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Two *Mallotus* species of different life histories adopt different defense strategies in relation to leaf age

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Abstract

Plants defend their leaves using multiple defense traits that change functions with leaf age. We examined the effects of leaf age on the development of multiple defense traits in two related *Mallotus* (Euphorbiaceae) species: young plants of the fast-growing *Mallotus japonicus* (Spreng.) Müll. Arg. and the slow-growing *Mallotus philippensis* (Lam.) Müll. Arg. Sequential leaves of the two species were measured for their leaf area, leaf mass/area, densities of trichomes and pellucid dots, extrafloral nectar volume, and the numbers of extrafloral nectaries and pearl bodies. *Mallotus japonicus* shifted its defense tactics from direct defense using trichomes and pellucid dots in young leaves to biotic defense using extrafloral nectar and pearl bodies in middle-aged leaves. In contrast, *M. philippensis* used direct, chemical defense throughout all leaf ages, together with the shift from indirect, biotic defense using extrafloral nectar in young leaves to direct, physical defense using leaf toughness in middle-aged leaves. These results strongly suggest that, in relation to life history, plants can alter optimal combinations of multiple defense traits with leaf age.

Keywords: biotic defense, chemical defense, defense shift, *Mallotus japonicus*, *Mallotus philippensis*, physical defense.

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Introduction

Plants in general are consumed by various herbivores. They have therefore employed diverse direct and indirect defense traits to reduce herbivory (Kant *et al*. 2004; Yamawo & Hada 2010; Agrawal 2011). Direct defense includes chemical defenses such as secondary compounds and toxins, and physical defenses such as trichomes and leaf toughness (Howe & Westley 1988; Schoonhoven *et al*. 1998). Indirect defense includes biotic defenses, such as extrafloral nectaries (EFNs), pearl bodies, and domatia, to attract predators and/or bodyguards against herbivores (Koptur 1992; Heil 2008).

Benefits and costs of such defense traits differ among them. Results of several studies suggest that the production cost of biotic (=indirect) defense traits is lower than that of direct defense traits (O'Dowd 1979; Katayama & Suzuki 2011). In *Ochroma pyramidale* (Cav. Ex Lam.) Urb., the cost of EFN production is about 1% of the total energy invested in leaves (O'Dowd 1979). By contrast, many reports describe that production costs of direct defense traits, such as trichomes, chemical substances, and leaf toughness, are high (Vickery & Vickery 1981; Strauss *et al*. 2002). In facultative ant–plant mutualism, the benefit or the efficiency of defense by biotic defense traits varies greatly compared with that by direct defense traits because the former depends on the abundance of ants in habitats (Giusto *et al*. 2001; Katayama & Suzuki 2011; Yamawo *et al*. 2012c). Therefore, the effect of biotic defense traits seems unstable even though its efficiency is higher than that of direct defense (Yamawo *et al*. 2012c).

Optimal resource allocation in plants to various defense traits has been presented in support of the "optimal defense theory" proposed by McKey (1974) and Rhoades (1979). The relative benefits of defense traits are affected by the value of defended organs, physiological cost of defense traits, and herbivory pressure. For example, leaf age affects the optimal defense tactics (Iwasa *et al*. 1996; van Dam *et al*. 1996; Lambdon & Hassall 2005; Hanley *et al*. 2007) because the leaf value, herbivory pressure, and physiological cost of defense traits change with the

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growth or aging of leaves (Meyer & Montgomery 1987; Kielkiewicz & van de Vrie 1990; Heil *et al*. 2004; Hanley *et al*. 2007). However, although the effects of plant age on defense tactics have been reviewed by Boege *et al*. (2011), the effects of leaf age on the development of multiple defense traits have been elucidated insufficiently.

Yamawo *et al*. (2012c) demonstrated that plants can shift defense tactics optimally with leaf age. In general, leaf value decreases with leaf age (van Dam *et al*. 1996). In the fast-growing *Mallotus japonicus* (Spreng.) Müll. Arg., middle-aged or mature leaves of low value are defended by biotic defense traits of low production cost, although their effect is unstable (Yamawo *et al*. 2012c). For *M. japonicus*, Yamawo *et al*. (2012c) suggested that the shift from expensive direct defense in young leaves to less expensive biotic defense in mature leaves might assure the high plant growth rate.

Such a time-shift in optimal defense tactics might occur differently even in related plants that have different life histories because the life history also affects the optimal defense strategy (Mole 1994; Agrawal 2007). Some studies predict that fast-growing plants should defend young leaves more efficiently than mature leaves (McKey 1974; van Dam *et al*. 1994). In contrast, slowly growing plants should defend mature leaves effectively as well as young leaves (Wesselingh 1995; van Dam *et al*. 1996) because they have leaves with greater longevity. Long-living leaves must be defended more effectively than short-living leaves (Newbery & de Foresta 1985; Wesselingh 1995; van Dam *et al*. 1996; Wright & Cannon 2001; Agrawal 2007).

In this study, we investigated the leaf defense traits of *Mallotus* plants (Euphorbiaceae), which includes both fast-growing and slow-growing species. *Mallotus japonicus*, a fast-growing pioneer species, is adapted to gaps and disturbed areas in temperate and subtropical regions of eastern Asia. This species has EFNs on its leaf edges and pearl bodies on its leaf surfaces and stems (Yamawo *et al*. 2012b) as biotic defense traits against herbivores (Yamawo *et al*. 2012c). Pearl bodies are known as lipid-rich particles (O'Dowd 1982) that are consumed by ants. Furthermore, *M. japonicus* has trichomes on its leaf surfaces as a physical defense and pellucid dots as a chemical defense. Pellucid dots typically contain toxic metabolic substances or essential oils (Wittstock & Gershenzon 2002; Sirikantaramas *et al*. 2008), and function as a general chemical defense on young leaves (Wittstock & Gershenzon 2002; Yamawo *et al*. 2012c). Although the pellucid dot contents of *M. japonicus* have not been identified, the pellucid dots of *Macaranga tanarius* (L.) Müll. Arg., a species in the Euphorbiaceae, contain the prenylated flavanone nymphaeol-C (Guhling *et al*. 2005).

Mallotus philippensis (Lam.) Müll. Arg., a slow-growing and shade-tolerant species that develops new leaves in autumn, is adapted to primary and secondary forests in tropical and subtropical regions of Asia and Australia (Sierra *et al*. 2005). Like *M. japonicus*, this species has EFNs as a biotic defense (Whalen & Mackay 1988) and pellucid dots as a chemical defense (Hussin *et al*. 1996). Trichomes are not apparent on leaves except for microscopic ones at stomata (Hussin *et al*. 1996). The mean lifespan of *M. philippensis* leaves is greater than 400 days (Ralhan & Singh 1987), which is three times longer than that of *M. japonicus* leaves (ca 130 days) (unpubl. data).

According to the optimal defense theory, *M. philippensis* is expected to defend its young and mature leaves nearly equally. Therefore, the use of physical, chemical, and biotic defenses might differ from that of *M. japonicus*. To confirm this point, we examined the effects of leaf age, not of plant age, on the development of multiple defense traits in both *M. japonicus* and *M. philippensis* plants. Our hypothesis is that optimal defense strategy using multiple defense traits differs between fast-growing and slow-growing species.

Materials and methods

Study area and date

Observations of two *Mallotus* species were conducted at forest edges and tree-fall gaps in Ishigaki Island, Japan (24°17′N, 123°53′E) during October 30 to November 1, 2009. The mean ± SD annual precipitation on Ishigaki Island during the past decade was 2041 ± 399 mm. The annual mean air temperature was 24.4 ± 0.2°C (Japan Meteorological Agency, http://www.data.jma.go.jp/obd/stats/etrn/index.php).

Estimates of defense traits in the field

Sample selection We randomly selected 30 young (=2-year-old) *M. japonicus* and 30 young (=2-year-old) *M. philippensis* plants of 20–45 cm height. For the top 10 leaves of each plant, the development of defense traits on leaves, leaf size, and leaf mass was examined. In both species, leaf positions were correlated strongly with leaf age (Yamawo *et al*. 2012c; unpubl. data).

Extrafloral nectar and pearl bodies The amount of extrafloral nectar and the number of pearl bodies produced by intact plants were not measured accurately because ants consumed or removed them continually. Therefore, on 30 October 2009, after removing all animals from each plant, an adhesive flycatcher ribbon was attached to the plant at ca 5 cm above the ground to block ant paths. Then, all extrafloral nectar and pearl bodies on every leaf were washed away with water. Each plant was then covered with a 0.5 mm meshed white cloth, which reduced sunlight by 10%. Twenty-four hours later, newly secreted extrafloral nectar on every leaf was collected with a 0.5-μL microcapillary tube to measure the nectar volume per leaf. The number of pearl bodies on every leaf produced in a day was also counted.

Leaf area and leaf mass The examined leaves of each plant were collected on 1 November, and were sandwiched in moisture-absorbing papers for 7 days. Then, the leaves were dried at 80°C for 3 days. The dried leaves were weighed to the nearest 0.1 mg using an electronic balance (BP211D; Sartorius, Goettingen, Germany). Each leaf was then scanned using an image scanner (PM-850A; Seiko Epson Corp., Suwa, Japan). Subsequently the area of each leaf was measured using a photo-image analyser (Scion Image; Scion Corp., Frederick, MD, USA). Finally, we calculated the leaf mass per area (LMA), which is highly correlated with leaf toughness (Wright & Cannon 2001; Hanley *et al*. 2007; unpubl. data).

Trichomes and pellucid dots The densities of trichomes and pellucid dots on each dry leaf were examined as follows. Two small areas near both sides of the midrib on the abaxial surface of the leaf were selected. The areas were roughly located on the one-fifth line of the leaf length. The trichomes and pellucid dots in each of the 23.7 mm^2 areas selected were counted under a microscope (40×). Microscopic trichomes at stomata were neglected. Then the densities (cm^{-2}) of these traits were calculated for each leaf.

Statistical analysis

A statistical analysis was performed using R Version 2.15.1 (R Development Core Team 2012). Analysis of variance by ranks (Friedman test) was employed for all sequential measures of leaf defenses: that is, leaf area, leaf mass per area (LMA), densities of trichomes and pellucid dots, the numbers of EFNs and pearl bodies, and the volume of extrafloral nectar. Thereafter, multiple pairwise comparisons (Steel–Dwass test) were performed.

Results

Leaf area and leaf mass

Leaves of *M. japonicus* developed gradually to their mature size until the third position from the tip ($P < 0.01$; Figure 1a). Leaf area did not increase below that position. The LMA did not differ among leaf positions ($P = 0.78$; Figure 1b). Leaves of *M. philippensis* developed to their mature size at the third position from the tip ($P < 0.001$; Figure 2a). The LMA of both first-year and second-year leaves did not differ among leaf positions within years ($P = 0.54$). Nevertheless, the LMA of the second year leaves was significantly greater than that of the first year leaves ($P < 0.001$; Figure 2b).

Trichomes

The densities of trichomes on *M. japonicus* leaves differed significantly among leaf positions ($P < 0.0001$; Figure 1c); trichomes on the first and second leaves were significantly denser than those on other leaves ($P < 0.01$) as previously reported by Yamawo *et al*. (2012c). On *M. philippensis* leaves, no trichomes were identified.

Pellucid dots

The densities of pellucid dots on *M. japonicus* leaves differed significantly among leaf positions ($P < 0.0001$; Figure 1d); pellucid-dot densities on the first and second leaves were significantly higher than those on other leaves ($P < 0.01$). In contrast, pellucid-dot densities on *M. philippensis* leaves did not differ significantly among leaf positions ($P = 0.55$; Figure 2c).

Extrafloral nectar

The number of EFNs on *M. japonicus* leaves did not differ among leaf positions ($P = 0.25$; Figure 1e). Nevertheless, the volume of produced nectar differed significantly among positions ($P < 0.0001$; Figure 1f); the second to fourth leaves produced larger quantities of extrafloral nectar than other leaves did ($P < 0.0001$). The number of EFNs on *M. philippensis* leaves did not differ among leaf positions ($P = 0.11$; Figure 2d) as those of *M. japonicus* did. In this species also, the volume of nectar produced on leaves differed significantly among positions ($P < 0.001$; Figure 2e). Unlike *M. japonicus*, the volume of extrafloral nectar on the first leaves was most abundant ($P < 0.001$), and it decreased rapidly with leaf position.

Pearl bodies

Pearl bodies on *M. japonicus* leaves were produced differently at different leaf positions ($P < 0.0001$; Figure 1g); they were significantly more numerous on the third to fifth leaves than on the other positioned leaves ($P < 0.05$). On *M. philippensis* leaves, no pearl bodies were produced.

Discussion

Results of this study demonstrate that *M. japonicus*, a fast-growing pioneer species, shifts its defense tactics with leaf aging from using direct defenses (trichomes and pellucid dots) in young leaves to biotic defenses (extrafloral nectar and pearl bodies) in middle-aged leaves, as reported by

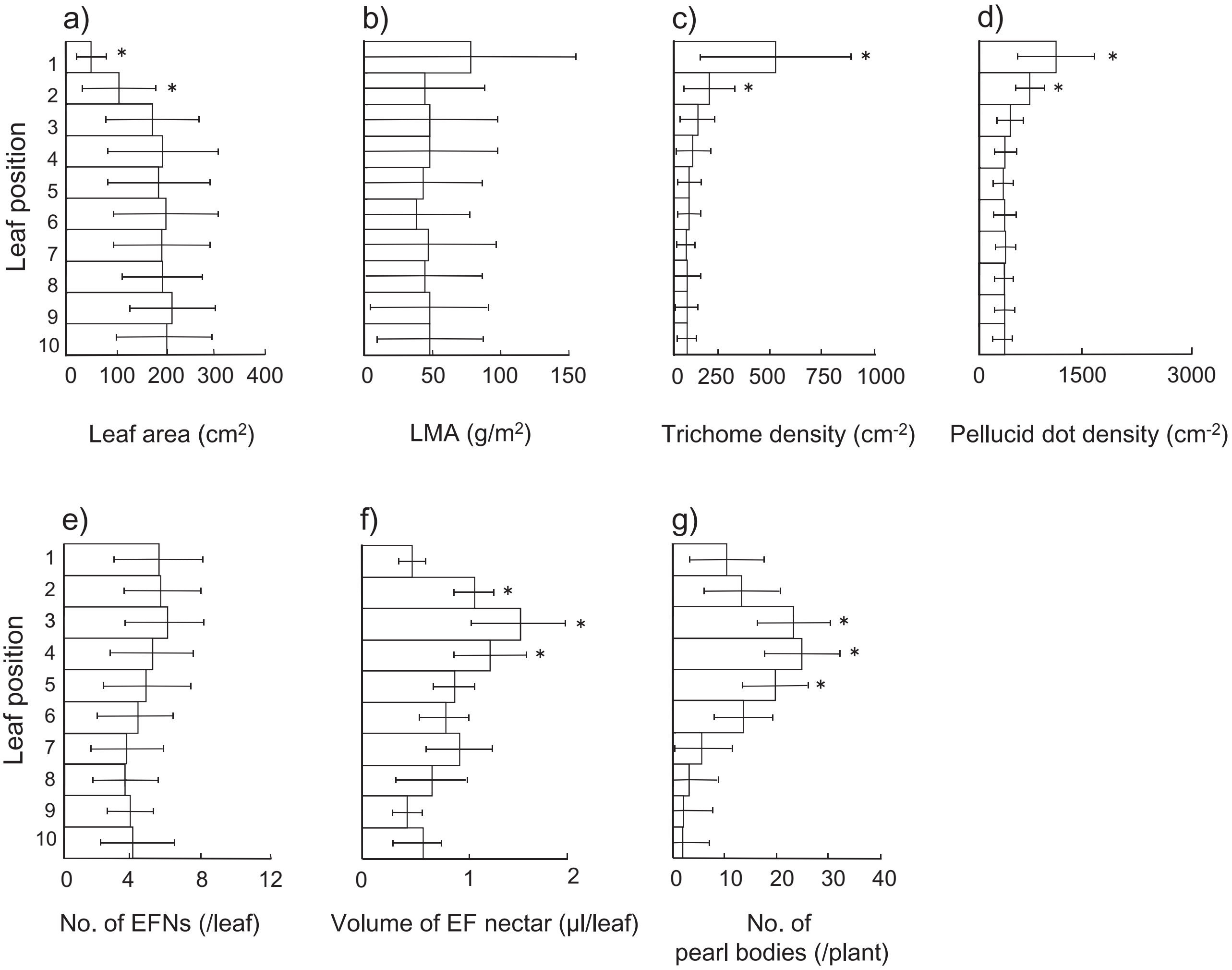

Fig. 1 Various leaf characteristics of *Mallotus japonicus* (Spreng.) Müll. Arg. at different leaf positions: (a) leaf area, (b) LMA (=leaf mass/area), (c) trichome density, (d) pellucid dot density, (e) number of extrafloral nectaries (EFNs), (f) volume of extrafloral nectar (EF nectar), and (g) number of pearl bodies. Bars represent SD ($n = 30$). Asterisks (*) denote significant differences (Steel–Dwass, $P < 0.05$).

Yamawo *et al*. (2012c). *Mallotus japonicus* does not increase LMA, which is highly correlated with leaf toughness (Wright & Cannon 2001), with leaf age. Presumably, it is beneficial for fast-growing plants to defend young, valuable leaves with direct defense traits because direct traits are more stable than biotic ones, but direct traits are costly, as demonstrated in many plants (Vickery & Vickery 1981; review in Strauss *et al*. 2002). Less-valuable middle-aged leaves are defended at low cost by biotic defense traits (O'Dowd 1979; Katayama & Suzuki 2011). The shift from direct to biotic defense with leaf aging supports the optimal defense theory, which posits that fast-growing plants maximize their fitness via such shifts (McKey 1974; Rhoades 1979; see Yamawo *et al*. 2012c).

In contrast, *M. philippensis*, a slow-growing and shade-tolerant species, uses direct chemical defense with a high density of pellucid dots on the leaves of all ages (=all leaf positions), indicating that this species continues to produce pellucid dots while developing leaves. Continuous high investment in a chemical defense of leaves in the slow-growing plant, *M. philippensis*, also supports the optimal defense theory (Wesselingh 1995; van Dam *et al*. 1996). In addition, young, upper leaves of *M. philippensis* secreted extrafloral nectar, which is the product of photosynthesis, as a biotic defense (Whalen & Mackay 1988). In general, the rate of photosynthesis depends on leaf nitrogen content (Feng 2008). The nitrogen content of young *M. philippensis* leaves is lower than that of mature leaves (Ralhan & Singh 1987). Therefore, the rate of photosynthesis of young leaves must be low. However, the additional biotic defense in young *M. philippensis* leaves requires much carbon. Therefore, the biotic defense in young *M. philippensis* leaves might be mediated by transporting newly assimilated carbohydrates from mature

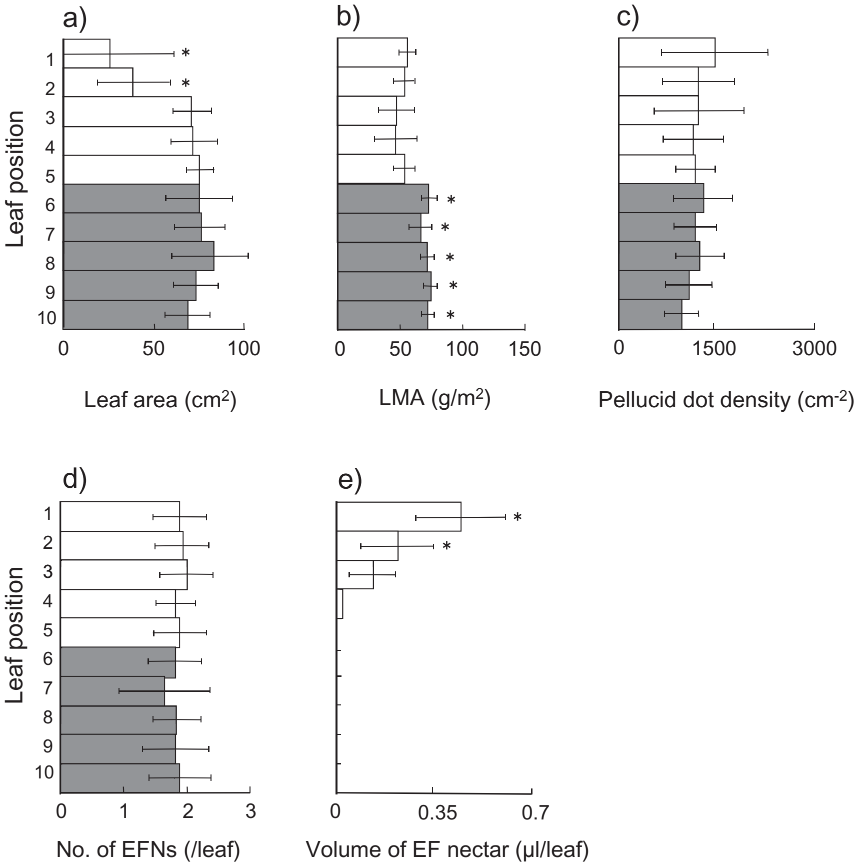

Fig. 2 Various leaf characteristics of *Mallotus philippensis* (Lam.) Müll. Arg. at different leaf positions: (a) leaf area, (b) LMA (=leaf mass/area), (c) pellucid dot density, (d) number of extrafloral nectaries, and (e) volume of extrafloral nectar (EF nectar). White rectangles, leaves produced in the study year; black rectangles, leaves produced in the prior year. Bars represent SD ($n = 30$). Asterisks (*) denote significant differences (Steel–Dwass, $P < 0.05$).

leaves to young leaves, as demonstrated in *Phaseolus lunatus* and *Ricinus communis* (Radhika *et al.* 2008). Moreover, the high LMA of mature leaves, which is consistent with the results of another study (Kikuzawa & Lechowicz 2011), indicates a shift from biotic defense to physical defense using leaf toughness with leaf aging.

Why does *M. philippensis* shift its defense tactics from biotic defense to physical defense with leaf age? In general, the value of young leaves is higher than that of mature leaves (Harper 1989; Heil *et al.* 2004; Lambdon & Hassall 2005). Therefore, plants should use physical defense in young leaves because its effect is more stable than biotic defense by ants. Nevertheless, young *M. philippensis* leaves adopt biotic defense using extrafloral nectar. Regarding this point, we argue the following. Young leaves cannot be mechanically tough because of the constraints of cell expansion and cell wall development (Kobayashi *et al.* 2008). Therefore, many plants use trichomes as a physical defense trait for young leaves (Strauss *et al.* 2002; Walters 2011). However, *M. philippensis* have no apparent trichomes on leaves to use for defense. Chemical defenses are commonly effective against generalist herbivores, but they are often ineffective against specialist herbivores (Handley *et al.* 2005; Blüthgen & Metzner 2007). Because young *M. philippensis* leaves are consumed not only by generalist herbivores but also by the specialist herbivore, *Pingasa ruginaria* (Lepidoptera: Geometridae; pers. obs.), nonchemical defense seems necessary. Biotic defense by ants does not inhibit leaf expansion and it is effective against widely diverse herbivores including specialist herbivores (Keeler 1977). For those reasons, *M. philippensis* is regarded as adopting chemical and biotic defenses on young leaves. These two defense traits might function compensatively against herbivores of different types. On expanded leaves with a long lifespan, defense by both pellucid dots and leaf toughness must be advantageous because these traits are stable.

Mallotus philippensis have no trichomes and pearl bodies, unlike *M. japonicus*. Many results of earlier studies suggest that habitat affiliation has shaped the evolution of plant defense traits (Kursar & Coley 2003; Fine *et al.* 2004, 2006; van Zandt 2007; Agrawal *et al.* 2009b). For example, in North American milkweeds, the primary driver of trichome evolution is apparently not herbivores (Agrawal *et al.* 2009a), but habitat conditions: species in drier habitats have higher trichome densities, although trichomes provide resistance to some herbivores (Agrawal & Fishbein 2006; Agrawal *et al.* 2009b). Young *M. japonicus* plants grow in various habitats such as open sunny sites, forest edges, and tree-fall gaps in forests. They can alter their defense tactics in response to the difference in various abiotic environmental factors including light, soil moisture, and nutrient conditions (Yamawo & Hada 2010; Yamawo *et al*. 2012a,b). In *M. japonicus* also, habitat affiliation might primarily have shaped trichomes as a defense trait, and herbivores and mutualist communities secondarily have driven their functions. Several years ago, Tanaka *et al*. (2008) reported that the trichome of a congener *Mallotus macrostachyus* functions as a desiccation-preventing trait as demonstrated in milkweed. In contrast to *M. japonicus* and *M. macrostachyus*, which grow under widely various habitat conditions, young *M. philippensis* plants grow under limited, nondry conditions (unpubl. data) such as forest floor and tree-fall gaps. This narrow range of habitats might not require *M. philippensis* to have trichomes and pearl bodies on leaves, but this species must defend its long-living leaves using pellucid dots and leaf toughness. Leaf toughness functions not only against herbivores but also against physical stress and microbes (Walters 2011). Then, shade-tolerant plants that have leaves with a long lifespan often impose the development of leaf toughness because their leaves are exposed to great physical and microbial stresses (Agrawal 2007; Kikuzawa & Lechowicz 2011).

In conclusion, we first demonstrated that defense strategies using multiple defense traits differ between the related species of different life histories. In the fast-growing species *M. japonicus*, which has leaves with a short lifespan, young leaves use direct defense, such as trichomes and pellucid dots, and middle-aged leaves use biotic defense, such as extrafloral nectar and pearl bodies. By contrast, in the slow-growing species *M. philippensis*, which has leaves with a long life-span, young leaves are defended with biotic defense using extrafloral nectar and middle-aged leaves are defended physically by toughening of leaves, together with direct chemical defense using pellucid dots throughout all leaf ages. These results suggest strongly that, in relation to leaf longevity, plants can adopt different optimal combinations of multiple defense traits with leaf age.

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