Scab formation and wound healing of plant tissue by soldier aphid

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In the social aphid *Nipponaphis monzeni*, a unique gall-repairing behaviour has been known: when a hole is made on the gall, many soldier nymphs discharge body fluid on the breach, which promptly solidifies and plugs the hole. Here, we experimentally investigated the subsequent fate of repaired galls and their inhabitants. Irrespective of natural repair by soldier nymphs or artificial repair with adhesive, repaired galls survived significantly better than non-repaired galls. Within a month after repair, the plant tissue around the hole proliferated and sealed up the hole. Many soldier nymphs were localized at the hole area and extermination of inhabiting aphids by insecticides aborted the gall regeneration, indicating that the gall regeneration requires inhabiting aphids, wherein soldier nymphs are likely to play a major role. This study provides an unprecedented case of scab formation and wound healing, which occurs at an animal–plant interface: scab derived from insect body fluid promptly plugs damaged plant tissue and subsequently the insects actively stimulate regeneration of the plant tissue, whereby the compromised plant tissue recovers. We suggest that the novel system may have evolved in the aphid lineage through enhancement and recruitment of the pre-existing capabilities of haemolymph coagulation and gall formation.

Keywords: social aphid; soldier nymph; gall repair and regeneration; wound sealing and healing

1. INTRODUCTION

The surface of animals is generally covered with a tough and impermeable structural barrier called skin, integument or epidermis. When the surface barrier is damaged, the animals quickly lose their body fluid, become vulnerable to mechanical stresses and easily infected with noxious micro-organisms, which would lead to fatal consequences. Hence, the ability to respond to injury and to repair damaged tissue is a fundamental property of all multicellular organisms (Jane *et al.* 2005; Gurtner *et al.* 2008).

In a wide array of animals including both vertebrates and invertebrates, wound repair and healing commonly entail two principal processes: rapid clotting and scab formation to seal the wound and subsequent tissue regeneration to heal the wound. When the surface barrier is breached, rapid clotting of body fluid occurs at the site of injury, which is followed by hardening and reinforcement of the clot to form a scab. The scab stops bleeding, physically protects the wound, prevents microbial infections and provides a scaffold for tissue regeneration. Subsequently, the cells around the wound proliferate, spread across the wound gap and restore the tissue integrity (Singer & Clark 1999; Gallo & Krasnow 2004).

Rapid scab formation and subsequent tissue regeneration are the common wound-healing processes found not only in mammals but also in insects and other invertebrates. However, the structure of their surface barrier, the composition of their body fluid, the biochemical components involved in their blood clotting and scab formation, and the restoration process of their surface tissue integrity are markedly different between those animal groups (Theopold *et al.* 2004; Jane *et al.* 2005). On account of the importance of wound sealing and healing for animals to survive in hostile environments, it has been assumed that the mechanisms for rapid scab formation and subsequent tissue regeneration have evolved repeatedly and independently in many animal lineages in a convergent manner. However, the conventional view was recently countered, at least partially, by the finding that both *Drosophila* and mouse commonly use the same transcription factor, *grainy head*, for initiation of their wound-healing processes (Mace *et al.* 2005; Ting *et al.* 2005). Considering that the wound responses in both vertebrates and invertebrates are integral to mounting innate immune responses at the front of the anti-microbial defence system (Jane *et al.* 2005; Gurtner *et al.* 2008), the diversity in processes and mechanisms of wound sealing and healing has become a focal topic in the emerging research field of the ecological and evolutionary immunology (Rolff & Siva-Jothy 2003; Siva-Jothy *et al.* 2005).

In this study, we report an unprecedented case of scab formation and wound healing, which occurs at an animal–plant interface: scab derived from insect body fluid promptly plugs damaged plant tissue and subsequently the insects actively stimulate regeneration of the plant

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tissue, whereby the compromised plant tissue recovers in a coordinated manner.

Many insects are known to form conspicuous galls on their host plant. The galls provide the inducer insects with isolated and exclusive habitat, constant and high-quality food supply, physical barrier against predators and mitigated environmental stresses such as desiccation and temperature fluctuation (Meyer 1987; Shorthouse & Rohfritsch 1992; Raman *et al.* 2005). The capability of gall formation, whereby the insects manipulate the plant growth and morphogenesis for their own sake, is no doubt an adaptive trait for the insects. Meanwhile, consisting of hypertrophied plant tissue and containing a large quantity of insects, the galls are potentially of high value as food source for herbivores and predators. Probably for that reason, some gall-forming aphids have evolved specialized altruistic individuals called 'soldiers' (Aoki 1987; Ito 1989; Stern & Foster 1996). Some of the social aphids produce soldier nymphs that are morphologically differentiated from normal nymphs and unable to grow, constituting a sterile caste, while other social aphids produce monomorphic soldier nymphs that are able to grow and reproduce. The social tasks of soldier nymphs include the colony defence prevalent among social aphids (Foster & Northcott 1994; Stern & Foster 1996), the housekeeping found in some gall-forming social species (Aoki 1980; Aoki & Kurosu 1989; Benton & Foster 1992) and the gall repairing known from *Nipponaphis monzeni* and *Pemphigus spyrothecae* only (Kurosu *et al.* 2003; Pike & Foster 2004).

The aphid *N. monzeni* (Aphididae: Hormaphidinae: Nipponaphidini) forms large globular galls on the tree *Distylium racemosum* (Sorin 1958). Kurosu *et al.* (2003) reported a spectacular, speedy and suicidal gall repair by monomorphic first-instar soldier nymphs of *N. monzeni*. When a hole was experimentally bored on the galls, many soldier nymphs came out, discharged a large amount of body fluid on the wounded plant tissue, mixed the fluid with their legs and plastered the fluid over the plant injury. The fluid soon became viscous and solidified, thereby plastering up the hole within an hour (movie 1 in the electronic supplementary material). Certainly, the self-sacrificing social behaviour quickly and successfully plugs the damaged galls, but the subsequent fate of the repaired galls has not been investigated. How about the survival of the repaired galls and their inhabitants? What will happen on the compromised gall tissue after the repair? How do the soldier nymphs behave in the repaired galls?

Here, we demonstrate that soldier nymphs of *N. monzeni* play active and dynamic roles in both the acute and chronic processes of the gall repair, namely scab formation over the hole and subsequent tissue regeneration beneath the scab, which provides an impressive case of sophisticated plant–herbivore interactions and extended plant phenotype due to insect manipulation in a social context.

2. MATERIAL AND METHODS

(a) Materials

Galls of *N. monzeni* formed on trees of *D. racemosum* were observed, experimentally manipulated and studied at Meguro and Shinkiba, Tokyo, Japan, from April to October in 2006–2008. After a series of experiments, the galls were collected,

brought to the laboratory at Tsukuba, Ibaraki, Japan, and subjected to further analyses.

(b) Experimental manipulation of galls

In the field, a small 2×2 mm square hole was bored on the wall of the galls using a fine edge of a chisel. In young spring galls, the hole was usually plastered up completely within 30 min by the body fluid of first-instar soldier nymphs, whereby completely repaired galls were prepared. For artificially disturbing the gall repair, the fluid on the edge of the hole was repeatedly removed by absorbing with tissue paper for 2 hours. Then, soldier nymphs stopped coming out and discharging the fluid, and the hole was kept open without further repair. For preparing artificially repaired galls, the hole was made and immediately filled with adhesive (Wood Glue, Konishi Co., Ltd; mainly consisting of 41% polyvinyl acetate). Preliminary experiments confirmed that the adhesive is not toxic to both the aphid and the plant tissue (data not shown).

(c) Insecticide treatment of galls

Chemically distinct two neurotoxic insecticides, mospiran (Nippon Soda) and sumithion (Sumitomo Chemical) were used. The main component of mospiran is acetamiprid, an agonist of the nicotinic acetylcholine receptor of insects. The main component of sumithion is fenitrothion, whose oxide inhibits the acetylcholine esterase of insects. Approximately 0.25 per cent aqueous solution of either mospiran or sumithion was spread on the whole surface of the galls by a brush. For control, distilled water was applied instead of the insecticide solution.

(d) Measurement of insect density

We cut the experimental galls and immediately took photos of the inside of their hole and non-hole areas. Numbers of soldier nymphs and non-soldier insects in 3×3 mm square were counted on the photos. For each of the experimental galls, a 3×3 mm square was set on the hole area, while three 3×3 mm squares were set on the non-hole area and subjected to averaging. The data of insect densities were statistically analysed with the software R v. 2.7.2 (R Development Core Team 2008). We adopted the generalized linear model (McCullagh & Nelder 1989) with a normal, gamma, inverse Gaussian or negative binominal distribution, which was selected according to the Akaike information criterion.

(e) Plant histology

Plant tissue specimens were dissected from the galls and fixed with FAA solution (1.9% formaldehyde, 5% acetic acid and 63% ethanol) for several months. The fixed tissues were dehydrated through the following water–ethanol series: 50 per cent ethanol for 2 days; 70 per cent ethanol for 2 days; 90 per cent ethanol for 2 days; and 100 per cent ethanol for 2 days twice. Then the samples were equilibrated with Technovit 7100 resin (Heraeus KULZER) as follows: 25 per cent resin diluted with ethanol for 1 day; 50 per cent resin for 2 days; 70 per cent resin for 2 days; 100 per cent resin for 5 days; and finally 100 per cent resin for 7 days. The samples were embedded in 100 per cent resin by an addition of the polymerizing reagent supplied by the manufacturer. Tissue sections were made on a rotary microtome (RM2165, Leica) with a tungsten knife, mounted on glass slides, stained with toluidine blue and observed under a light microscope.

Figure 1. Galls of *N. monzeni*. (a) A small young gall in early spring. (b) A premature gall in late spring. (c) A mature gall in summer. (d) The inside of a small young gall, greenish and thin-walled. (e) The inside of a premature gall, brownish and thin-walled. (f) The inside of a mature gall, with extremely thick and lignified wall. (g) A premature gall on which an experimentally bored hole was repaired by soldier nymphs. Arrow indicates the repaired hole. (h) An enlarged image of the repaired hole. Note that some soldier nymphs are left outside (arrows) while other soldier nymphs are buried in the solidified body fluid (arrowheads), which are all destined to die. (i) A premature gall with a naturally repaired hole (arrow), which was probably bored by a lepidopteran larva. (j) A dead experimental gall. The gall was experimentally bored and immediately repaired by soldier nymphs. After 30 days, however, the gall was found dead with another hole (arrow). (k) A premature gall on which an experimentally bored hole was filled with adhesive. Arrow indicates the artificially plastered hole. (l) A mature gall in autumn, on which an exit for the migration of winged insects was formed.

3. RESULTS

(a) General observation

In the research fields, galls of *N. monzeni* were found on *D. racemosum* trees with many galls of other species belonging to the same aphid tribe Nipponaphidini, including *Nipponaphis distyliicola*, *Neothoracaphis yanonis*, *Monzenia globuli* and *Quadrartus yoshinomiya*. Among them, the galls of *N. monzeni* were the largest in size (up to 8 cm in diameter) and the smallest in number (usually none or a few per tree). The gall-repairing behaviour was observed in no other aphid species than *N. monzeni*. The galls were small in early spring (early April; figure 1a), rapidly expanded during late spring (late April to early June; figure 1b) and reached maturity in summer (late June

and on; figure 1c). The galls were completely closed with no opening from spring to summer, but in late autumn (usually November) when many winged adult aphids for migration emerged, an opening spontaneously appeared on the wall by an unknown mechanism (figure 1l). In the mature summer galls, the wall was over 3 mm in thickness, highly lignified and extremely hard (figure 1f). In the young spring galls, by contrast, the wall was thin and soft (figure 1d,e). Ito & Hattori (1983) reported that young galls of nipponaphidine aphids are vulnerable to invasion by specialized lepidopteran larvae such as *Nola innocua*. In the fields, we certainly observed that galls of *N. distyliicola* and *N. monzeni* were occasionally tunnelled by the moth larvae (M. Kutsukake 2006, unpublished data).

Table 1. Survivorship of the experimental galls of *N. monzeni* whose hole was either repaired or non-repaired.

repair status	experimental treatment	survived after ~1 month	dead after ~1 month
hole repaired	nine spring galls, naturally repaired	7	2
	eight spring galls, artificially repaired	7	1
	five summer galls, artificially repaired	4	1
subtotal	22	18 ^a	4 ^a
hole not repaired	two spring galls, naturally repair failed	0	2
	five spring galls, artificially repair disturbed	0	5
	five summer galls, without repair	1	4
subtotal	12	1 ^a	11 ^a
total	34	19	15

^aThe subtotal numbers were subjected to a Fisher's exact probability test ($p < 0.001$).

(b) Active repairing behaviour of soldiers and survival of repaired galls in spring

On 28 April 2006, we bored a hole on 11 young galls of *N. monzeni* on *D. racemosum* trees in the field. In the spring season, the galls were premature, relatively small in size and with thin (approx. 1.2 mm) and soft wall tissue (figure 1b,e). In all the galls, first-instar soldier nymphs actively performed gall-repairing behaviour: discharging body fluid on the hole, mixing the fluid with their legs and plastering the fluid onto the hole. The fluid promptly became viscous and solidified, whereby the hole was plastered up (figure 1g,h). Here, we note that several natural galls were found with an already repaired hole (figure 1i). Colour of the solidified body fluid was initially whitish (cf. figure 1g,h), but soon turned into black (cf. figure 1i). The hole was completely filled up in nine galls, while the repair was incomplete in two galls wherein the hole was filled only partially. After approximately a month, on 29 May 2006, we inspected the survival of the experimental galls. Out of the nine completely repaired galls, seven survived and had grown up, while two had died with another small hole that was possibly made by a lepidopteran larva after the experimental treatment (figure 1j). Both of the two incompletely repaired galls were found to be dead.

(c) Artificial gall repair resulted in survival of damaged galls

On 28 April 2006, we also bored a hole on eight young galls of *N. monzeni*, and the hole was immediately filled with adhesive (figure 1k). After approximately a month, on 29 May 2006, seven out of the eight galls survived and had grown up, whereas one gall was dead.

(d) Disturbance of gall repair caused high mortality of damaged galls

On 15 May 2008, we bored a hole on five young galls of *N. monzeni*, on which we disturbed the gall repair by absorbing the nymphal fluid with tissue paper. As a result, the gall repair failed and the hole remained open. After approximately three weeks, on 5 June 2008, all the five galls were dead.

(e) Absence of repairing behaviour of soldiers and high mortality of damaged galls in summer

On 28 August 2006, we bored a hole on five galls of *N. monzeni*. In the summer season, the galls were mature, large in size and with thick (approx. 4.0 mm) and hard wall tissue (figure 1c,f). In all the galls, no nymphs

exhibited the gall-repairing behaviour, and thus the hole remained open. On 12 October 2006, we inspected the survival of the experimental galls: four out of the five galls had died, while one gall was still alive with aphids inside. In the live gall, some debris was found stuck in the hole.

(f) Artificial gall repair improved survival of damaged summer galls

On 28 August 2006, we also bored a hole on five mature galls of *N. monzeni*, and the hole was immediately filled up with adhesive. On 12 October 2006, four out of the five galls were alive whereas one gall was dead.

(g) Significantly better survival of repaired galls than non-repaired galls

Table 1 summarizes the survivorship of the experimentally manipulated galls after approximately one month in the fields. Out of the 22 galls that were plugged either by soldier nymphs or with adhesive, 18 galls survived whereas only 4 galls died. Out of the 12 galls that were not plugged but kept open, only 1 gall survived while 11 galls died. The difference in survivorship between the repaired galls and the non-repaired ones was statistically significant (Fisher's exact probability test, $p < 0.001$).

(h) Maintenance of secretion plug and regeneration of plant tissue following gall repair

When we examined the inside of the young spring galls that had been repaired by soldier nymphs approximately a month ago, notably scar of the plant tissue due to the hole was almost unrecognizable, suggesting that plant tissue around the injury had regenerated and covered the hole. In order to observe the wound-healing process, we prepared six completely repaired galls on 23 April 2007, and harvested them 0, 2, 9, 14, 22 and 30 days later, respectively. The plant tissue around the hole grew and extended towards the centre, gradually covered the injury and finally fused to recover the smooth inner wall (figure 2a–e). Figure 3 shows the histology of the plant tissue during the wound-healing process. Just after the repair, the hole was tightly plugged by the solidified body fluid of soldier nymphs (figure 3a,b). After 9 and 14 days, it was observed that the plug was partially detached from the plant tissue, probably because of growth of the gall (figure 3c,d). At this stage, inner surface of the plug was connected to the plant tissue by a thin layer, which consisted not of plant cells but of plug material (figure 3c,d, arrowheads). After 14 and 22 days, the plant tissue around the hole remarkably grew and became

Figure 2. Regeneration process of plant tissue after gall repair by soldier nymphs of *N. monzeni*. (a–e) The inside of premature spring galls on which a hole was bored and subsequently repaired by soldier nymphs; (f) outside and (g) inside of a premature spring gall on which a hole was bored and immediately filled with adhesive; (h) inside of a mature summer gall on which a hole was bored and immediately filled with adhesive. (a) 2 days after repair, (b) 9 days after repair, (c) 14 days after repair, (d) 22 days after repair, (e) 30 days after repair, (f) and (g) 30 days after repair (artificially repaired gall) and (h) 45 days after repair (summer gall).

thick (figure 3d,e, arrows). After 30 days, the thickened plant tissue around the hole fused, thereby sealing up the hole completely (figure 3f). When we inspected the artificially repaired spring galls with adhesive, similar regeneration patterns were observed (figure 2f,g). Meanwhile, when we examined the artificially repaired summer galls with adhesive, no such tissue regeneration occurred (figure 2h).

(i) *Aggregation behaviour of soldier nymphs on gall-regenerating area*

On the inner surface of the regenerating galls, we observed an interesting pattern of insect distribution. In the process of plant tissue regeneration, many soldier nymphs aggregated on the regenerating area beneath the hole (figure 4a), whereas no such aggregation was seen after the hole had been sealed up by the plant tissue (figure 4b). In order to confirm the pattern quantitatively, we prepared 20 completely repaired galls on 28 April 2008, cut them 3, 13 and 30 days later and recorded the densities of soldier nymphs and non-soldier insects on the hole and non-hole areas. In the regenerating galls 3 and 13 days after repair, the densities of soldier nymphs on the hole area were significantly higher than those on the non-hole area (figure 4c). No such pattern was seen in the post-regeneration galls 30 days after repair (figure 4c). Non-soldier insects exhibited no such aggregation on the hole area (figure 4d).

(j) *Extermination of inhabiting aphids aborted gall growth and regeneration*

In an attempt to examine the possibility that inhabiting aphids are responsible for the gall tissue regeneration, we performed a series of experiments using two chemically distinct insecticides: the neonicotinoid mospiran and the organophosphate sumithion. On 23 May 2007, we prepared 15 completely repaired galls, out of which 5 galls were treated with 0.25 per cent mospiran solution, 5 with 0.25 per cent sumithion solution and 5 with distilled water. Preliminary experiments revealed that topical application of these insecticides resulted in extermination of aphids in the galls within a few days with no visible acute effects on the plant tissues (data not shown). After approximately a month, on 28 June 2007, all the galls were alive, and we harvested, cut and inspected

them. The insecticide-treated galls were certainly alive and fresh, but their growth was remarkably suppressed in comparison with that of the water-treated control galls (data not shown). In the insecticide-treated galls, inside of the solidified plug was not covered by the plant tissue at all (figure 5a,b). The plant tissue around the hole exhibited no remarkable and orderly growth, but irregular callus-like tissue formations were found on the edge of the hole (figure 5d,e). In the water-treated control galls, by contrast, the hole was completely and smoothly covered with regenerated plant tissue (figure 5c,f).

4. DISCUSSION

In the spring galls whose thin and soft wall is vulnerable to infestation with lepidopteran larvae (figure 1b,e), first-instar soldier nymphs actively performed gall-repairing behaviour (cf. figure 1g,h; movie 1 in the electronic supplementary material). Plugging of the hole by soldier nymphs improved the survival of the galls, whereas disturbing the gall repair resulted in high mortality of the galls (table 1). Artificial plugging of the hole with adhesive also improved the survival of the galls (table 1). These results indicate that the prompt plugging of the gall breach is certainly important for the survival of the aphid colonies in the galls. In *N. monzeni*, soldier nymphs not only attack predators but also repair their galls, probably in order to intercept further invasion of predators and also to prevent desiccation and other environmental stresses for the gall inhabitants.

By contrast, in the summer galls whose wall is very thick and highly lignified (figure 1c,f), invasion of predators is unlikely to occur under natural conditions. Possibly for that reason, first-instar nymphs exhibited no gall-repairing behaviour in this season. Notwithstanding this, the holed summer galls suffered high mortality while artificial plugging of the hole improved their survival (table 1), supporting the idea that the gall breach is fatal for the aphid colonies and to be repaired as soon as possible.

Certainly, the solidified body fluid of soldier nymphs quickly and successfully plugged the gall breach, but the repair may be just an emergency measure. The galls consist of plant tissue and exhibit remarkable growth during the life cycle of the aphid colonies (cf. figure 1a–c). Thus, it is expected that the plug may soon or later detach

Figure 3. Plant histology during the gall regeneration process. (a) The day of repair (0 day), (b) 2 days after repair, (c) 9 days after repair, (d) 14 days after repair, (e) 22 days after repair and (f) 30 days after repair. Out, outside of the gall; in, inside of the gall; S, solidified secretion of soldier nymphs plugging the hole. Asterisks indicate the outer surface of the repaired hole. Arrows highlight the growing plant tissue around the hole, whereas arrowheads indicate the plug material connected to the plant tissue.

Figure 4. Aggregation of soldier nymphs on the regenerating gall area. (a) Inner surface of a young spring gall 13 days after repair. Whitish small individuals are soldier nymphs while dark larger individuals are non-soldier insects. Many soldier nymphs densely aggregate on the regenerating hole area (arrow). (b) Inner surface of a young spring gall 30 days after repair. Only a few soldier nymphs are seen on the hole area (circle). Densities of (c) soldier nymphs and (d) non-soldier insects on the hole and non-hole areas in the regenerating galls (3 and 13 days after repair) and the post-regeneration galls (30 days after repair). Means and standard deviations are shown. Open and filled bars indicate mean densities on the hole and non-hole areas, respectively. Sample sizes are shown in parentheses. Asterisks indicate statistically significant differences (generalized linear model; $p < 0.05$), while ND means no significant difference.

from the plant tissue owing to the expansion of the hole in accordance with the gall growth. How can the aphid deal with the problem? Monitoring of the inside of the plugged

galls (figures 2 and 3) unveiled previously unknown aspects of gall-repairing activities performed by soldier nymphs of *N. monzeni*.

Figure 5. Aborted regeneration in insecticide-treated galls. Inside view of the hole of (a) a mospiran-treated gall, (b) a sumithion-treated gall and (c) a water-treated control gall. Tissue section of the hole area of (d) a mospiran-treated gall, (e) a sumithion-treated gall and (f) a water-treated control gall. All these galls were inspected 36 days after repair by soldier nymphs. Out, outside of the gall; in, inside of the gall; S, solidified secretion of soldier nymphs plugging the hole. Asterisks indicate the outer surface of the repaired hole. Arrows point callus-like tissue formations on the edge of the hole.

Firstly, we found that, in the galls one to two weeks after repair, a thin layer was formed to bridge the plug and the surrounding gall tissue, which was not made of plant cells but of plug material (figure 3*c,d*). We also found that many soldier nymphs were aggregating on the plugged area inside the galls (figure 4*a,c*). These observations suggest that soldier nymphs are continuously maintaining the secretion plug during the gall growth, probably by discharging and plastering their body fluid onto the gap between the plug and the gall tissue.

Secondly, we found that, in the galls three to four weeks after repair, the plant tissue around the hole proliferated, upheaved and finally sealed up the hole (figure 3*e,f*). The proliferation of the plant tissue was prominent around the edge of the hole (figures 3*e* and 5*c,f*), where many soldier nymphs were localized (figure 4*a,c*). When aphids in the galls were killed by insecticides, regeneration of the plant tissue was aborted (figure 5). These results suggest that, following the prompt plugging of the gall breach by the discharged body fluid of soldier nymphs, (i) proliferation of the plant tissue around the breach is induced to restore the inner wall of the gall, (ii) in this way, the unstable secretion plug is reinforced by a more stable barrier of the regenerated plant tissue and (iii) the compensatory growth of the plant tissue requires inhabiting aphids, wherein soldier nymphs gathering around the gall breach are likely to play a major role.

What induces the compensatory growth of the plant tissue around the gall breach is an intriguing problem. One possibility is that the solidified aphid body fluid plugging the hole somehow induces the proliferation and migration of the adjacent plant cells. In mouse and *Drosophila*,

coagulated body fluid and dead cells at the site of injury, namely clot or scab, emanate diffusible molecules such as uric acid, histone, fibroblast growth factor, etc., which are involved in proliferation, migration and rearrangement of surrounding epithelial cells and play important roles in wound healing (Galko & Krasnow 2004; Jane *et al.* 2005). However, in the gall repair of *N. monzeni*, such a pathway is unlikely to operate, because the plant tissue regeneration was observed in the experimental galls whose hole was artificially filled with adhesive (figure 2*f,g*). Another, and more likely, possibility is that aphids, particularly soldier nymphs gathering around the gall breach, stimulate and induce the proliferation and regeneration of the plant cells, probably by injecting bioactive substances through their stylet. It has been generally thought that, although the molecular mechanisms have been totally unknown, gall-inducing insects excrete and inject hormone-like or growth-factor-like chemicals into their host plant in a spatio-temporally highly controlled manner, thereby inducing elaborate hypertrophy and malformation of the plant tissue leading to the gall formation (Meyer 1987; Shorthouse & Rohfritsch 1992; Raman *et al.* 2005). In this context, it may be notable that some soldier aphids are known to inject bioactive substances, including a venomous protease, into the body of natural enemies (Kutsukake *et al.* 2004).

The finding that killing of aphids by insecticides aborted not only gall regeneration but also gall growth (cf. figure 5) is suggestive of a link between the gall regeneration activity and the gall-forming ability of *N. monzeni*. At ordinary times, plausibly, not only soldier nymphs but also non-soldier insects excrete and inject

gall-forming factors into the plant tissue and contribute to the gall growth. Meanwhile, when a breach is made on the gall, soldier nymphs gather around the site of injury and inject gall-forming factors in a concentrated manner, whereby the plant tissue around the breach is preferentially stimulated to proliferate and regenerate.

Gall regeneration via stimulation of plant growth has been also reported from a phylogenetically distinct social aphid *P. spyrothecae* (Aphididae: Eriosomatinae: Pemphigini; Pike & Foster 2004). When a hole was bored on intact galls of *P. spyrothecae*, compensatory plant growth occurred around the breach, which finally resulted in complete or partial closure of the hole. On the ground that soldier nymphs are preferentially distributed near the gall opening, it was suggested that the soldier nymphs mainly contribute to the closure of the gall breach (Pike & Foster 2004; Pike 2007). In *P. spyrothecae*, however, no prompt gall repair occurs, it takes 10 days or more to seal up the gall breach and the breached galls are vulnerable to predators and often perish during the susceptible period (Pike & Foster 2004). In this respect, the gall-repairing system of *N. monzeni*, namely the rapid sealing by discharged body fluid and the subsequent healing by stimulation of plant growth, appears more effective and sophisticated than that of *P. spyrothecae*. Of course, the difference between the gall-repairing strategies must be relevant to and constrained by various biological aspects of these social aphids. For example, galls of *N. monzeni* contain thousands of insects (Kurosu *et al.* 2003), whereas galls of *P. spyrothecae* harbour only 500 or so individuals (Benton & Foster 1992). Needless to say, the prompt gall repair by discharged body fluid, which sacrifices a large number of soldier nymphs, is expected to be highly costly for the aphid colonies. Such a defensive strategy may be adoptable only by the social aphid with large colony size. Anyway, it appears plausible that the capability of inducing gall tissue regeneration has evolved independently in these social aphid lineages by recruiting their original gall-forming ability.

Upon wounding, insect body fluid rapidly coagulates to form clot, and the clot is subsequently hardened and melanized to form scab, thereby sealing up and stabilizing the injury. Previous studies have characterized several cellular and molecular components involved in the process including (i) degranulation or disintegration of haemocytes called coagulocytes, which leads to the establishment of the soft clot, (ii) degranulation or disintegration of haemocytes called oenocytoids (or crystal cells in *Drosophila*) that releases components of the prophenol oxidase (PPO) cascade, (iii) mounting of the PPO cascade wherein a series of enzymes are activated to produce quinones and allied reactive molecules, (iv) covalent cross-linking of the soft clot by the reactive molecules leading to formation of the hard scab and (v) melanization of the scab as a consequence of the activated PPO cascade (Theopold *et al.* 2004; Cerenius *et al.* 2008). It should be noted that the discharged body fluid of soldier nymphs was initially whitish (cf. figure 1g,h) but soon became dark in colour (cf. figure 1i), which is suggestive of activation of the PPO cascade in association with the body fluid coagulation. Kurosu *et al.* (2003) histologically described that the body fluid of soldier nymphs is full of peculiar large globular cells, which are probably highly specialized and hypertrophied haemocytes for the purpose of gall

repair, although their cellular identity is presently unknown. On the basis of these results and circumstances, although speculative, we propose the hypothesis that soldier nymphs of *N. monzeni* upregulate the coagulation mechanisms in their body fluid, discharge the enhanced biochemical and cellular processes outside their body and use the clot/scab-forming activities for the purpose of the social colony defence. Whether these cellular and molecular mechanisms are actually activated and used for that purpose is of great interest and deserves future studies.

Our study revealed that soldier nymphs of *N. monzeni* take care of their exclusive habitat, the gall, in an elaborate and dynamic manner. When encountering predators, soldier nymphs attack them. When the gall is damaged, soldier nymphs repair the breach using their own body fluid. During the growth of the repaired gall, soldier nymphs maintain the secretion plug. Around the gall breach, soldier nymphs aggregate and stimulate the plant tissue, thereby inducing regeneration of the gall breach. It has been generally thought that social systems in aphids are much simpler than those in bees, wasps, ants and termites (Aoki 1987; Ito 1989; Stern & Foster 1996). The conventional view may certainly be true, but the sophisticated gall formation, repair and maintenance found in *N. monzeni* illuminate the potential that some social aphids can attain such a high level of social intricacy comparable with the nest building, repair and maintenance found in hymenopteran and isopteran social insects (Wilson 1971; Costa 2006).

In mammals, insects and other invertebrates, wound reactions commonly entail two principal processes: rapid clotting and scab formation to seal the wound, and subsequent tissue regeneration to heal the wound (Jane *et al.* 2005; Gurtner *et al.* 2008). Certainly, the gall-repairing processes in *N. monzeni* superficially look analogous to the wound-sealing and -healing processes, but there is a fundamental difference: the clot/scab is derived from the insect while the regenerating tissue originates from the plant and the processes proceed outside the insect body at the insect-plant interface. The unique gall-repairing system of *N. monzeni* provides an intriguing case as to how cellular and molecular immune mechanisms can be recruited for a novel ecological function in a social context.

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