of leaves and galls was strong. Furthermore, $F_v/F_m$ values and Chl content were used to investigate whether the photosynthesis performance of galls and host leaves of various PS-related proteins were different, with the finding that non-galled and galled leaves had a similar $F_v/F_m$ value (0.77) and Chl content (2000 ~ 2134 μg/g DW) in all tested proteins, while cup-shaped galls contained a similar ChlF value (0.705) and trace amounts (close to zero) of Chl in all proteins. $F_v/F_m$ values and Chl content were significant higher in the two leaf types than in galls of all proteins (Huang *et al.,* 2015). However, no significant differences in Fv/Fm values and Chl content were observed between non-galled and galled leaves, indicating that host leaf PS efficiency might not be affected at all by insect galling activity. These results can be explained by a stronger fluorescence intensity in the chloroplasts of host leaves than in leaf-derived galls, and by the pigment-protein complexes of PSI and PSII in galls being deficient over the lifetime of the gall (Yang *et al.,* 2003). Furthermore, the deficiency of Chl in LHC proteins results in incomplete light-harvesting by PSI and PSII, and $F_v/F_m$ values might be much lower in galls than in leaves or even that no photosynthesis took place at all. Galls might have a much lower PSII energy transduction efficiency than non-galled leaves, exhibit a depressed PSII efficiency, and suffer physical damage to their PSII reaction centers.

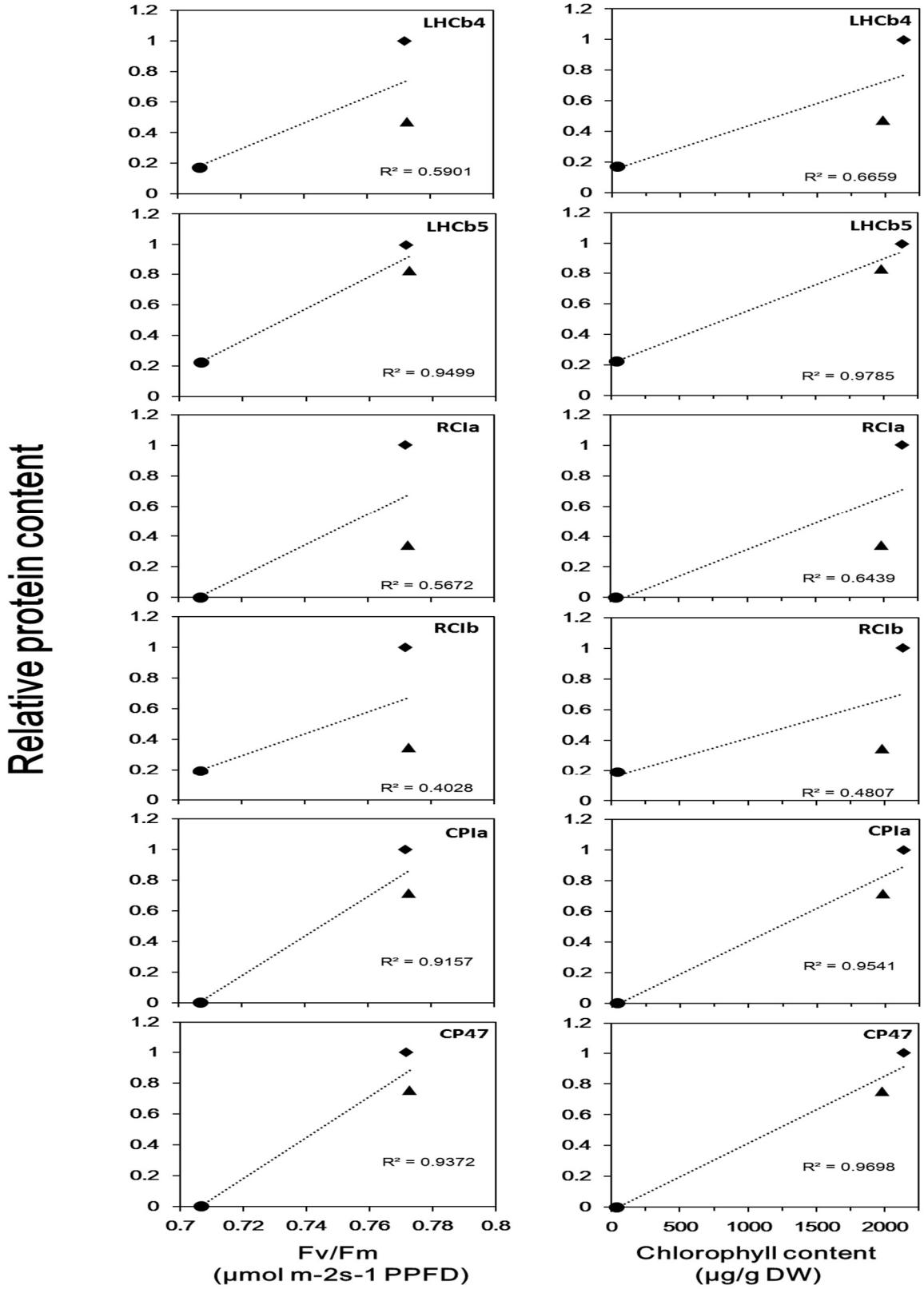


**Fig. 2.** Relationships between photosystem-related protein content and Fv/Fm value (left panel) and chlorophyll content (right panel). These protein quantities are presented as ‘‘relative’’ values for comparing of relative protein contents among leaves and galls. The regression line was calculated from the pooled results for all protein contents of non-galled leaves (◆), galled leaves (▲), and gall tissues (●).

Plants adapt photosynthesis to a certain degree in response to the prevailing environment, and the sensitivity of photosynthesis to stress varies among plant species and cultivars. Photoprotective reactions prevent the formation of reactive excited states and photoinhibition. The photoinhibition of photosynthesis is characterized by a reduction in the quantum yield of photochemistry and a decrease in ChlF, which entails not only the inhibition of PSII but also increases the thermal de-excitation of excited Chl (Demming-Adams *et al.,* 1996). The photosynthetic rate, PSII efficiency, and Chl content of plants can be estimated by leaf ChlF (Weng *et al.,* 2006). Measuring the yield of ChlF gives specific information about photochemical efficiency and heat dissipation. ChlF components can be used to measure different functional levels of photosynthesis, and changes in ChlF can also be used to quickly assess plant physiological responses during stress (Laing *et al.,* 2000). Eco-physiological studies of leaf ChlF have become popular because of their simplicity and non-destructiveness to leaves. Nevertheless, insect gall infections reduce the photosynthetic efficiency that is detected using ChlF (Huang *et al*., 2011). The contents of pigment-protein complexes decrease throughout their lifetimes, and they are sensitive to temperature, irradiation, and photoperiod (Lu *et al.* 1995). The observed similarities between the ChlF properties of insect-induced galls and Chl-deficient mutants suggest that galls might also be sensitive to temperature, light intensity, and photoperiod. There is much literature on the ChlF properties of leaves, with much of it relating to physiological studies and the large-scale remote sensing of vegetation (Gay *et al.,* 2008). Aldea *et al.* (2006) found lower PSII efficiency by ChlF ($F_v/F_m$) in galls and galled leaves compared to uninfected leaves. A scale insect on leaves of *Ilex aquifolium* also caused a higher PSII energy transduction efficiency, as indicated by ChlF, in affected tissues relative to uninfected tissues (Retuerto *et al.,* 2004). Traditional methods of pigment analysis, through extraction and spectrophotometric or HPLC measurements, require the destruction of measured leaves and are time-consuming and expensive. In contrast, the measurement of Fv/Fm is simple, rapid, and can be applied across spatial scales (Sims and Gamon, 2002). Positive and significant ($p < 0.05$ and $R^2 = 0.9157$~$0.9499$) correlations were found between $F_v/F_m$ and the LHC-b5, CP-1a, and CP47 of galls and leaves (Fig. 2). Our results indicate that it is possible to use $F_v/F_m$ as a tool for studying the eco-physiology of galls that occur seasonally in conducting large-scale remote sensing vegetation studies.

In conclusion, the proteins associated with photosynthesis were expressed differently in galls and leaves. The amount of all proteins in cup-shaped galls and galled leaves were obviously lower than in non-galled leaves, indicating that gall formation depresses PSII efficiency and physically damages PSII reaction centers. The responses of Chl content and Fv/Fm to PS-related proteins in galls and leaves showed that LHC-b5, CP-1a, and CP47 contents had a significantly higher correlation with Chl content and Fv/Fm values than other proteins, and therefore the Fv/Fm value represents a novel and useful parameter for examining the photosynthesis-related proteins of galls and leaves and can be utilized as a tool to easily and quickly study the eco-physiology of galls.

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**Supplementary materials are available from Journal Website.**