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Life time deficiency of photosynthetic pigment-protein complexes CP1, A1, AB1, and AB2 in two cecidomyiid galls derived from *Machilus thunbergii* leaves

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Abstract

Two kinds of cecidomyiid galls induced by *Daphnephila* on *Machilus thunbergii* Sieb. & Zucc. leaves at various developmental stages, *i.e.*, young, growing, and mature, were analyzed for their biochemical composition of photosynthetic pigment-protein complexes located in thylakoid membranes using the Thornber and *MARS* electrophoretic fractionation systems. Both kinds of galls were totally deficient in the pigment-protein complexes CP1, and A1, AB1, and AB2 through the whole period of gall formation. Immunoblotting of antibody against light-harvesting complex 2b (LHC2b) apoprotein confirmed this deficiency in gall's lifetime, which never recovered under any condition. Electron microscopy demonstrated that already at the early developmental stage the gall chloroplasts had thylakoid morphology like that in a normal leaf.

Additional key words: chlorophyll; chloroplast; immunoblotting; leaf age; ultrastructure.

Introduction

Insect galls are the transformed abnormal growth tissues of infected plants, in which the gall maker resides (Williams 1994). Although all plant organs are subject to insect galling, about 75 % of insect galls form on the leaf (Dreger-Jauffret and Shorthouse 1992, Yang and Tung 1998). In spite of the appearance diversity, most of the leaf galls must originate from leaf cells containing chloroplasts, in which photosynthetic pigments are located. Traditionally, the knowledge of photosynthesis in higher plants was based on the study of green leaf, and relatively few studies used chlorophyll-deficient mutants.

Morphology and anatomy of insect galls have often been studied (Dreger-Jauffret and Shorthouse 1992, Meyer 1987, Williams 1994) but only rarely the physiological changes in plant tissues in response to gall inducers have been evaluated. These include changes in pH, cell division polarity, nuclear and nucleolar hypertrophy, excess free amino acids and sugars, and the presence of hydrolytic enzymes such as amylase and protease (Mani 1992, Rohfritsch 1992).

Net photosynthetic rate (P_N) measured in the galls directly or estimated from radioactive labelling experiments is usually much lower than in normal leaf tissues (Andersen and Mizell 1987, Larson and Whitham 1991, Burstein *et al*. 1994, Larson 1998, Dorchin *et al*. 2006). Only a scale insect on leaves of *Ilex aquifolium* induced a higher P_N in affected tissues (Retuerto *et al.* 2004, Dorchin *et al*. 2006). All these measurements of gas exchange were made on leaf galls, making it difficult to differentiate between values recorded in the galls and those in surrounding leaf tissues. Little is known to date about the photosynthetic potential of chlorophyll-containing galls that are induced in other parts of the plant, such as stems or buds (Dorchin *et al*. 2006). However, studies on the impacts of gall-formers on host photosynthesis did not suggest any general trends; a range of effects from negative to positive was reported (Andersen and Mizell 1987, Fay *et al*. 1993, Bagatto *et al*. 1996, Larson 1998, Retuerto *et al*. 2004, Dorchin *et al*. 2006). Relatively little work has been conducted on chloroplasts of infected leaves. Three studies deal with the agranal thylakoid membrane distributed in the stroma (Rey 1973, 1974, 1992). Some photosynthetic pigment-protein complexes, such as A1, AB1, AB2, and CPI, are totally missing at mature stage, but the gall chloroplast still has normal grana and thylakoid morphology (Yang *et al*. 2003).

The above data were obtained by analyzing the galls collected from their mature stage. A further question

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arose on whether the pigment-protein complexes were deficient at the beginning of gall formation. Therefore we analyzed the biochemical composition of pigment-protein complexes of thylakoid membrane isolated from young (Y), growing (G), and mature (M) cecidomyiid gall chloroplasts of *M. thunbergii* Sieb. & Zucc. leaves, respectively. The same unique pattern of pigment-protein complex, which is different from that in normal chloroplast, was discovered in all stages of gall.

Materials and methods

Plant and gall: The green obovate and red oval-pointed galls induced by *Daphnephila* spp. (Diptera, Cecidomyiidae), with various sizes at three various developmental stages and residing on the lower epidermis of *Machilus thunbergii* Sieb. & Zucc. (Lauraceae) leaves were collected from Chung-Cheng Mountain of the Yangmingshan National Park in northern Taiwan during different seasons (Fig. 1). The two galls take about six months to develop and grow from deposition to maturity. Y, G, and M galls were collected in October and December of 2002 and March of 2003, respectively. The galls were detached from the infected mature leaves, and the surrounding healthy leaf tissue was trimmed to avoid contamination. All experiments were conducted on both galls, and all results were the same, so only the data of the green obovate gall is presented in this article. Similar results were also found in the gall growth seasons of 2001–2002 and 2003–2004.

Pigment-protein complexes: Thylakoid membranes were purified from both leaf and detached galls by differential centrifugation and were further analyzed for their constituent pigment-protein complexes by solubilization with SDS and electrophoresis on the gel of Thornber and *MARS* fractionation systems (Markwell 1986). The system of Thornber resolves two pigment-protein complexes, termed CP1 and CP2, in addition to a zone of free pigment (FP). The *MARS* system resolves four pigment-protein complexes, named A1, AB1, AB2, and AB3, besides the free pigment zone (Markwell 1986).

Detection of LHC2 apoproteins: The fractionated pigment-protein complexes in the Thornber and *MARS* gels (Markwell 1986) were directly trans-blotted onto the nitrocellulose paper. Non-fat dry milk was used as a blocking agent. The immunoblots were incubated with antibodies against LHC2a, LHC2b, and LHC2c apoproteins of *Ficus microcarpa* leaf, and visualized by means of a goat anti-mouse IgG conjugated with alkaline phosphatase and the NBT-BCIP (nitro blue tetrazolium chloride-5-bromo-4-chloro-3-indolyl phosphate) chromogenic detection system as described by Leary *et al*. (1983). Only the data of the LHC2b immunoblot are presented here because data of all three LHC2 apoproteins are the same.

Transmission electron microscopy: The material was prepared according to the method of Spurr (1969). The inner part of gall at various developmental stages and central part of leaf were collected and cut into small cubes of the size of 1 mm^3 pieces, which were then put into a buffer solution containing 0.1 M phosphate buffer (pH 7.3) and 2.5 % glutaraldehyde, and underwent prefixation for 2–4 h at 4 °C. After this, washing was done twice using 0.1 M phosphate buffer of 5 % sucrose solution, 15 min each time, and then using 0.1 M phosphate buffer solution containing 1 % osmic acid to postfix for 1–2 h. Again, the sample was washed twice with 0.1 M phosphate buffer of 5 % sucrose. The sample was dehydrated with a series of ascending acetone concentrations, 50, 50, 70, 70, 90, 90, and 100 % 15 min each, which was followed by resin/acetone (1/2), resin/acetone (1/1), and resin/acetone (2/1) for 1, 2, and 4 h, respectively. Finally, 100 % resin was used overnight before being taken out, and then 100 % resin for 1–3 h. After embedding, it was placed in a 70 °C oven for polymerization for 8–48 h. Ultracut E machine (*Reichert–Jung*, Australia) was applied to cut samples into 60–90 nm chips, which were salvaged with a grid covered with film. Double-dyeing in uranyl acetate and 0.4 % lead citrate followed. The thylakoid morphology was examined with a *Philips CM 100* transmission electron microscope at 75 kV.

Results and discussion

Gall appearance and greenness: Two galls, green obovate and red oval-pointed, at three developmental stages, *i.e*. Y, G, and M, were approximately 1.5, 3.5, and 5.5 mm in diameter, respectively (Fig. 1). The fruit-like galls were formed on the lower epidermis of leaves. In green obovate gall the inner area was as green as that of higher plant leaf. In contrast, the red oval-pointed gall was less green and showed reddish purple inside (data not shown). During their lifetime, galls grew under shade. Their chlorophyll (Chl) *a/b* ratios were between 2.5 and 2.8, *i.e*. in the range of normal leaf (usually around 3).

Pigment-protein complexes: The system of Thornber electrophoresis showed that the infected leaves at any stages of *M. thunbergii* contained both the CP1 and CP2 pigment-protein complexes commonly found in higher plants while the two insect galls, at all developmental stages, contained only CP2 (Fig. 2). CP1 is derived from

photosystem (PS) 1 and contains mainly Chl *a*, while CP2 contains both Chl *a* and *b* and is derived from PS2 (Markwell 1986). The *MARS* electrophoretic system resolves only AB3 of the three pigment-protein complexes containing Chl *a* and *b* present in the normal thylakoid membranes of leaf at any development stage (Fig. 2). The pigment-protein complex A1 is a constituent part of the core of PS1, and AB1, AB2, and AB3 are derived from light-harvesting complex 2 (LHC2). The two cecidomyiid galls at any of the developmental stages of *M. thunbergii* are totally deficient in the pigment-protein complexes CPI, A1, AB1, and AB2, and no deficiency was recovered at any time or under any condition.

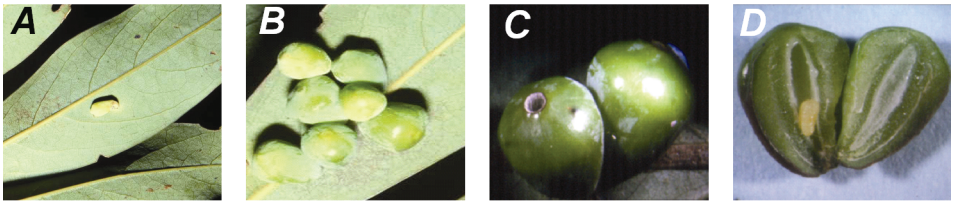

Fig. 1. The obovate cecidomyiid galls caused by *Daphnephila* sp. at various developmental stages on the leaf of *M. thunbergii*. *A*, *B*, and *C* are young (Y), growing (G), and mature (M) galls, respectively. *D* is an inside profile of mature gall. A yellow larva resides in the lower space of the left half gall chamber.

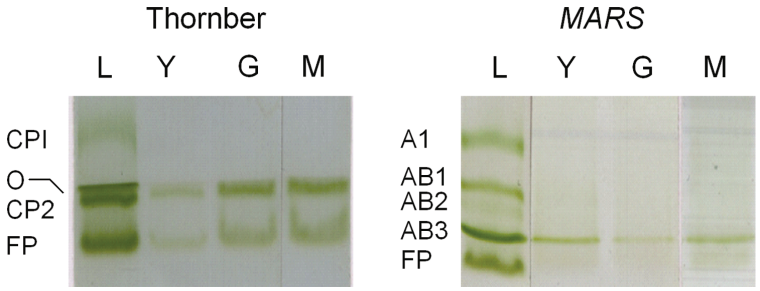

Fig. 2. Pigment-protein complexes of thylakoid membranes isolated from the obovate cecidomyiid galls induced by *Daphnephila* sp. at various developmental stages on the leaf of *M. thunbergii*. An oligomeric form (O) of the CP2 complex is visible migrating between CPI and CPII. L, leaf; Y, young gall; G, growing gall; M, mature gall.

LHC2 apoproteins: To estimate their content in the pigment-protein complexes of the two gall chloroplasts, three antibodies against LHC2a, LHC2b, and LHC2c of *F. microcarpa* were used. The pattern of pigment-protein complexes was further confirmed by western blotting of antibody against LHC2b apoprotein (Fig. 3). Only CP2 and its oligomeric form (O), and AB1, AB2, and AB3 contained the three LHC2 apoproteins. The two galls at all developmental stages contained only CP2 and AB3, in which LHC2b apoprotein resides.

The pigment-protein complex pattern of the insect gall is the same as that of mungbean testa (Yang *et al*. 1995) and black bean testa (Lu 2000) at all developmental stages, and neither of them is found in the normal chloroplast. The deficiency of pigment-protein complexes in insect-induced gall derived from infected leaf and in the green tissue of mungbean and black bean seed-coats is an interesting coincidence. The protein content of the inner gall tissue and that of non-gall plant tissues surrounding cynipid galls is very different (Schörogge *et al*. 2000). The former might involve the ectopic expression of seed-specific proteins. However, these proteins are not derived from leaves.

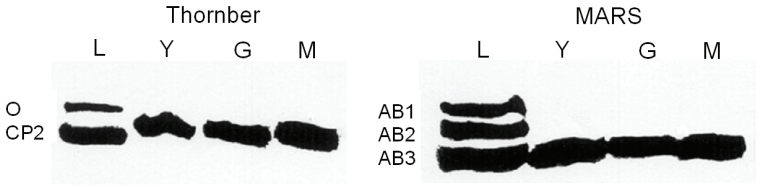

Fig. 3. Immunoblotting of antibody against LHC2b apoprotein in the fractionated pigment-protein complexes isolated from various developmental galls analyzed by Thornber and *MARS* electrophoretic systems. L, leaf; Y, young gall; G, growing gall; M, mature gall.

Thylakoid morphology: Ultrastructural study showed that the chloroplast of gall at all stages on the leaf of *M. thunbergii* had normal grana and thylakoid morphology and was the same as that of other higher plant leaves (Fig. 4). Our results reconfirmed the previous report (Yang *et al*. 2003) and even the gall chloroplast thylakoid at very early developmental stage still expressed normal morphology. The number of paired thylakoid membranes per granum in gall chloroplast increases as gall grows. According to Rey (1973, 1974) the chloroplasts in the gall of *Pontania proxima* Lep-infected willow leaf never contain starch, but a bundle of tubules appears in their stroma which is very often isolated in a stretched lobe. We observed similar thylakoid morphology, but at the very early stages of gall growth and development and not at the studied stages (data not shown). The results also

show that besides the LHC2 complex, other factors may get involved in grana formation.

Chl-deficient mutants—reported in barley, maize, pea, rice, soybean, sugar beet, wheat, sweetclover, *Arabidopsis thaliana*, *Chlamydomonas*, and other plants—reveal similar characteristics such as reduction in Chl content, a higher ratio of Chl *a/b*, an immature ultrastructure of thylakoid membrane, marked changes in pigment-protein complexes, and general sensitivity to temperature, irradiance, and photoperiod (Yang *et al*. 1993). However, the cases of three Chl-deficient mutants (sweetclover, barley, and rice) are inconsistent with the above pattern. These mutants are deficient or lacking in LHC2 complex, but still have normal stacking grana (Nakatani and Baliga 1985, Quijja *et al*. 1988, Yang and Chen 1996). Our previous study of a red oval-pointed cecidomyiid gall derived from *M. thunbergii* leaf was a fourth exception (Yang *et al*. 2003).

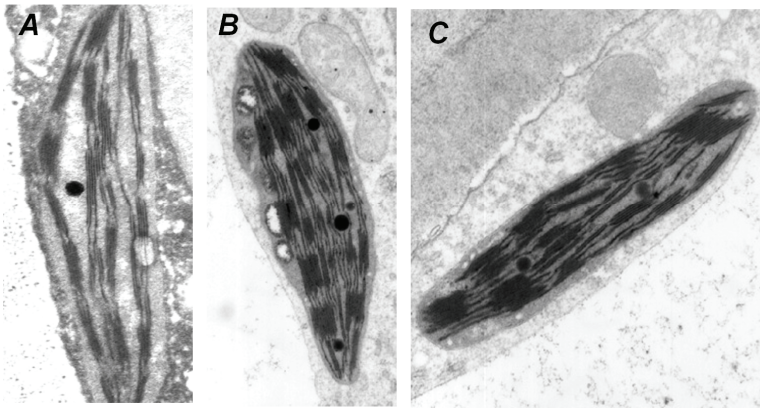

Fig. 4. Ultrastructural morphology of chloroplast thylakoid membrane of the obovate cecidomyiid galls at various developmental stages on the leaf of *M. thunbergii* galls. *A*, young gall; *B*, growing gall; *C*, mature gall.

An integration of the data in literatures and the present study lead to conclude that insect-induced galls, mungbean testa, and black bean testa are a kind of Chl-deficient mutant of leaf or a non-leaf green tissue with abnormal morphology. However, while the Chl *a/b* ratios of the insect-induced gall and mungbean testa are below the average, between 2.5 and 3.0, of leaf, those of Chl-deficient mutants are higher than 4, and some are indefinite (Yang *et al*. 1993). The Chl *a/b* ratio of the insect-induced gall, about 2.5, is close to that of leaf while those of mungbean and black bean testa, both about 0.7, are much lower.

Insect-induced galls are transformed from leaves infected by insect and contain abnormal pigment-protein complex composition of PS1 and PS2. The pigment-protein complexes of gall are not remnant components of gall formation, because CP1, A1, AB1, and AB2 are missing throughout the life of the gall. The incomplete organization of PS1 and PS2 may affect the gall photosynthetic functions of light-harvesting, energy transfer, and photochemical energy conversion performed in pigment-protein complexes. More researches should be conducted in the near future on the galls' photosynthesis.

Herbivorous insects cause the deficiency of pigment-protein complexes in an oval-pointed cecidomyiid gall of *M. thunbergii* leaf at a very early stage of development. The two insect-caused galls contain low amounts of LHC2, but still possess normal grana stacking and thylakoid morphology. That is, factors waiting to be explored other than LHC2 may also be involved in the grana stacking of chloroplast thylakoid. However, many questions remain unanswered, such as how frequently the deficiency phenomenon of pigment-protein complexes occurs in other insect galls, how the herbivorous insects induce the lack or deficiency of photosynthetic pigment-protein complexes, and what the physiological effect of this deficiency is.

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