Medicago truncatula Mutants Demonstrate the Role of Plant Calcium Oxalate Crystals as an Effective Defense against Chewing Insects[1]

Kenneth L. Korth*, Sarah J. Doege, Sang-Hyuck Park, Fiona L. Goggin, Qin Wang, S. Karen Gomez[2], Guangjie Liu, Lingling Jia, and Paul A. Nakata

Department of Plant Pathology (K.L.K., S.J.D., S.-H.P., Q.W., S.K.G., G.L.) and Department of Entomology (F.L.G., L.J.), University of Arkansas, Fayetteville, Arkansas 72701; China National Rice Research Institute, Hangzhou 310006, China (G.L.); and United States Department of Agriculture Agricultural Research Service Children's Nutrition Research Center, Baylor College of Medicine, Houston, Texas 77030 (P.A.N.)

Calcium oxalate is the most abundant insoluble mineral found in plants and its crystals have been reported in more than 200 plant families. In the barrel medic *Medicago truncatula* Gaertn., these crystals accumulate predominantly in a sheath surrounding secondary veins of leaves. Mutants of *M. truncatula* with decreased levels of calcium oxalate crystals were used to assess the defensive role of this mineral against insects. Caterpillar larvae of the beet armyworm *Spodoptera exigua* Hübner show a clear feeding preference for tissue from calcium oxalate-defective (*cod*) mutant lines *cod*5 and *cod*6 in choice test comparisons with wild-type *M. truncatula*. Compared to their performance on mutant lines, larvae feeding on wild-type plants with abundant calcium oxalate crystals suffer significantly reduced growth and increased mortality. Induction of wound-responsive genes appears to be normal in *cod*5 and *cod*6, indicating that these lines are not deficient in induced insect defenses. Electron micrographs of insect mouthparts indicate that the prismatic crystals in *M. truncatula* leaves act as physical abrasives during feeding. Food utilization measurements show that, after consumption, calcium oxalate also interferes with the conversion of plant material into insect biomass during digestion. In contrast to their detrimental effects on a chewing insect, calcium oxalate crystals do not negatively affect the performance of the pea aphid *Acyrthosiphon pisum* Harris, a sap-feeding insect with piercing-sucking mouthparts. The results confirm a long-held hypothesis for the defensive function of these crystals and point to the potential value of genes controlling crystal formation and localization in crop plants.

Plants have a multitude of biochemical and physical defenses, both preformed and wound induced, that protect them from consumption by insect herbivores. Different classes of phytochemicals fend off insects by serving as antifeedants, toxins, or by interfering with insect digestion. Leaf hairs, a thick cuticle, or thorns can all serve as physical deterrents to feeding, depending on the feeding style of the herbivore. Because of their known qualities as irritants to humans (Bradbury and Nixon, 1998; Salinas et al., 2001) and their formidable appearance, needle-like raphide crystals of calcium oxalate in plants have been suggested to be physical deterrents to herbivore feeding (Whittaker, 1970).

Calcium oxalate is the most abundant and widespread insoluble mineral found in plants. Calcium oxalate crystals occur in well over 200 plant families and in some plant tissues have been reported to comprise as much as 80% of the dry weight (Franceschi and Horner, 1980). In spite of their common occurrence among plants, the physiological functions of these crystals are not fully understood, but it could be that they play multiple roles. In addition to a possible role in defense, calcium oxalate could serve as a means of regulating internal calcium stores, as a sink for toxic oxalic acid, or as a way to maintain ionic balance (Webb, 1999). For example, crystals in the legume *Phaseolus vulgaris* play an important role in regulating levels of bulk calcium (Jáuregui-Zùñiga et al., 2005).

Several lines of evidence support the hypothesis that calcium oxalate can act in defense against feeding insects. Calcium oxalate produced by fungi (Binns, 1980) or applied externally (White, 1997) has been shown in mushroom production systems to protect fungi from feeding damage by sciarid flies. In plants, a specialist leafminer species that attacks American holly (*Ilex opaca* Ait.) avoids feeding on cell types that contain calcium oxalate (Kimmerer and Potter, 1987). Calcium oxalate has been suggested as a constitutive form of

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[2] Present address: Boyce Thompson Institute, Tower Road, Ithaca, NY 14853.

* Corresponding author; e-mail kkorth@uark.edu; fax 479–575–2771.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Kenneth L. Korth (kkorth@uark.edu).

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defense against bark borers in conifers. A survey of 46 conifer species suggests that there is an inverse relationship between abundance of calcium oxalate crystals and the number of aggressive bark beetle species that feed on the conifer (Hudgins et al., 2003). A defensive role for calcium oxalate has also been proposed in the desert lily (*Pancratium sickenbergeri* C. et Barbey), where herbivores of three types (mammalian, insect, and snail) all avoid feeding on tissues that contain raphide crystals (Ward et al., 1997; Ruiz et al., 2002). Furthermore, mechanical wounding of leaves can sometimes lead to increases in crystal density in leaves of some species, leading to the proposal that this could be a form of induced defense (Molano-Flores, 2001). Not all previous studies, however, provide consistent evidence of a defensive role for calcium oxalate. A comparison of related species of Ficus showed that mechanical wounding can lead to an increase, decrease, or no change in calcium oxalate depending on the species (Xiang and Chen, 2004). Furthermore, the same study showed that herbivory rates of insects in the field did not necessarily correlate with calcium oxalate levels. All previous studies that have shown insect avoidance of calcium oxalate have compared different plant species or different tissue types within the same species. This leaves open the possibility that differences in herbivore feeding or performance were due to some other physical or biochemical distinction between tissues or species.

The mineral precipitation of calcium oxalate within a species is tightly controlled, leading to specific sizes, shapes, and localization of crystals (Franceschi and Horner, 1980; Bouropoulos et al., 2001). A specific calcium-binding protein has been identified in the crystal matrix of water lettuce (*Pistia stratiotes*), suggesting that crystal formation is associated with the structure or action of specific proteins (Li et al., 2003). The crystals can be found in various forms, most commonly appearing as raphides, prismatic blocks, bunches of small precipitants called crystal sand, or as multifaceted druse crystals (Franceschi and Nakata, 2005). In the species *Medicago truncatula* Gaertn., calcium oxalate accumulates predominantly as prismatic crystals in a sheath surrounding secondary veins of the leaf, and as much smaller crystals in mesophyll cells of older leaves (Nakata and McConn, 2000). *M. truncatula* has emerged as a valuable model system for study of legume biology and plant interactions with symbiotic microbes (Cook, 1999). This annual diploid species is a relative of the forage crop alfalfa (*Medicago sativa*). Characteristics of mutant lines of *M. truncatula* with altered crystal formation demonstrate the genetic control of crystal formation (Nakata and McConn, 2000; McConn and Nakata, 2002). The nonallelic mutations in the calcium oxalate-defective (*cod*) lines *cod*5 and *cod*6 result in severely reduced levels of calcium oxalate compared with those in the parental wild-type line A17, although levels of total calcium in each line are not substantially different (Nakata and McConn, 2000). The *cod*5 mutant line does not show measurable growth differences compared to wild-type plants (Nakata and McConn, 2003). Although multiple roles for calcium oxalate formation in wild-type *M. truncatula* cannot be ruled out, the similarities between mutant and wild-type growth and development suggest that the crystals are not necessary for many of the previously proposed functions (e.g. regulating excess calcium, tissue support, etc.).

The availability of single gene mutations that lead to altered levels of calcium oxalate crystals provides a valuable set of tools to determine the role of this mineral in insect defenses. For these studies, we utilized the chewing insect *Spodoptera exigua* Hübner, a lepidopteran pest that feeds on a variety of plant species, including many important crops. To ascertain the role of calcium oxalate crystals in insect defense, we compared insect feeding and growth on wild-type *M. truncatula* with two independent *cod* mutant lines that have severely decreased levels of calcium oxalate.

RESULTS

Insect Feeding Avoidance of Calcium Oxalate

We noted that, in the early stages of growth, *S. exigua* larvae avoid feeding on tissues containing calcium oxalate crystals. Neonates and early instar larvae scrape the leaf surface, often leaving behind a cell layer and the secondary veins (Fig. 1A). As young larvae age, they continue to feed mostly on interveinal tissues. This continues until about the third instar, when their increasing size (15–20 mm in length) necessitates that they begin to consume all leaf materials, including secondary veins and the midvein (Fig. 1B). Similar to the localization in *M. sativa* (Ward et al., 1979), the calcium oxalate crystals of *M. truncatula* are concentrated along these secondary veins and their prismatic shapes are clearly visible when viewed with a light microscope equipped with a cross-polarizing filter (Fig. 1C). In the secondary veins of *cod*5 and *cod*6 mutants of *M. truncatula*, these crystals are virtually absent (Nakata and McConn, 2000).

The avoidance of tissues containing calcium oxalate by feeding insects (Fig. 1, A and B) led us to test whether larvae had a measurable liking for genotypes deficient in this mineral. Larvae were caged overnight with equal amounts of leaf tissue from each of two genotypes, and the fresh weight of remaining tissue from each genotype was determined at the end of the trial. The ratios of the weight of remaining tissue were calculated, and a value of 1.0 would indicate no preference. In these two-way choice tests, third-instar larvae showed a clear and measurable preference for *cod*5 and *cod*6 tissue over that of wild-type A17 (Fig. 1, D and E). However, insects did not show a preference for *cod*5 or *cod*6 when those two genotypes were compared (Fig. 1E).

Plant Calcium Oxalate Effects on Insect Growth

In experiments comparing insect growth on A17 and the *cod* mutants, it was apparent that larvae grew

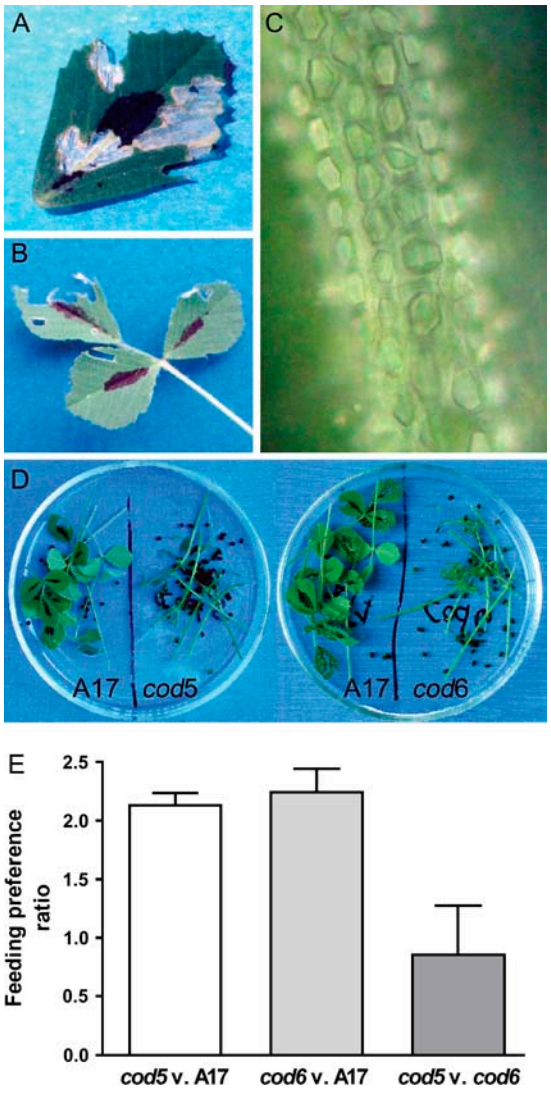

Figure 1. Insect feeding preferences on crystal-containing leaves indicate a defensive role for calcium oxalate. A, Neonates and early instar larvae scrape the surface of leaves. B, Older larvae tend to feed between secondary veins when possible, avoiding tissues that contain crystals. C, Sheaths surrounding the secondary vascular strands in *M. truncatula* contain the prismatic calcium oxalate crystals that are visible with polarized light. D, Typical results of two-way choice tests comparing *S. exigua* feeding on A17 with the *cod*5 and *cod*6 mutants. Two larvae were caged overnight in each dish with equal fresh weights of leaves from two genotypes as labeled. E, Feeding preferences for *cod*5 and *cod*6 tissue were quantified by comparing the fresh weights of uneaten tissue within a dish. Larvae showed a significant preference for *cod* tissue over A17 (*t* test; $P < 0.005$, $n = 5$), but did not show a preference for *cod*5 or *cod*6 when those two genotypes were compared (*t* test; $P > 0.75$, $n = 5$). A value of 1.0 would indicate no preference; error bars indicate SE.

to a larger size on lines with reduced amounts of calcium oxalate (Fig. 2A). Measurement of larval weight over time confirmed that *S. exigua*, reared from the neonate stage on intact *cod*5 or *cod*6 plants, grew to a larger size than those reared on A17 plants (Fig. 2B). These weight differences were relatively small at the earliest stages of insect growth (second instar) that were measured, but increased dramatically over the course of larval development. This is possibly due to the feeding style of early instar larvae, which avoid tissues high in calcium oxalate. The data also suggest that larvae feeding on *cod*5 and *cod*6 begin to pupate earlier than larvae reared on A17 (Fig. 2B). The pupae resulting from such comparisons are significantly larger when reared on tissue lacking calcium oxalate. Pupae from larvae reared on *cod*5 or *cod*6 grow to nearly twice the mass of larvae that feed on the wild-type tissue (Fig. 2C).

Larval mortality rates were also measured over the course of development. The data show that, at later stages of larval growth, after third instar, mortality rates are higher when insects feed on detached leaves from wild-type plants as compared with a diet of *cod* leaves (Fig. 2D). Development of insects was delayed when they were reared on detached leaves, as compared with intact plants. This accounts for the different time scale as seen in the data shown in Figure 2B.

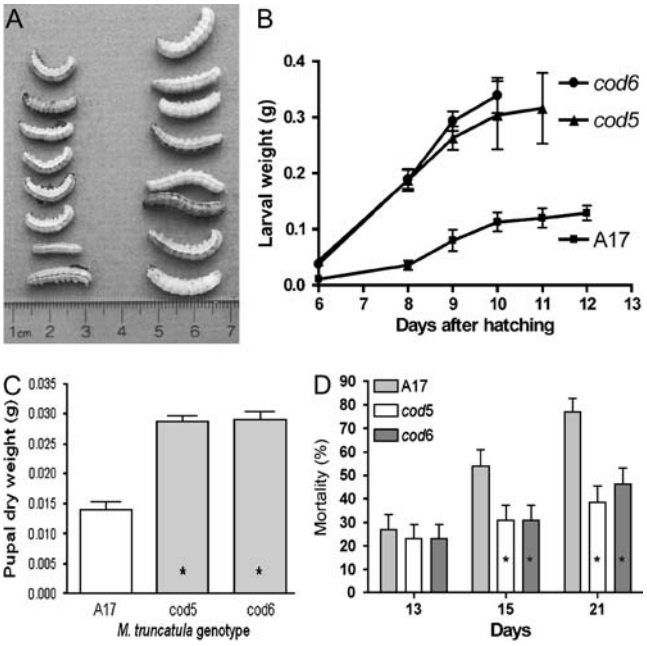

Figure 2. Insect larvae grow larger on tissue lacking calcium oxalate. A, Typical larvae collected after feeding on the wild-type line A17 containing calcium oxalate (left) and the *cod*5 line with decreased levels of calcium oxalate (right). B, Insect larvae grow larger on mutant lines that lack calcium oxalate. Average fresh weights of *S. exigua* larvae reared on *cod*5, *cod*6, or the wild-type line A17. Weight determination was halted when larvae entered early stages of pupation. C, Average pupal dry weight of larvae reared on each of the three genotypes. Bars with asterisks are significantly different from the values on A17 (ANOVA; $P < 0.001$; A17, $n = 13$; *cod*5, $n = 37$; *cod*6, $n = 28$). D, Larvae of *S. exigua* suffer higher mortality rates when reared on detached leaves of the wild-type line A17, as compared to mortality rates of insects feeding on *cod*5 or *cod*6. Data represent mean + SEM; bars with asterisks on a given day indicate no significant difference in values (ANOVA; $P > 0.10$, $n = 60$). Error bars on each graph indicate SE.

General Plant Defenses in *cod* Mutants

A possible explanation for the enhanced insect growth and feeding preference on *cod*5 and *cod*6 mutants could be that these mutants are unable to mount normal wound-induced defense responses. We examined the transcript levels of a well-characterized wound-responsive gene in A17 and *cod* mutants. The terpene synthase 1 (*Tps*1) gene encodes a cytosolic sesquiterpene synthase in *M. truncatula*. Transcripts for this gene are strongly and rapidly induced by insect herbivory, insect oral factors, and the plant wound hormone jasmonic acid (Gomez et al., 2005). Following herbivory, transcripts for *Tps*1 accumulate to high levels in leaves of *cod* mutants, as they do in A17, 20 h after the initiation of insect feeding (Fig. 3A). These data suggest that the perception and signaling pathways for responses to insect feeding are operating normally in *cod* mutants.

The performance and feeding patterns of *S. exigua* demonstrate the role of calcium oxalate in defense against a chewing insect. We also compared the performance of a sucking insect, the pea aphid (*Acyrthosiphon pisum*), on A17 and *cod*5 and *cod*6. Aphids (15 per plant) were added and maintained on intact plants for 7 d, and then the original aphids and their offspring were counted. The data show that, unlike with *S. exigua*, there is no negative effect of *M. truncatula* calcium oxalate on the performance of the pea aphid (Fig. 3B).

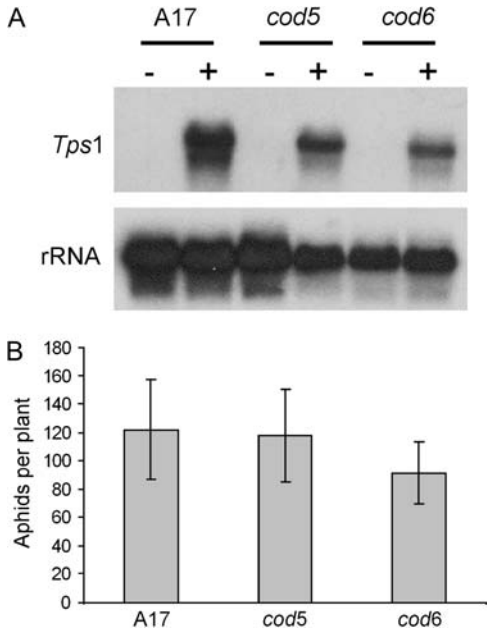

Figure 3. A, A total RNA blot was probed with a sequence encoding a wound-inducible *Tps*1 from *M. truncatula* (Gomez et al., 2005). Induction of *Tps*1 occurs in the *cod* mutants, as it does in the A17 wild type, 20 h after initiation of feeding by *S. exigua* larvae. The same blot was reprobed with an 18S ribosomal RNA clone as a loading control. B, Populations of a sucking insect, the pea aphid, are not negatively affected by calcium oxalate in the wild-type line A17. Data represent mean ± SEM, which did not differ significantly between mutant and wild-type plants (ANOVA; $P > 0.10$).

Calcium Oxalate Effects on Insect Digestion

Chewing insects prefer to eat tissue lacking calcium oxalate, so their enhanced growth on mutant lines might be due to increased food consumption. It is also possible that the decreased growth of lepidopteran larvae on wild-type plants could be affected by antinutritive properties of the calcium oxalate crystals. No-choice gravimetric consumption and food utilization measurements (Waldbauer, 1968; Sharma and Norris, 1991) were carried out to test these possibilities. Consumption indices (CI) and growth rates (GR) confirmed that insects consume more foliage and grow more rapidly when feeding on *cod* mutants than on A17 (Table I). The approximate digestibility (AD) indices, based on the proportion of ingested food that is excreted as frass, suggest that the digestion of tissue from all three genotypes is comparable. However, measurement of efficiencies of conversion of ingested food (ECI) and digested food (ECD) show that, in *S. exigua*, the leaves of both *cod*5 and *cod*6 are converted to insect biomass at a much higher level than are leaves of A17 (Table I). These findings suggest that calcium oxalate crystals act as an antinutritive defense as well as a feeding deterrent.

Calcium Oxalate as an Abrasive during Feeding

To determine whether there are physical effects of calcium oxalate crystals on insect mouthparts, larvae were reared on different diets and their mandibles were examined by scanning electron microscopy (SEM). At least six individuals reared on each food source were examined, and representative results are presented (Fig. 4). The mandibles of *S. exigua* larvae that feed on an artificial diet retain teeth with sharp points and a serrated edge (Fig. 4, A and B). The artificial diet in this case is a soft agar, casein, and wheat germ-based mixture (Bio-Serv). Larvae that feed on A17 leaves from the neonate stage through the fifth instar have mandibles with noticeable wear because they lose the serrated edge of teeth that are shortened and smoothed (Fig. 4, C and D). When fed on a diet of *cod*5 leaves, larvae retain a sharper point and remnants of a serrated edge more similar to those fed on an artificial diet (Fig. 4, E and F). When examined by SEM, the gut peritrophic membrane of *S. exigua* larvae does not show any obvious visible damage after feeding on A17 leaves when compared to a diet of *cod*5 leaves or artificial diet (data not shown).

DISCUSSION

Soluble oxalic acid is a strong acid and its effects as a toxin in animals are well documented. The ingestion of plants with high levels of oxalic acid can sometimes

Table I. *Consumption and food utilization of M. truncatula genotypes by S. exigua larvae feeding for 72 h on detached leaves (based on dry-weight measurements)*

Values with the same letters within a column indicate no significant difference; $P > 0.05$.

Genotype	CI	GR	AD	ECI	ECD
A17	3.63x	0.229x	82.4x	6.28x	7.62x
*cod*5	3.97y	0.361y	75.6x	9.08y	12.01y
*cod*6	4.20z	0.366y	77.7x	8.72y	11.22y

have lethal effects, often due to renal failure, on grazing animals (Von Burg, 1994) and humans (Hodgkinson, 1977). Soluble oxalic acid has also been shown to have inhibitory effects on sucking insects, such as planthoppers (Yoshihara et al., 1980) and aphids (Massonie, 1980). The levels of soluble oxalate in *cod*5 (1.0 mg g^{-1} dry weight) and *cod*6 (0.6 mg g^{-1} dry weight) are only slightly lower than the levels in wild-type A17 (1.4 mg g^{-1} dry weight), whereas the levels of insoluble calcium oxalate are dramatically lower in these *cod* mutants (Nakata and McConn, 2000).

When oxalic acid forms a complex with calcium, the resulting crystals are highly insoluble in an aqueous environment. These crystals are well known to sometimes cause injury in animals, including humans. Calcium oxalate in the leaves and corms of many edible aroid species will cause severe swelling of the mouth and throat if foods are not properly prepared before consumption (Bradbury and Nixon, 1998). The crystals in species such as daffodil (Julian and Bowers, 1997) and agave (Salinas et al., 2001) can act as irritants when they come into contact with human skin. A dramatic example of calcium oxalate as a skin irritant exists in the stinging plant *Tragosia ramosa* (Thurston, 1976), where a specialized cell on the leaf surface contains a needle-shaped crystal. When disturbed, the grooved crystals from this plant can be embedded in animal skin and channel a colocalized toxin to the wound site, causing pain in the affected area. Although it seems likely that there are also incidents of combined effects of calcium oxalate crystals and toxins in deterring insects, these have not been well documented.

Based upon insect growth, survival, and food utilization, it is clear that calcium oxalate contributes as an effective defense against chewing insects in *M. truncatula*. Because levels of soluble oxalate are similar in *cod*5, *cod*6, and A17, the data indicate that the striking differences we observe in insect performance are due to differences in insoluble calcium oxalate crystals and not to oxalic acid levels. Herbivory-induced transcript accumulation for *Tps*1 occurs in *cod* mutants as it does in A17 (Fig. 2D), suggesting that these lines possess the normal recognition and signaling processes for wound-induced defenses. These observations, along with the fact that *cod*5 and *cod*6 are nonallelic mutations with a severe deficiency of calcium oxalate as their common trait, provide strong evidence that increased insect performance on these mutants is due to the lack of calcium oxalate crystals.

Plants have multiple means of active defense against insects, and so the potent negative role of calcium oxalate on insect performance could possibly be due to synergistic effects of the crystals with some other plant component. For example, *M. truncatula* has abundant levels of defensive glycosylated triterpenoids (saponins) that also likely have important functions in defense (Huhman and Sumner, 2002).

The data presented here suggest that calcium oxalate has defensive activity by several modes of action. Feeding-choice tests show that *S. exigua* larvae can detect and will avoid, if possible, *M. truncatula* leaf tissue containing calcium oxalate crystals. This finding in itself indicates that plant calcium oxalate serves a defensive role. The striking abrasive effects that the crystals have on insect mandibles (Fig. 4) suggest that calcium oxalate acts, at least in part, by a physical means to deter insect chewing.

The physical abrasion of insect mandibles by calcium oxalate is reminiscent of a study of the effects of these crystals on humans. Prehistoric human populations in the Lower Pecos region of Texas, whose diets relied heavily on plant species high in calcium oxalate, demonstrated significant dental wear likely due to these crystals (Danielson and Reinhard, 1998). Other minerals in plants have also been shown to act as physical abrasives to chewing insects. There is a negative

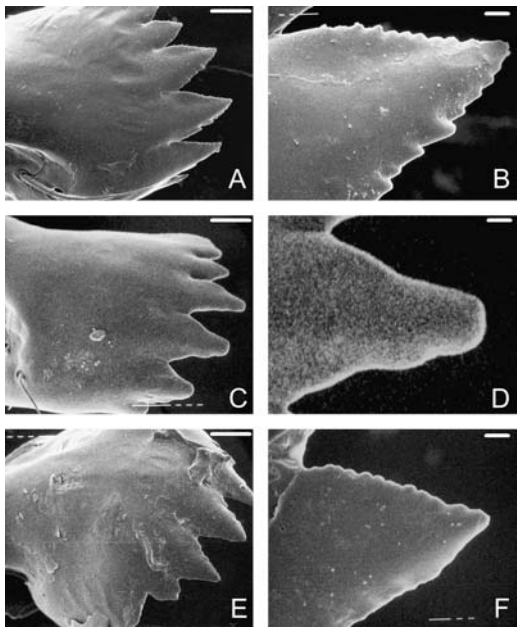

Figure 4. Prismatic crystals of calcium oxalate in *M. truncatula* have abrasive effects on mandibles of chewing insect larvae. SEMs of mandibles taken from fifth-instar *S. exigua* larvae reared on an artificial diet (A and B), or wild-type A17 (C and D) or *cod*5 (E and F) plants. A whole mandible (left column) and an individual tooth (right column) of each is shown. Sizing bars in the top right are 100 μm (left column) and 10 μm (right column).

correlation between levels of silica and susceptibility of rice (*Oryza sativa*) plants to the Asiatic rice borer, and the mandibles of this insect suffer significant wear when feeding on high-silica varieties (Djamin and Pathak, 1967). Performance of the pea aphid, which feeds on phloem sap via a piercing-sucking mechanism, was not reduced on line A17 when compared to feeding on *cod*5 or *cod*6. Taken together, our data indicate it is likely that the defensive function of calcium oxalate crystals in plants, especially as a deterrent to feeding by chewing insects, is due in part to their physical properties.

Comparisons of food consumption show that *S. exigua* larvae feeding on *cod*5 and *cod*6 eat more leaf tissue than comparable larvae feeding on A17 (Table I). Observing that insects consume more leaf tissue of one type, however, is not sufficient to conclude that this will necessarily lead to enhanced insect growth. In fact, increased food consumption is a typical response of chewing insects on food sources that actually have lower nutritive value (Tabashnik and Slansky, 1987).

In addition to being a physical deterrent to feeding, calcium oxalate interferes with the assimilation of *M. truncatula* leaf material in *S. exigua*, as shown by food utilization measurements (Table I). Based on our data, we cannot determine whether such digestive interference is due to an effect of the size or shape of the crystals in the insect gut or to some biochemical effect that calcium oxalate has on lepidopteran digestion. It is clear, however, that the overall effects on insect food utilization are quite strong. Leaf material from wild-type leaves is converted much less efficiently to insect body mass than plant tissue lacking calcium oxalate.

Several models suggest that if an insect's growth rate during development varies based on environment (e.g. temperature, diet), then it is generally advantageous to grow larger and develop faster, although there is often a tradeoff between these traits (Nylin and Gotthard, 1998). Based on such models, it appears that insects feeding on tissue lacking calcium oxalate would have a distinct advantage over insects feeding on wild-type *M. truncatula*. Although we did not measure insect fitness, it is worthwhile to note that positive correlations between lepidopteran body mass and fecundity or overall fitness have been reported (Lill, 2001; Iyengar and Eisner, 2002).

Calcium oxalate is a very common and widespread mineral in plants and can be abundant in some tissues of important food plants, such as grape (*Vitis vinifera*), spinach (*Spinacia oleracea*), and soybean (*Glycine max*; Massey et al., 2001). Most previous support for the defensive role for calcium oxalate has focused on needle-like raphide crystals. Here we show conclusively that naturally formed calcium oxalate crystals, even the prismatic-shaped crystals in *M. truncatula*, serve an effective defensive role against chewing insects. Many possibilities have been suggested for the biological role(s) of calcium oxalate in plants (Franceschi and Nakata, 2005). Detailed characterization of the *cod*5 mutant shows that a severe reduction in crystal formation does not significantly alter growth or development (Nakata and McConn, 2003). Therefore, at least in *M. truncatula*, defense against chewing insects is the only biological role for calcium oxalate crystals that is thus far supported by experimental evidence.

Given the strong negative effects of calcium oxalate on insect performance and its abundance in so many different plant species, this mineral probably represents an underappreciated form of effective natural plant defense. Modification of calcium oxalate in crop plants could potentially serve as an environmentally friendly means to improve plant defenses. Identification of the plant genes responsible for crystal size, shape, and localization could therefore prove to be very valuable in the selection or development of plants with enhanced levels of insect resistance.

MATERIALS AND METHODS

Feeding-Choice Tests

Spodoptera exigua eggs were obtained from the Gast Rearing Laboratory (U.S. Department of Agriculture Agricultural Research Service) and allowed to hatch on artificial diet (Bio-Serv). The isolation and initial characterization of mutants *cod*5 and *cod*6 have been described (Nakata and McConn, 2000). The wild-type *Medicago truncatula* line A17 was the parental line that gave rise to *cod* mutants and was used as the control treatment in all experiments. Independent two-way choice experiments were performed to measure insect preferences for *cod*5 or *cod*6 mutant tissue compared with A17 or with each other. Individual larvae were placed in a petri dish containing equal amounts of leaf material from each of two genotypes as indicated. Third-instar larvae of similar size were selected and allowed to feed at will for 20 h. Weight of leaf material was measured before and after the feeding period. Dishes containing plant material were kept together in larger, humidified containers to prevent desiccation of the leaf material. Five replicate tests were conducted for each comparison. Ratios for each phenotype comparison were then calculated on the basis of the fresh weight of material consumed. A value of 1.0 would indicate that equal amounts of tissue from each genotype were consumed; a one-sample *t* test was used to determine whether values were significantly different from a theoretical mean of 1.0.

Insect Growth and Mortality

Plants were maintained in a growth chamber at a constant temperature of 22°C with a 16-h-light/8-h-dark cycle. Insects, five per plant, were caged on intact 4- to 6-week-old plants. Larval weight measurements were taken at daily intervals initiated when larvae were large enough to easily withstand handling, at a minimum of 5 to 6 d after hatching, and continued until pupation. When larvae near pupation, they have reduced fluid levels and so their fresh weight decreases. To indicate the approximate timing of early stages of pupation, fresh weights are not shown after the time point when average weights on a genotype started to decrease. Pupae were collected, dried, and weighed. Weights were compared via ANOVA (A17, $n = 13$; *cod*5, $n = 37$; *cod*6, $n = 28$).

For mortality measurements, neonate larvae, 60 per genotype, were individually caged in 12-well plates with detached leaves of the plant line indicated and maintained at constant humidity at 22°C. Fresh leaves of 4- to 6-week-old plants were added for food as needed. Numbers of surviving larvae were recorded daily. Survival rates were compared via ANOVA for each day independently.

RNA-Blot Analysis

Four third-instar larvae per plant were added to plants and allowed to feed at will for 20 h. Insects were removed and damaged leaves were collected and stored at −80°C. Total RNA was isolated from leaves using TriReagent (Molecular Research), and 15 μg of total RNA from each sample were

separated on 1% agarose formaldehyde gels. Accumulation of transcripts by RNA-blot hybridization was measured using standard techniques (Sambrook et al., 1989) and ^{32}P-labeled probes as described previously (Gomez et al., 2005).

Aphid Feeding Studies

Pea aphids (*Acyrthosiphon pisum* Harris), 15 per 6-week-old plant, were added and 14 plants per genotype were arranged in a complete random design in a growth chamber at a constant temperature of 22°C with a 16-h-light/8-h-dark cycle. Surviving adult aphids and their offspring were counted after 7 d and data were analyzed by ANOVA.

SEM

Insects were reared from the neonate to the fifth-instar stage on either artificial diet or on intact A17 or *cod*5 plants. At early fifth instar, larvae were euthanized and mandibles were removed. Mandibles were gold coated and subsequently viewed with an ISI-60 SEM. The results shown in Figure 4 are representative of the consistent appearance of mandibles observed from each of at least six individuals reared on each food source.

Food Utilization Measurements

Food consumption and utilization analyses were carried out as described (Waldbauer, 1968; Sharma and Norris, 1991). Second-instar larvae were weighed and caged individually with 3 g fresh weight of detached leaf material. Cages were kept together in a moist chamber at 24°C. For each plant genotype, 15 larvae were measured. After 72 h, insects, leaves, and frass were weighed, then dried and weighed again to determine dry weights. Initial larval dry weights were estimated based on fresh weights of the larvae using an experimentally determined value of 17.29% as the dry-weight component of each. Mean daily larval weights were calculated from the initial and final larval weights. Data were analyzed by ANOVA and value means were compared using LSDs.

Values were calculated as follows.

$$\text{CI} = \text{food ingested}/(\text{days} \times \text{mean larval weight})$$

$$\text{GR} = \text{weight gained}/(\text{days} \times \text{mean larval weight})$$

$$\text{AD} = (\text{food ingested} - \text{frass})/\text{food ingested}$$

$$\text{ECI} = \text{weight gained}/\text{food ingested}$$

$$\text{ECD} = \text{weight gained}/(\text{food ingested} - \text{frass})$$

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