Medicago truncatula interaction with insects

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Abstract

Medicago truncatula is attacked by a broad range of insect herbivores including sieve-element feeders, cell content feeders, and chewing insects. In *M. truncatula*, genetic resistance against insects has to date been identified only against aphids: single dominant resistance genes have been identified conferring resistance against the pea aphid, bluegreen aphid, and spotted alfalfa aphid. Aphids are readily maintained in the laboratory because of their clonal reproduction, and because they can be maintained on intact plants, excised leaves, or on artificial diet. Aphid host plant resistance studies should include host selection assays, measures of aphid growth and development, and also effects on plant vigor. Direct measurements of aphid feeding behavior can also be accomplished using the electrical penetration graph (EPG) technique. Recent studies of aphid-induced gene expression in *M. truncatula* implicate the octadecanoid pathway in resistance against the bluegreen aphid. Bioassays of cell content feeders and chewing insects generally focus on the performance of the herbivores, measuring individual development or population growth. Defence gene expression studies have also implicated jasmonic acid signaling and the octadecanoid pathway in defence against chewing insects. Calcium oxalate crystals may also function in chewing insect defence.

1 Overview of insects attacking *Medicago truncatula*

Medicago truncatula, like other legumes, is host to a broad range of arthropod herbivores (Edwards and Singh 2006). For example in Australia, where it is grown as a pasture species, a number of insect and mite species are recognised as pests on *M. truncatula*. In the establishment phase, seedlings are attacked by redlegged earth mite (*Halotydeus destructor*), the blue oat mite (*Penthaleus major*), bluegreen or blue alfalfa aphid (*Acyrthosiphon kondoi*), spotted alfalfa aphid (*Therioaphis trifolii maculata*), sitona weevil larvae (*Sitona discoideus*), and the brown pasture looper (*Ciampa arietaria*). The bluegreen aphid, sitona weevil adults, and the lucerne flea (*Sminthurus viridis*) can also cause serious damage when the plants are more established. Other insect species with more generalist feeding habits can be occasional pests, such as the pasture day moth (*Apina callisto*) and the cotton bollworm (*Helicoverpa armigera*). Other species, such as *Spodoptera littoralis*, known to feed on relatives of *M. truncatula* also feed successfully on *M. truncatula* under laboratory and glasshouse conditions.

The assemblage of herbivores attacking *M. truncatula* include sieve-element feeders (aphids), cell content feeders (mites, lucerne flea), and chewing insects (sitona weevil, moth larvae). Thus, *M. truncatula* should be a useful model to study mechanisms of defence in legumes against all three feeding strategies. However, to date genetic resistance has been identified only against aphids in *M. truncatula*. This has produced cultivars with resistance to two cosmopolitan aphid genera that are especially important pests of *Medicago* spp.: *Acyrthosiphon* (including bluegreen aphid and pea aphid) and *Therioaphis* (including spotted alfalfa aphid and spotted clover aphid). The presence of genes for aphid resistance in multiple genetic backgrounds provides a useful system for discovering mechanisms of defence against these insects (Klingler et al., 2005; Gao et al., 2006a; Gao et al., 2006b). Defence against chewing insects has also been studied in the *M. truncatula* reference genotype A17 (Leitner et al., 2005; Bede et al., 2006) and in mutants deficient in calcium oxalate crystals (Korth et al., 2006).

2 Aphids

Overview

Among piercing-sucking insects, aphids are especially important pests of agriculture. They are exquisitely adapted to feed from individual sieve elements, the phloem sap-conducting cells joined end-to-end to form contiguous sieve tubes. Aphids cause plant damage both by the direct removal of nutrients and by transmitting the majority of insect-vectored plant viruses. Many aphid species cycle annually between sexual and asexual modes of reproduction, or have lost the sexual mode of reproduction altogether. In a typical agricultural setting, aphid pests spend most or all of the growing season as parthenogenetic, viviparous females with a generation time of about one week. All parthenogenetic aphids are born as wingless nymphs, but can develop into either alate (winged) or apterous (wingless) morphs at maturity. A single individual of the migratory alate morph can land on a crop plant and initiate a colony that grows to thousands of genetically identical descendants within one month. A colony's ratio of alate to apterous adults can depend heavily on the suitability of the host plant, with the proportion of alate adults increasing with decreasing host suitability (due to overcrowding of aphids or the presence of genetic plant resistance).

In comparison to pathogens and to a lesser degree chewing insects, little is known about how plants defend themselves against aphids and other phloem-feeding insects. *M. truncatula* is arguably the best plant model available for studying plant defense against aphids. Breeders at the South Australian Research and Development Institute (SARDI) in Adelaide, Australia, have screened a large collection of *M. truncatula* germplasm from around the world and identified accessions with resistance to the three major aphid pests of *Medicago* spp. worldwide: the spotted alfalfa aphid (*Therioaphis trifolii* form *maculata*), the bluegreen or blue alfalfa aphid (*Acyrthosiphon kondoi*) and the pea aphid (*A. pisum*). These accessions have been used as donor parents in backcrossing programs at SARDI to generate multiple aphid-resistant and near-isogenic susceptible lines (Crawford et al, 1989).

The single dominant gene, *AKR*, conditions resistance to the bluegreen aphid (Klingler *et al.* 2005) and possibly the pea aphid. This trait involves feeding deterrence and phloem-specific, inducible suppression of aphid development (Klingler *et al.* 2005). Several genes in the octadecanoid pathway are predominantly or exclusively up-regulated in response to aphid feeding in genotypes containing *AKR* compared to closely related genotypes lacking the gene (Gao *et al.* 2006). A separate dominant gene, *TTR*, conditions resistance to the spotted alfalfa aphid (Klingler *et al.* submitted). This gene not only suppresses spotted alfalfa aphid growth and development, but also controls how the plant responds locally and systemically to this aphid's feeding (Klingler *et al.* submitted).

2.1 Laboratory rearing of aphids

The asexual mode of aphid reproduction facilitates laboratory rearing and experimentation. A single aphid, collected from the field, can initiate a colony of clonal descendants that can be maintained in a controlled environment indefinitely, with relatively little maintenance aside from watering, fertilizing, and occasional replenishing of the colony host plant. Appropriate environmental conditions for rearing aphids can be considered the same as those best for growth

of the host plant. To maintain a colony, an ideal host variety is genetically uniform, susceptible to colonization and relatively tolerant of high aphid numbers for many days before dying or declining excessively in food quality. If a colony develops to thousands of individuals on a large, healthy plant while only producing a small proportion of alatae, this is an indication that the host variety is well-suited for that particular aphid clone. For studies of *M. truncatula*-aphid interactions we have used *Medicago sativa* (alfalfa or lucerne) and *Trifolium subterraneum* (subclover) as hosts of *Acyrthosiphon* and *Therioaphis* colonies. Individual aphids can be moved from one location to another by gently picking them up with a fine paint brush. In some cases, moistening the brush with water can help force the aphid to adhere to the brush during transfer.

Bulk rearing

Aphids are easily reared on plants maintained in growth cabinets or glasshouses. Single plants are usually sufficient to provide aphids for standard experiments. The plants supporting aphid colonies should have a fine mesh covering, or be placed in insect-proof cages to prevent parasites or predators from invading. Ants will often be attracted to the honeydew being produced by aphids in culture, which can be controlled by placing the pots or cages on supports whose bases are placed into water. Aphid bulk rearing colonies require an ongoing supply of new plants, which should be provided as existing plants succumb to aphid feeding pressure. For cultures in cages, new plants can simply be placed adjacent to existing plants. For cultures maintained in pots, infested material can be cut and transferred to new plants. Most aphid species will independently walk off of dying plant material and move onto adjacent fresh material, without direct intervention.

Excised leaf cultures

Genetic stocks of aphids can be maintained for long periods using excised trifoliate leaves embedded in 1.5-2% agar containing liquid fertilizer. Each aphid clone can then be cultured independently and securely in single inverted 55mm diameter Petri dishes with a disc of filter paper in the lid to absorb excess moisture and honeydew (Edwards 2001, http://www.princeton.edu/~dstern/PlatesProtocol.htm). To reduce the risk that clones will be lost, we maintain duplicate cultures in different growth cabinets. Cultures should be transferred to new plates every 10-14d at 15-20°C, or every 4-6 weeks at 10°C.

Artificial diets

Artificial diets have been developed for some aphid species, including the pea aphid (Auclair 1965). The health of aphid cultures maintained on artificial diets will usually begin to deteriorate after 2-3 generations. The diet is also labor-intensive to make and maintain. As such, artificial diets are most often used to provide a medium to allow nutrient or toxin manipulation in experiments, or when a controlled, 'neutral' food source is needed prior to experimentation.

Experimental cohorts

Many experiments require cohorts of aphid of near-equal age. This can be achieved most easily by collecting adult aphids from bulk rearing cultures and placing them on excised leaves or artificial diet for 24-48h.

2.2 Field testing of aphid resistance

It is difficult to achieve an even distribution of aphid infestations in field situations, so it is not a preferred method for screening un-replicated populations, such as F_2 progeny. Rather than rely on natural aphid populations, we will generally provide aphid-infested source plants distributed among the test rows. We use the following design:

- Plants of each genotype are grown in 2 m-long plot rows in a randomized block design with at least 4 plots/genotype.
- 2-4 weeks after germination, one heavily-infested source plant is placed in the centre of each group of four rows. Aphids should be developing into the alate (winged) dispersal morph on heavily-infested plants.
- Eight weeks later, aphid damage in each plot is visually rated using a graduated 0-10 scale, where 0 represents no visual damage, and 10 represents a dead plant.
- Aphid numbers can also be determined by taking all above-ground plant material from a section (eg. 15 cm long) of each row. Aphids can be removed from plants by washing the plant material in hot water over a 200 μm sieve.

2.3 Glasshouse and laboratory analysis of *M. truncatula* populations for aphid resistance

This technique can be used to rapidly screen accessions for genetic variation in resistance, or for studies of the inheritance of resistance. By allowing aphids to move among plants the technique will measure antixenosis (non-preference or avoidance of a host by the aphid), as well as resistance based on antibiosis and/or plant tolerance. The optimal conditions for identifying genetic variation for resistance among plants depend on plant age, initial infestation pressure and infestation duration; these will likely vary with aphid species, environment, and the mechanism of resistance. It is advisable to conduct pilot experiments where possible to determine these optimal conditions. The protocol below has been adapted successfully for genetic analysis of *AKR*-mediated resistance to bluegreen aphid (Klingler *et al.* 2005), and *TTR*-mediated resistance to spotted alfalfa aphid (Klingler *et al.* submitted).

Plant preparation

- Prior to experiments, seeds are scarified and germinated in the dark on moist filter paper at 4°C for 10 to 14 d to synchronize radical growth before transfer to soil.
- Plants are grown individually in 1.2 L pots, randomly assigned to a glasshouse benchtop or screening cage and placed in contact with one another to allow aphids to move among plants.
- Known susceptible and resistant plants should be interspersed among the test plants as controls.

Aphid infestation and scoring methods

- Two weeks after sowing, two apterous (unwinged adult) aphids are placed on each seedling, and are allowed to develop, reproduce, and move freely among plants.
- After ~3 weeks, a subjective **damage** score (1-5 or 1-10 scale) is given to each plant, based on the amount of leaf damage and aphid-induced stunting. The damage scale should be

standardised using the interspersed control plants. This is the least labor-intensive and most efficient of scoring methods, but may not be suitable for all resistance mechanisms.

- After 2-4 weeks, a score of **aphid numbers** (1-5 or 1-10 scale) is given to each plant, based on a sliding scale of aphids/plant or aphids/growing tip (eg. 0, 1-5, 6-20, 21-50, >50).
- After ~5 weeks, effects of aphid damage on **plant growth** can be recorded from the above-ground fresh weight of each plant and number of pods per plant.

2.4 Aphid performance assays on intact plants

In these bioassays, aphids are confined to individual, whole plants or portions of plants; no choice among hosts is provided. In this situation, aphids may be forced to feed on plants or on tissues that would not normally be accepted as a suitable host. This design allows for more controlled analyses of individual plants – such as comparing different feeding sites, comparing local to systemic plant responses, and measuring effects of pre-treatments such as prior aphid infestation. Aphid performance can be quantified as total colony biomass per plant after 2-3 weeks of infestation. Aphids are first removed from plants with a fine brush and the total colony weight is recorded. The aphid-free plant can have its fresh or dry weight recorded. Aphid biomass as a function of plant biomass can indicate the degree of plant tolerance if aphid-free plants of the same genotype are grown in the same environment and compared with infested plants.

Alternatively, if investigators have access to a microbalance, the growth of a group of synchronously aged nymphs (a cohort) can be monitored over the course of a few days (less than one aphid generation). This method, described below, can be used to predict colony performance over the long-term.

Aphid development on individual plants

- Plants are grown in individual 0.9 to 1.2 L pots.
- Pre-weighed cohorts of 10 early-instar nymphs (0-48 h old) are placed on each plant, confined to the pot by enclosing the plant in a mesh sleeve or equivalent enclosure that allows reasonable air exchange. Alternatively, aphids can be confined to particular leaves or stems with smaller mesh sleeves or cages (see pre-infestation protocol below).
- After 4-6 d (prior to nymph production), the number and total weight of surviving aphids is recorded. Aphid performance can be calculated using a population growth rate (PGR) statistic, which is the per diem difference between the logarithms of the initial and final weights of aphids placed on the plant (Edwards 2001). This statistic combines the effects of the plant on aphid growth and survival.

Aphid development on previously infested plants

- To measure the effects of prior aphid infestation on resistance, we use a clear plastic cylinder cage of 35mm diameter and 80 mm length with slotted, gas-permeable sponge disks at each end. These cages can be placed around central stem regions, or around distal stems/leaves. The wooden stake is used to support the stem and cage.
- For the pre-infestation treatment, 20 adult aphids are placed into the cage for a period of 48 h.
- For controls, empty cages are used.

- PGR of pre-weighed nymphs is measured as above.

2.5 Excised Shoot Bioassays

Excised shoot bioassays allow higher throughput and require less space than whole plant bioassays. However, aphid performance on excised shoots or leaves does not necessarily correspond to performance on intact plants, so caution should be used when interpreting results. We use excised shoot assays predominantly to characterise resistance mechanisms, in particular to determine whether a mechanism is tissue-autonomous.

Excised shoot assay

- Individual trifoliate leaves, or stem tips with three nodes, are excised from plants and inserted into agar supplemented with soluble fertilizer in an inverted 90 mm diameter Petri dish according to Milner (1982). Each dish contains filter paper on the lower side to absorb condensate and aphid honeydew.
- A pre-weighed cohort of 5 early instar nymphs is placed on to each of at least six replicate leaves or stems.
- After 5-7 d, the number and weight of surviving aphids and any new nymphs produced are recorded to calculate the population growth rate (PGR) as described above.

2.6 Artificial diet bioassay

Preparation of diet sachets

- Artificial diets can either be nutritionally complete, or a basic sucrose solution. In either case, the pH and the sucrose level must suit the aphid species being tested. Of all aphids attacking *M. truncatula*, a complete aphid diet has been developed only for the pea aphid, *Acyrthosiphum pisum* (eg. Auclair 1965). To date, we have not been able to identify a diet or sucrose solution that is suitable for bluegreen (blue alfalfa) aphid (*A. kondoi*). The spotted alfalfa aphid (*Therioaphis trifolii* form *maculata*) is reported in Miles & Oertli (1993) to feed on a 15% sucrose solution, pH 6.8.
- Prepared artificial diets should be frozen until needed. Diet sachets should be prepared immediately prior to experimentation to reduce the likelihood of fungal infections.
- Diet sachets consist of two layers of stretched Parafilm® with 150-200ul of solution sandwiched between. Alternatively, more temporary diet sources can be created by using plastic cover slips rather than a second layer of stretched Parafilm®. Aphids will feed on the solution by inserting their stylets through the parafilm.

Determining the bioactivity of crude extracts or candidate compounds

- The test arena consists of a 36 mm diameter plastic Petri dish top.
- After aphids are placed inside the Petri dish we stretch a layer of Parafilm® over the top, pipette 160ul of solution onto this layer, then stretch the top layer of Parafilm® over the solution to seal.
- An experiment usually consists of 10 replicates each of a control and three treatment concentrations, which requires a total of 200 nymphs.

- To collect these early instar nymphs, 48 h before the experiment we add 10 apterae (wingless adults) to a series of these arenas containing only the diet solution. Generally around 30-40 nymphs are produced per arena, but this will vary by species.
- Nymphs are collected into treatment groups of 5 and placed into gelatine capsules. After their total weight is determined using a microbalance, the nymphs are randomly assigned to control or treatment sachets, and the arena is covered with an identical Petri dish top and secured to the based with alligator clips.
- After 5 d in a 20^{o}C incubator, the aphids are removed and the survival and weight of the aphids in each sachet are recorded. Total weight gain, or mean relative growth rate (Edwards 2001) can be recorded.

2.7 Winged adult host choice assay

Determining preference of foraging aphids between two alternative plant genotypes

- Plants of each genotype are grown individually in 1.2 L pots.
- Two 21-day-old plants of each genotype are randomly assigned to insect-proof cages.
- Pots are spaced so that no leaves touch other plants.
- At least six replicate cages should be used. We use cages of dimensions 38cm (L) X 28cm (W) X 46cm (H) covered with fine, light-transmitting mesh on the top and on three sides, and with a sliding Perspex cover on the remaining side.
- A 5 cm Petri dish platform is placed centrally in the cage, equidistant from all test plants. Petri dish should be suspended at least 10 cm above the soil level of the plants.
- At least 24 alatae (winged adult aphids) are placed on the platform in each test cage and allowed to choose among the host plants in the cage for 48 h.
- The number of aphids on each plant is observed at 3 h, 6 h, 24 h, and 48 h after release.
- For each time point, the pooled number of aphids on each host plant genotype can be compared using chi-square analysis or paired *t*-tests.

2.8 Electrical Penetration Graph (EPG) analysis of aphid feeding behavior

For the electrical penetration graph technique, a low voltage is applied between an electrode in the soil of a potted *M. truncatula* plant and a wire attached to a feeding aphid. An electrical circuit is achieved when the aphid's stylets are inserted into the plant. Variation in the level of resistance in this circuit during aphid feeding results in a pattern of waveforms, which have been correlated to the tissue location of the stylet tip and the feeding behaviour of the aphid (Tjallingii 1987). Comparisons of aphid feeding between resistant and susceptible plants can help to characterise the location and mechanism of resistance.

- Each test aphid is starved for approximately 1h as a 2- to 4-cm length of gold wire is attached to its dorsal surface using sliver conductive paint.
- The other end of the wire is connected to a Giga-4 (four channel) or Giga-8 (eight channel) DC amplifier and 10^{9}-Ω input resistance (Wageningen Agricultural University, The Netherlands) in an electrical circuit that also includes the host plant via an electrode placed in the soil.

- Aphid behaviour is monitored for 6-16 h, depending on the aphid species and the resistance level in the test plants. Some aphid species begin to feed immediately, whereas others will 'settle' for hours before beginning to probe the plant.
- Waveform patterns are scored as non-probing, pathway activities, salivation, phloem ingestion, xylem ingestion, and cell puncture events (Tjallingii & Esch 1993) using the EPG analysis software MacStylet 2.0 (Febvay *et al.* 1996) or Probe (Wageningen Agricultural University, The Netherlands).

2.9 Plant defense responses to aphids

Plants

- Seeds are scarified, bleached and germinated in the dark on moist filter paper for two days before planting into soil.
- Plants are grown in individual 0.9 L pots in growth cabinets with 16 L (22°C):8 D (20°C) under high pressure sodium and fluorescent lamps (280 $\mu E\ m^{-2}\ s^{-1}$).
- Plants are watered every second day with half-strength Hoagland's solution.

Aphid infestation

- Two mature, fully expanded trifoliate leaves from the primary stem of individual plants (3-4 weeks old) are infested with 15 late instars or adults, and caged in linen mesh supported by a bamboo stick.
- For non-infested control plants, two similar leaves are caged without aphids.
- The cages should be air- and light-transmittable, and must be shown to have no effect on defence gene transcript level.
- After disassembling the cage, aphids are brushed off using a fine paint brush; the non-infested control also receives a similar extent of brushing.

Tissue collection

- Leaflet and petiole samples are removed from 6 h to 72 h after aphid infestation.
- Leaflets are separated from petioles at harvest. Leaflets or petioles from two trifoliate leaves per plant are combined for each sample.
- All samples are frozen in liquid N_2 and stored at -80°C until RNA isolation.

RNA isolation and cDNA synthesis

- It is generally not easy to get a high yield of RNA from petiole tissues compared to leaves. Extensive grinding of the petiole tissues using a rough pestle can increase the yield dramatically.
- RNA isolation is performed using the Purescript RNA isolation kit (Gentra Systems, Minneapolis).
- Genomic DNA contamination is removed using the TURBO DNA-free kit (Ambion).
- The absence of genomic DNA contamination was confirmed by PCR using a primer pair flanking an intron region of a trehalase gene (Salzer *et al.*, 2000).
- RNA integrity was checked on a 1.5% (w/v) agarose gel.

- One to two microgram of total RNA is routinely used for first-strand cDNA synthesis using SuperScript III Reverse Transcriptase (Invitrogen).
- When a large quantity of RNA or cDNA is needed for an experiment, such as a microarray experiment using petioles, but it is difficult to obtain such a quantity through the ordinary RNA isolation and cDNA synthesis methods, the BD SMART PCR cDNA Synthesis Kit (BD Biosciences Clontech) is highly recommended.

PCR primer design

- Putative defence-related genes are identified in *M. truncatula* by BLASTN or TBLASTN searching of the TIGR Medicago Gene Index database (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=medicago) with query sequences derived from characterised Arabidopsis genes and EST tentative consensus annotations.
- Sequences of gene family members are aligned to enable design of specific primer sets using the Multiple Sequence Alignment application (AlignX) of the Vector NTI Suite program (InforMax, Inc., North Bethesda, MD), based on the Clustal W algorithm.
- Tentative consensus (TC) sequences are chosen according to abundance in defence or stress-related libraries shown in the TIGR MtGI database (http://tigrblast.tigr.org/tgi/). Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) is used with primer picking parameters modified to suit the real-time quantitative PCR conditions (Bio-Rad, Bulletin 2593).
- BLASTN analysis of TIGR MtGI is performed on the primer sequences to confirm specificity of amplification.

Real-time quantitative PCR conditions and analysis

- Real-time quantitative polymerase chain reactions (RT-qPCR) are performed using an iCycler^{TM} (Bio-Rad Inc.).
- Reactions are set-up according to (Klok *et al.*, 2002) with the thermal profile: 95°C for 2.5 min, 40 cycles of 95°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec, followed by a melt curve program of 70-95^{o}C with 0.5^{o}C increase per cycle.
- In order to compare data from different PCR runs or cDNA samples, C_T values for all selected genes are normalized to the C_T value of a tubulin gene (TC106434; forward primer: 5'-CCTGTTGCCGGTTCATAATC-3'; reward primer: 5'-CCCAAACATAGATTGCTGCTT-3'), whose expression remained constant among various aphid-infested and non-infested tissues.
- Relative gene expression is derived from using $2^{-\Delta CT}$, where ΔC_T represents C_T of the gene of interest minus C_T of tubulin.
- The significance in difference between ratios is analyzed and compared by the LSD test using GenStat 6.2 (Lawes Agricultural Trust, Rothamsted Experimental Station).

3 Chewing insects

Overview

The interaction between chewing (including cell-content feeding) insects and their host plants is not as intimate as it is for phloem-feeding insects. Chewing insects, such as caterpillars, have the

crudest feeding strategy of insect herbivores, using well-develop external mouthparts (mandibles, maxillae) to cut and chew leaf material in the presence of oral secretions prior to ingestion. Jasmonic acid (JA) signaling has been strongly implicated in chewing insect defence in a number of plants (Kessler & Baldwin 2002, and references within), often with additional, more specific responses mediated by insect-derived factors. JA accumulation has been observed in *M. truncatula* after chewing insect feeding (Leitner *et al*. 2005), and genes in the terpenoid biosynthesis pathway are induced by insect-derived factors in the regurgitant of *Spodoptera exigua* caterpillars (Gomez *et al.* 2005; Bede *et al.* 2006).

In addition to being serious pests on *M. truncatula* and related species in the field, chewing insects can be useful in laboratory studies of plant responses to herbivory. The size of lepidopteran caterpillars means that it is easy to handle and monitor individuals, and the relative simplicity of rearing some species makes them attractive models. Methods for measuring insect performance on plants and for caging and handling chewing insects have been well described (Smith, 2005).

Cell-content feeders, such as thrips and mites, use specialized mouthparts (stylets) to puncture epidermal cells of the host plant and ingest the cellular contents, often resulting in a chlorotic lesion. Physical and chemical defenses have been implicated in successful cell-content feeder defense in other plants. In *M. truncatula*, plants respond differently to chewing insects and cell-content feeders (Leitner *et al.* 2005). Salicylic acid (SA) is more involved in the response to cell-content feeders, and the local JA response is delayed compared to chewing insects (Leitner *et al.* 2005).

All studies to date investigating defense against chewing or cell-content feeding arthropods have examined compatible interactions, so it is not yet possible to associate these induced responses with successful defense. This should be clarified in the future as resistant or defense-deficient mutants become available.

3.1 Laboratory rearing of chewing insects

Established protocols exist for rearing lepidopteran species such as *S. littoralis* (cotton leafworm) and *S. exigua* (beet armyworm), and each species will readily feed on *M. truncatula* (Leitner, et al., 2005; Gomez et al., 2005). These species are generalist feeders that can inflict severe damage on crops throughout the world. Because of their voracious appetites for consuming foliage, it can sometimes be difficult to grow enough plant material to maintain large populations of caterpillars in the lab. Therefore, artificial diets are widely used to sustain lab colonies. Powdered preparations of artificial diets for *Spodoptera* are commercially available (Bio-Serv, Frenchtown, New Jersey, USA). Another advantage of artificial diets is that they can be easily amended with known quantities of candidate compounds for study of effects on insect feeding patterns or growth. Newly hatched larvae (neonates) can be reared until pupation on artificial diet in small plastic storage boxes or individually in insect feeding cups available from scientific suppliers. Although larvae can readily be grown together, overcrowding can lead to poor growth and an increased threat of disease. Once formed, pupae are generally placed in larger screened vessels to allow flight and mating of the emerging adults, with a 10% solution of honey supplied as a food source. A substrate, such as intact plants, hanging strips of filter paper, or a cloth covering the cage, is generally provided for egg-laying. Eggs of *S. exigua* are laid in masses of 50-125 eggs,

which can then be transferred to artificial diet for hatching. The life cycle of *S. exigua* can be as short as 28 days from egg to adult depending on growth conditions. As long as a suitable food source is supplied, temperature is the primary environmental factor that determines growth rate. Lab colonies are normally maintained from 22-26^{o}C.

Other chewing insects are specialist feeders, with a narrow host range that can include *Medicago* species. The alfalfa caterpillar (*Colias eurytheme* L.) is the larval stage of a sulphur butterfly and feeds on several species within the Fabaceae, but this species is more difficult to rear in captivity requiring intact plants as food material (Wang and Porter, 2004). Populations of the alfalfa weevil (*Hypera postica* Gyllenhal) can be maintained on artificial diet (Hsiao and Hsiao, 1974) or caged plants; both larvae and adults readily feed on *M. truncatula* foliage and stems.

Cell content feeding insects are generally maintained using protocols similar to those described above for bulk rearing of aphids (2.1.13.2.1).

3.2 Performance bioassays

Chewing insects
Assessing the effects of plant material on insect growth and survival can be performed via several established methods (Smith, 2005). One of the most common methods is to simply track the growth of larvae (by measuring mass) and mortality levels (counting survivors) over time (Korth, et al., 2006). These types of assays are best performed on intact plants, where practical, and can provide an easy measure of negative or positive effects of plants on insect performance. However, multiple plant characters can affect overall insect growth, and so insect weight gain does not always provide a complete picture of how plant defenses are influencing insects. For example, antifeedants might cause lower insect weights due to lower consumption rates, whereas toxic plant compounds might directly cause lower weights by interfering with insect metabolism. Although other methods have been applied, insect utilization of food materials can be assessed through gravimetric measurement of inputs and outputs (Waldbauer, 1968).

- Second instar larvae are weighed and caged individually in small chambers (4 x 10 cm each) that allow air exchange, with 3 g fresh weight of detached leaf material. A wire grid (~0.5 cm grid) above the surface of each chamber allows frass to drop through and away from food material.
- Cages are placed together in a moist chamber at 24^{o}C to prevent leaf material from excessive drying. For each plant treatment, at least 15 larvae-plant sets should be measured.
- At a suitable time after initiation of the experiment (48 or 72 hr), when there is still food material remaining available to the insects, the larvae, leaves, and frass from each chamber are collected separately and weighed. If moisture content of the leaves did not change over the course of the experiment, then fresh weights can be used for calculations of food utilization. An alternative approach is to dry all materials and weigh each, and then dry weights are used. Larval and plant dry weights at the outset of the experiment need to be estimated. This is done based on measurement of the dry weight component of a separate sampling of larvae and plants, by comparing fresh vs. dry weights.

- The methods for calculating consumption indices, growth rates, and digestion efficiencies are well established (Waldbauer, 1968) and have been successfully applied in M. truncatula (Korth, et al., 2006).

Cell content feeding insects
Because of their size, it is generally less practicable to assay cell content feeders using the growth and survival of individuals. Performance of these herbivores is generally assayed by measuring population growth over several generations.

- Initiate populations on enclosed potted plants with 25 (eg. mites) to 50 (eg. lucerne flea) individuals.
- Plants should be monitored regularly to be sure that sufficient leaf material remains to support the herbivore population. Experiments are generally terminated after 4-6 weeks.
- A damage score of 0-10 (0: no damage; 10: dead plant) is recorded for each pot, based on the amount of leaf damage and the overall health of the plant.
- All plant material is then cut at soil level and stored in a paper bag at 15°C until processing.
- The soil surface and plant material of each pot is inspected to collect all surviving individuals, which are stored in alcohol.
- The individual insects can later be counted (and staged using microscopy, if desired) to provide population numbers for each pot.

3.3 Plant defense responses to chewing insects

Plant preparation
If plant responses are to be measured, the feeding habits of lepidopteran larvae require that plants are large enough to sustain damage but still have tissue remaining for analysis. About four weeks after sowing, plants are ready for use although older plants can also be used and will provide more material. Plants, potted individually, are maintained in essentially the same conditions as for aphid assays (2.1.13.2.3). Because chemical pesticide use could interfere with treatments, it is important to keep plants free of insect pests by careful cultural practice (e.g., contained plant growth, cleaning up debris, using insect-free soil). It is important to maintain control plants under the same conditions as experimental plants, but because plant-derived volatile signals can affect wound responses in long-term experiments, plants should be kept at a distance if possible. Healthy, fully expanded leaves are generally the tissue of choice for gene expression analyses. Regardless of the tissue used, it is important to select developmentally similar tissue if multiple treatments or time points are to be compared. The amount and time of feeding damage, the timing of tissue collection, and insect stage can all affect the extent of plant responses (reviewed by Kessler and Baldwin, 2002), and so consideration should be given to each of these factors and consistency in treatments is essential.

Long-term feeding treatments
- Individual plants are surrounded with a transparent cylinder with open ends. A one- or two-liter clear beverage bottle with the ends removes is a convenient device for this. One open end is placed over the plant and onto the soil, and the other end is covered with a mesh fabric such as cheesecloth. Non-damaged control plants are caged in an identical manner.

- For overnight feeding assays, ten to twelve second-instar (~1.5 cm) larvae can be caged together on a plant about six weeks of age. Alternatively, four to six fourth-instar (~2.5 cm) larvae can be used. This level of infestation should result in heavy damage, but there will still be abundant tissue available for collection. Larval stage can affect plant responses, so insects of similar size should be selected. Insects are added in the late afternoon (1500 hr) and tissue is collected mid-morning of the following day (1000 hr).
- Damaged leaves are typically selected for gene expression analysis. However, with this high level of sustained damage, most of the plant is probably experiencing systemic responses and this should be kept in mind for interpretation of results.
- Standard solutions and methods for RNA isolation and analysis (e.g., TriReagent, MRC, Cincinnati, OH, USA) are used for characterization (see section 2.1.13.2.9).

Short-term feeding treatments

- Systemic wound responses in *M. truncatula* and other plant species are lower with short term feeding (<2 hr) by caterpillars. However, such feeding does result in strong local wound responses. Individual larvae can be caged on one or a few trifoliates using small clip cages (Smith, 2005).
- To encourage timely commencement of feeding, larvae are starved for 2-4 hr prior to placing them on plants. This is done by simply caging the larvae individually in the absence of food.
- At the end of the desired feeding period, typically two hours, damaged leaves are collected for analysis of local wound responses. Undamaged leaves on the same trifoliate can be used to measure systemic responses.
- Leaf tissue for RNA isolation and gene expression analysis is treated as described above.

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