its extensive *S* haplotype-associated polymorphism (*19*, *20*). SLG has been shown to migrate on SDS-PAGE as a cluster of molecular mass forms (Fig. 2D) (*21*). However, only one of these SLG_6 forms bound to SCR_6 beads, suggesting a degree of specificity in the observed SLG_6-SCR_6 binding. Additional evidence for specificity is provided by the finding that treatment of S_2S_2 stigma extracts with SCR_6 beads failed to pull down detectable levels of SLG_2 or of the *S*-locus related *SLR1* and *SLR2* gene products (Fig. 2D), all of which exhibit only 65% amino acid sequence identity to SLG_6. SLG is thought to function, at least in some cases, as an accessory molecule that enhances the SRK-mediated SI response (*3*), possibly by contributing to the stabilization and proper maturation of SRK (*18*). The interaction observed between SCR and SLG suggests that some forms of SLG might also function in ligand binding. However, the physiological importance of this relatively weak interaction remains to be determined.

Our results demonstrate that SCR interacts with the ectodomain of SRK. Apparently, the SRK-SCR interaction does not require additional components specific to the stigma and pollen surfaces, because the interaction was observed between recombinant proteins purified from *Nicotiana* leaves and bacteria. The data indicate that specificity in the SI response results from *S* haplotype-specific molecular interaction of SCR and SRK, which would selectively trigger activation of self SRK and a pollen-inhibitory chain of events. Analysis of receptor-ligand interactions demonstrated by SRK-SCR and by CLV1-CLV3 of *Arabidopsis thaliana* (*22*) should provide useful paradigms for the study of transmembrane receptor signaling and of the function and regulation of small diffusible peptide ligands in plants.

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11. The SRK_6 ectodomain carrying a COOH-terminal FLAG epitope was cloned into the Potato Virus X (PVX)-derived vector pP2C2S′ (*23*). In vitro transcripts produced with the RiboMAX system (Promega, Madison, WI) were used to infect 4-week-old *N. benthamiana* plants. Infected leaves showing disease symptoms were ground in liquid nitrogen, resuspended in buffer [30 mM Tris-HCl pH 7.5, 75 mM NaCl, 10% v/v glycerol, 5 mM ascorbate, 2.5 mM potassium metabisulfite, 1 mM phenylmethylsulfonyl fluoride (PMSF), aprotinin (10 μg/ml), leupeptin (10 μg/ml), and pepstatin A (1 μg/ml)], and then $eSRK_6$-FLAG was purified on FLAG M2 affinity gel (Sigma, St. Louis, MO). Recombinant SCR_6 and SCR_{13} (without the signal peptide) carrying a COOH-terminal myc-His_6 were produced using the pBAD/gIIIB vector (Invitrogen, Carlsbad, CA) and were purified on Ni–nitriloacetic acid (Ni-NTA) affinity agarose (Qiagen, Valencia, CA).
12. Recombinant SCR proteins were assayed on stigmas collected from *S*-locus homozygotes, which are generated by manual pollination in immature buds before the developing buds acquire the ability to reject self-pollen. For these bioassays, which were modified from (*24*), carrier pollen coat protein (50 ng) was extracted with cyclohexane (*15*) from pollen that is genetically unrelated to the stigmas and pollen used for pollination, mixed with 300 ng of SCR_6-myc-His_6 or SCR_{13}-myc-His_6, and applied to S_6S_6 or $S_{13}S_{13}$ stigmas. Preliminary experiments showed that 300 and 60 ng of SCR applied to a stigma activated the incompatibility response and 30 ng were partially effective, whereas 3 ng and 0.6 ng were ineffective. Treated stigmas were allowed to dry for ~1 hour before pollination, and pollen tube growth was visualized by ultraviolet fluorescence microscopy (*5*). Controls included untreated stigmas, S_6S_6 and $S_{13}S_{13}$ stigmas treated with carrier pollen coat protein alone or with non-self SCR, and S_2S_2 stigmas treated with carrier pollen coat protein and the preparation of SCR used in the experimental samples.
13. SCR proteins were resolved on 15% SDS-PAGE and detected with antibodies to myc (Invitrogen). For detection with antibodies to SCR_6 generated against SCR_6–glutathione *S* transferase (SCR_6-GST) fusion proteins, reducing agents were omitted because they quenched antibody binding. $eSRK_6$ and stigma proteins were resolved on 7.5% SDS-PAGE and detected with antibodies to S domain: monoclonal MabH8, which detects SRK_6, SRK_{13}, SLG_6, and SLG_{13} (*17*, *18*); polyclonal antibodies to SLR2, which detect SLR2 and SLG_2 (*25*); and SLR1-specific polyclonal antibodies (*21*). Protein bands were visualized with horseradish peroxidase–conjugated secondary antibodies (Sigma) and the ECL+ system (Amersham Pharmacia Biotech, Piscataway, NJ).
14. ELISA was carried out according to (*26*). Microtiter plate wells were coated with 0.5 μg of $eSRK_6$ and then were incubated with increasing amounts of SCR_6-myc-His_6 or SCR_{13}-myc-His_6. After incubation with antibodies to myc and alkaline phosphatase–conjugated secondary antibody (Sigma), the reaction was developed with 100 μl of Sigma Fast pNPP substrate (Sigma), and absorbance was measured at 405 nm in an ELISA plate reader.
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Resistance to an Herbivore Through Engineered Cyanogenic Glucoside Synthesis

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The entire pathway for synthesis of the tyrosine-derived cyanogenic glucoside dhurrin has been transferred from *Sorghum bicolor* to *Arabidopsis thaliana*. Here, we document that genetically engineered plants are able to synthesize and store large amounts of new natural products. The presence of dhurrin in the transgenic *A. thaliana* plants confers resistance to the flea beetle *Phyllotreta nemorum*, which is a natural pest of other members of the crucifer group, demonstrating the potential utility of cyanogenic glucosides in plant defense.

Cyanogenic glucosides are a group of amino acid–derived secondary metabolites that are widely distributed in the plant kingdom (*1*, *2*). When the plant tissue is disrupted by herbivore attack, the cyanogenic glucosides are degraded into a sugar, a keto compound, and hydrogen cyanide (HCN). This cyanogenesis confers protection against some, but not all, herbivore attacks (*1*, *3*). Insects feeding on cyanogenic plants may have evolved mechanisms to detoxify or to sequester cyanogenic glucosides (*4*–*6*), which in turn protect the insect against predators (*7*). To assess the effect of cyanogenesis, it is necessary to study insects that have not coevolved with cyanogenic glucosides. Such insects are found among those that specifically feed on cruciferous plants, which do not produce cyanogenic glucosides. To render investigations in such an experimental system possible, we transferred the pathway for cyanogenic glucoside biosynthesis into the cruciferous plant

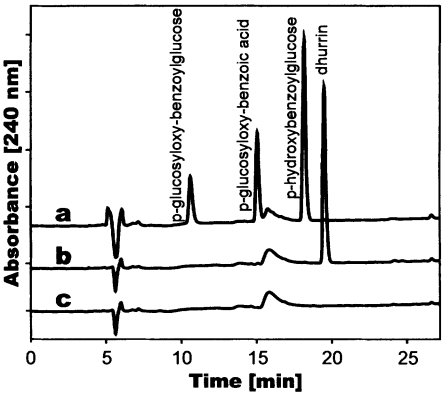

Fig. 1. HPLC analysis of *A. thaliana* plants containing either the entire dhurrin biosynthetic pathway or the cytochrome P450–catalyzed part. a, CYP79A1 and CYP71E1; b, CYP79A1, CYP71E1, and sbHMNGT; c, wt. Each UV trace represents equal amounts of leaf material.

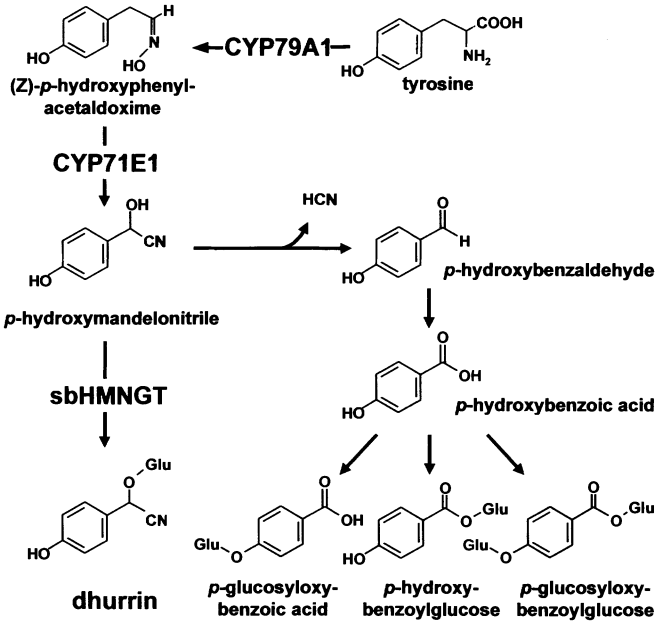

Fig. 2. *A. thaliana* plants expressing both CYP79A1 and CYP71E1, but not sbHMNGT, accumulate glucosides of benzoic acid, a product of *p*-hydroxymandelonitrile decomposition.

species *Arabidopsis thaliana* by genetic engineering and studied the effect of cyanogenic glucosides in plant defense against the crucifer-specialist flea beetle *Phyllotreta nemorum*.

Biosynthesis of the cyanogenic glucoside dhurrin in *Sorghum bicolor* is highly channeled (*8*) and catalyzed by two multifunctional microsomal cytochromes P450 (CYP79A1 and CYP71E1) (*9–13*) and a soluble UDPG-glucosyltransferase (sbHMNGT) (*14*) [Web fig. 1 (*15*)]. We transformed *A. thaliana* plants expressing the genes encoding CYP79A1 and CYP71E1 (*16*) with a recombinant plasmid conferring gentamycin resistance and encoding sbHMNGT (*17*). Newly generated plants containing the dhurrin pathway were kanamycin- and gentamycin-resistant, which facilitated their selection.

The strategy resulted in several independent lines that were examined for their ability to synthesize dhurrin. Upon administration of radiolabeled tyrosine to detached leaves and analysis of methanol (MeOH) extracts by thin-layer chromatography, one predominant radioactive product comigrating with authentic dhurrin was observed. High-performance liquid chromatography–mass spectrometry (HPLC-MS) analyses (*18*) showed that the product had the same retention time, ultraviolet (UV) spectral properties, and molecular mass as the dhurrin standard (Fig. 1).

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Four-week-old transgenic *A. thaliana* plants contained as much as 4 ± 0.5 mg of dhurrin per gram of fresh weight (gfw) (*19*). This level of dhurrin is similar to that found in seedlings of *S. bicolor* (*20*), demonstrating the ability to efficiently integrate the dhurrin biosynthetic pathway into *A. thaliana*. Transgenic *A. thaliana* lines containing ~1 mg of dhurrin/gfw displayed no apparent phenotypic differences as compared with the wild-type (wt) plants, and lines containing higher levels of dhurrin had only small reductions in growth [Web fig. 2 (*15*)]. Therefore, the diversion of tyrosine toward dhurrin biosynthesis and the storage of dhurrin did not cause inherent metabolic problems. As with other plants containing cyanogenic glucosides, the transgenic *A. thaliana* plants released high levels of HCN, up to ~2 μmol/gfw, upon tissue damage (*21*). Likewise, dialyzed protein extracts from *A. thaliana* hydrolyzed dhurrin (*22*). Therefore, an endogenous β-glucosidase with dhurrin hydrolyzing activity is present in *A. thaliana*.

The expression of the CYP79A1 gene in *A. thaliana* results in the production of *p*-hydroxybenzylglucosinolate as a result of metabolic cross talk between the pathways for cyanogenic glucoside and glucosinolate synthesis (*23*). *A. thaliana* plants expressing all three dhurrin biosynthetic pathway genes also accumulated *p*-hydroxybenzylglucosinolate, although at much lower levels (*22*), whereas dhurrin was the only product seen to accumulate in high abundance (Fig. 1). As with *S. bicolor* (*8*), pathway intermediates in these *A. thaliana* plants were hardly detectable. Thus, although all three sorghum sequences were driven by the cauliflower mosaic virus (CaMV) 35*S* promoter in the transgenic *A. thaliana* plants, the individual enzyme activities of the pathway for dhurrin biosynthesis seemed to be adequately balanced. Transgenic plants lacking sbHMNGT produce *p*-glucosyloxy-benzoylglucose, *p*-glucosyloxy-benzoic acid, and *p*-hydroxybenzoylglucose (Fig. 1). Radiolabeling experiments showed that these glucosides are derived from *p*-hydroxymandelonitrile that had decomposed into HCN and *p*-hydroxybenzaldehyde, of which the latter had been finally oxidized into *p*-hydroxybenzoic acid (Fig. 2). None of the hundred or more predicted *A. thaliana* (*24*) secondary plant product glucosyltransferases are capable of converting the aglycone *p*-hydroxymandelonitrile into the corresponding cyanogenic glucoside in planta. Thus, the glucosyltransferase activity provided by sbHMNGT is necessary to obtain dhurrin production in transgenic *A. thaliana*.

The flea beetle *P. nemorum* (Coleoptera: Chrysomelidae: Alticinae) accepts wt *A. thaliana* as a food source (*25*) and as a specialist crucifer-feeder is not expected to have encountered cyanogenic glucosides during its recent evolutionary history. In choice tests with adult beetles (*26*), the consumption of leaf-disc material from transgenic *A. thaliana* plants containing dhurrin was compared to consumption of wt plants (Fig. 3A). The beetles consumed up to 80% less of the transgenic leaf-disc material (D ± 95%, confidence limit = 0.70 ± 0.10). Consumption of leaf-disc material from the transgenic lines expressing the two cytochrome P450 genes (CYP79A1 and CYP71E1) (D = 0.06 ± 0.16), or the UDPG-glucosyltransferase gene (sbHMNGT) (D = 0.03 ± 0.06), or containing the two empty expression vectors (D = −0.18 ± 0.23) was not significantly different from the consumption of

leaf-disc material from wt plants. Therefore, the deterrent effect was directly attributable to the presence of dhurrin.

Normally, after hatching from eggs laid in the soil, the larvae of *P. nemorum* climb a plant in search of a suitable site for initiation of a leaf mine. In nonchoice bioassays with newly emerged flea beetle larvae (*27*), the presence of dhurrin in the transgenic plants reduced the number of leaf mines initiated (G = 190.0, df = 5, $P < 0.0001$) (Fig. 3, B and C). All larvae that did not initiate mines died. Of the larvae that did initiate mines, a higher mortality was observed on leaves containing dhurrin (G = 149.9, df = 5, $P < 0.0001$) (Fig. 3C). As for the adult insects, mine initiation and larval survival on transgenic plants expressing the cytochrome P450 genes only, the glucosyltransferase gene only, or containing the two empty expression vectors were not significantly different from those on wt plants (mine initiation rates: G = 4.39, df = 3, $P > 0.05$; survival rates: G = 1.93, df = 3, $P > 0.05$).

Thus, the pathway for biosynthesis of the cyanogenic glucoside dhurrin can be transferred from sorghum into the acyanogenic model plant *A. thaliana* by the use of genetic engineering. The accumulation of substantial amounts of dhurrin does not appear to pose any inherent physiological problems for the transgenic *A. thaliana* and confers resistance to the flea beetle *P. nemorum*, demonstrating that cyanogenic glucosides can promote plant defense. Such engineering of cyanogenic glucosides into acyanogenic crop plants may prove useful for pest control purposes.

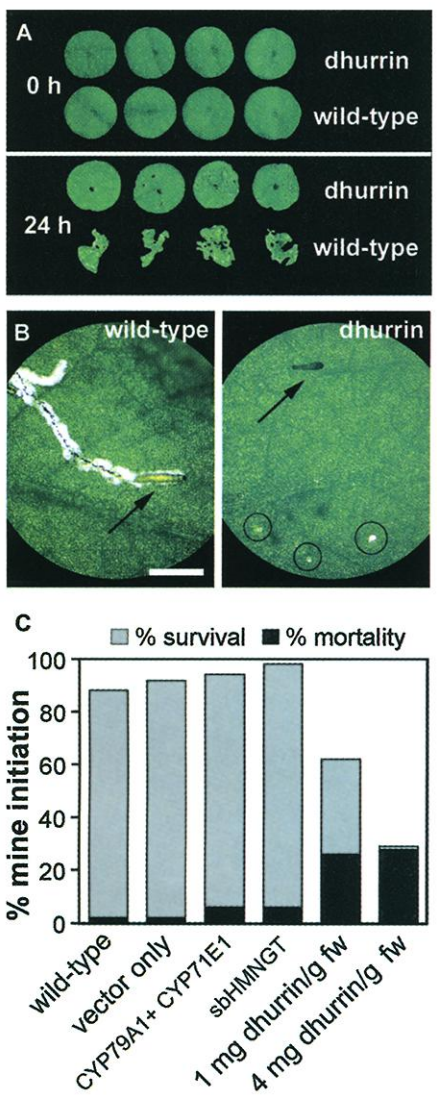

Fig. 3. *A. thaliana* leaves containing dhurrin inhibit flea beetle and larvae feeding. **(A)** Adult beetles fed extensively only on leaves containing no dhurrin. **(B)** Larvae (indicated by arrows) frequently initiated no mines on leaves containing dhurrin, although attempts were made to feed (indicated by circles). Scale bar, 2.5 mm. **(C)** Nearly all larvae (98%) presented to leaves containing about 4 mg of dhurrin/gfw died.

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17. The cDNA encoding sbHMNGT was excised with Eco RI and Xba I from pcDNAII sbHMNGT (*14*) and ligated into pRT101 (*28*). The sbHMNGT cDNA and the CaMV 35*S* promoter and polyadenylation site were then excised with Hind III and ligated into pPZP221 (*29*). *A. thaliana* plants [ecotype Columbia (Col-0)] containing CYP79A1 and CYP71E1 (*16*) were transformed with the floral dip method, (*30*) using *Agrobacterium tumefaciens* C58C1/pGV3850 transformed with the pPZP221-sbHMNGT plasmid. Transgenic offspring were selected as described (*16*) with the addition of gentamycin (200 mg/l).
18. Leaves from 4-week-old plants grown in soil (*16*) were extracted in boiling 85% (v/v) MeOH for 10 min. The MeOH was evaporated in vacuum and water-soluble material was recovered after the addition of water and centrifugation (at 15,000*g* for 5 min). Aliquots were analyzed by HPLC with a Supelcosil LC-ABZ$^+$Plus column (size 250 × 4 mm; Supelco, Bellefonte, PA) equilibrated in 4% (v/v) CH_3CN and 0.1% (v/v) HCOOH (flow rate, 0.5 ml/min). After a sample application, the column was washed for 2 min to remove glucosinolates and other ions. Elution was initiated with a linear gradient developed over 40 min to a concentration of 32% (v/v) CH_3CN and 0.1% (v/v) HCOOH, after which a steeper gradient to 80% (v/v) CH_3CN and 0.1% (v/v) HCOOH was applied. Elution profiles were monitored at wavelengths of 200 to 300 nm. Components of interest were subsequently introduced into a Bruker Esquire-LC ion-trap mass spectrometer. For MS analysis, HPLC solvents contained 50 μM NaCl, the mass spectrometer was run in positive ion mode, and the [mass + Na]$^+$ adduct ions were used for identification. Dhurrin (Extrasynthèse, Genay, France), *p*-glucosyloxy-benzoic acid, and *p*-hydroxybenzoylglucose (a gift from L. Heide, Universität Tübingen, Germany) were used as authentic standards.
19. Dhurrin levels were determined by analysis of MeOH-extracted leaf tissue by HPLC (*18*), with amygdalin (Sigma) as an internal standard. Four individual measurements were carried out for each transgenic line, and dhurrin levels were calculated by peak integration and reference to a standard curve constructed with authentic dhurrin.
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27. Bioassays and statistical analyses of data from experiments with flea beetle larvae were performed as described (*31*). Individual leaves, from plants as described (*26*), were placed in plastic vials (25 ml) together with a piece of moist filter paper. One neonate larva was transferred to each vial, and after 3 days at 24° ± 1°C, the survival or death of each larva was recorded, as well as the number of mines made. Fifty or more replicates were made per plant line.
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