Epidermal Cell Fate Determination in *Arabidopsis*: Patterns Defined by a Steroid-Inducible Regulator

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The *Arabidopsis* mutant *ttg* lacks both trichomes (epidermal hairs) and anthocyanin pigments. Trichomes and anthocyanins are restored by the constitutive expression of the maize transcriptional regulator (R). The expression of an R-glucocorticoid receptor chimeric protein results in a steroid hormone–dependent, conditional allele of R that functions in whole *Arabidopsis* plants. The response of the chimeric protein to pulses of hormone was used to define the pattern and timing of trichome formation on the developing leaf epidermis. Each adaxial epidermal leaf cell appears to have an equal probability of differentiating into a trichome; there is a temporal zone of decision for trichome cell fate that proceeds as a wave from the tip to the base of developing leaves.

The determination of cell fate is a fundamental aspect of normal development, and the precise choice of cell fate in multicellular organisms is a complex process. Determining how, when, and where cells make a fate choice provides a framework for understanding the ontogeny of individual organisms and for elucidating how specific structures are determined. Conditional mutations in regulatory genes that allow controlled modulation of pattern development have been instrumental in defining temporal and spatial controls. The use of permissive and nonpermissive conditions, which otherwise have little effect on the organism, can be used to determine precisely when and where a cell fate choice is made.

The *Arabidopsis* regulatory mutation transparent testa glabra (*ttg*) affects multiple sets of target pathways, including several that alter the pattern of epidermal cell fate determination. Plants expressing *ttg* lack trichomes, anthocyanins, seed coat pigment, and mucilage (*1*) and produce excess root hairs (*2*). All *ttg* defects are reversed by expression of the maize regulatory factor R (*2*, *3*). In maize, the transcription of anthocyanin genes requires R, together with a second regulator, C1 (*4*). The R protein contains acidic and basic helix-loop-helix (bHLH) domains (*5*). The bHLH domain shares homology with the DNA binding and protein dimerization motif of the MYC family of mammalian transcriptional regulators.

The rat glucocorticoid receptor (GR) functions in cultured cells of heterologous species including yeast (*6*), *Drosophila* (*7*), and tobacco (*8*). The steroid binding domain of the rat glucocorticoid receptor contains a regulable inactivation function; this domain can confer hormone inducibility on heterologous regulatory factors (*9*). When this domain is fused to the adenovirus E1A transcription factor, transcription from an E1A-regulated promoter becomes strictly hormone-dependent. Similarly, fusions of the human protooncogenes *c-myc* and *c-fos* as well as the human immunodeficiency virus–type 1 (HIV-1) transactivator Rev to the hormone binding domain of rat GR confer steroid-dependent transformation, transcription, or transactivation (*10*). Fusions between MyoD and the hormone binding domain of human GR give steroid-dependent myogenesis of mammalian cells in culture (*11*).

To determine whether such fusions would function in *Arabidopsis* plants, a fusion between R (*5*) and GR was constructed (Fig. 1A). When expressed in plants, this fusion produces the full-length, 610–amino acid R protein followed by a 2–amino acid linker and amino acids 512 through 795 of the GR receptor, containing the ligand binding domain (*12*, *13*). This chimeric protein was placed under the control of the largely constitutive cauliflower mosaic virus 35S promoter (35S) (*14*) in pKYLX71 (*3*, *15*). The expression construct was introduced into *Arabidopsis* by *Agrobacterium*-mediated transformation (*16*). Transformants were selected by their resistance to kanamycin, and expression of the transferred genes was monitored by RNA blot analysis.

Eight independent lines expressing R-GR were produced. Seeds from selfed transformants were plated on germination media (*16*) with and without 1 μM dexamethasone (Dex, Sigma), a synthetic glucocorticoid. Six lines showed low background levels of R-dependent anthocyanin and trichome production without Dex. All six showed hormone-dependent induction of both anthocyanins and trichomes. Because line 1857 showed very low background expression and had strong induction, it was analyzed in detail. There is very slight anthocyanin production in this R-GR transformant in the absence of the steroid, observed as a faint band of red pigment on the hypocotyl just below the cotyledons (*17*). Two lines showed no R-dependent phenotypes with or without Dex.

Similar fusions between R and the ligand binding domain of the estrogen receptor (R-ER) were constructed and transformed into *Arabidopsis*. These transformants also showed ligand-dependent induction of anthocyanins and trichomes. All the transformants showed substantial background expression and were not analyzed in detail.

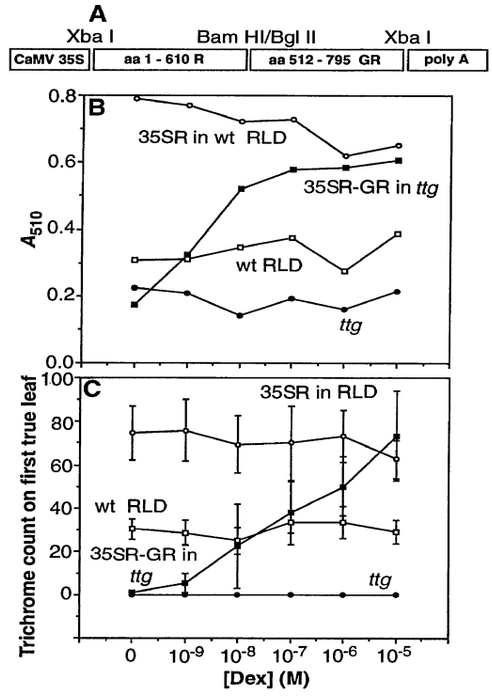

Fig. 1. Diagram of the R-GR fusion construct and quantification of phenotypes. (**A**) The R-GR translational fusion and plant transcription signals are shown: cauliflower mosaic virus 35S promoter, amino acids 1 through 610 of the maize regulator R, two linker amino acids, amino acids 512 through 795 of the rat GR, and a plant polyadenylation signal. (**B**) Optical densities of extracts of Dex-treated seedlings. Seeds were plated on germination media (*16*) containing increasing concentrations of Dex as shown. Three days after plating (Fig. 2), 50 mg of seedling tissue was extracted with 100 μl of acidic methanol (*3*, *27*) and absorbance was measured at 510 nm. The *Arabidopsis* strains and transgenes were as shown except that *ttg* and wild-type (wt) RLD were transformed with the parent vector that lacked an insert. (**C**) Trichome number on Dex-treated seedlings. Seeds were plated as in (B). The number of trichomes was counted on the first true leaf to expand after the development of cotyledons. Each data point is the average of 10 seedlings.

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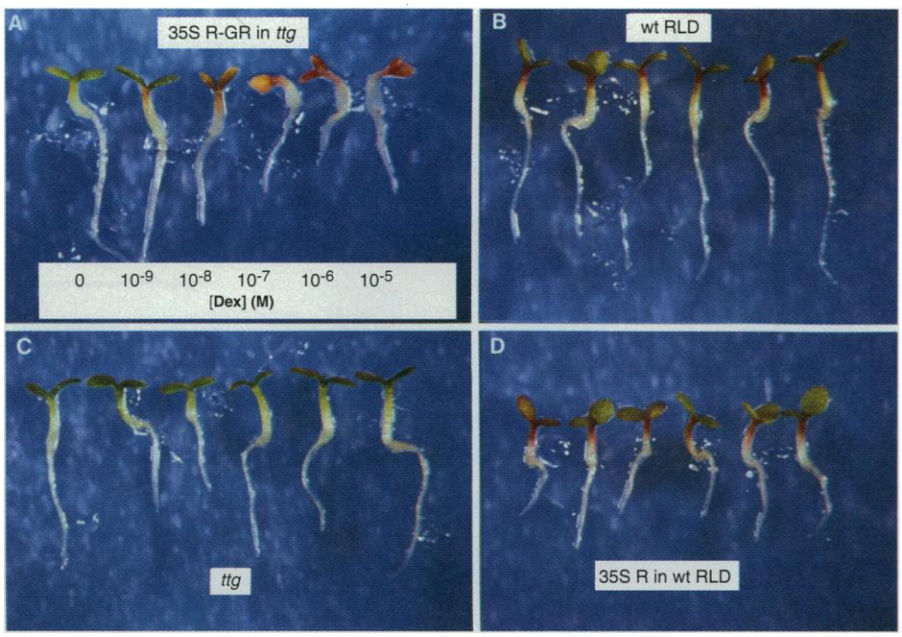

Fig. 2. Effect of increasing concentrations of Dex. Seeds were planted as in Fig. 1. Three days after planting, seedlings from different concentrations as shown in (A) were lined up for comparison. (**A**) The *ttg* mutation transformed with p35S R-GR. (**B**) Wild-type (wt) RLD transformed with pKYLX71. (**C**) The *ttg* mutation transformed with pKYLX71. (**D**) Wild-type RLD transformed with p35SR. The concentrations of Dex in panels (B) to (D) are the same as shown in (A).

Anthocyanin production is regulated by Dex concentration in R-GR seedlings (Fig. 2). The *ttg* mutant expressing the R-GR fusion responds to as little as 1 nM Dex with visible anthocyanin accumulation (Figs. 1B and 2A). These data confirm the hormone-dependent induction of pigment. The R-GR expressing seedlings also show a hormone-dependent inhibition of growth rate. A similar growth rate inhibition is observed in plants expressing high concentrations of R (compare Fig. 2, B and D). This inhibition may result from the overproduction of anthocyanins. Gross overproduction of R is also toxic to *Petunia* (*18*).

The control seedlings are unaffected by Dex. They include the wild-type Rschew (RLD) line (Fig. 2B) and *ttg* (Fig. 2C), both transformed with the parent plasmid lacking an insert. These results demonstrate that pigment induction depends on the R-GR fusion and does not require the wild-type TTG product or the *ttg* mutation. The *ttg* or wild-type RLD that were transformed with a constitutively expressed R construct (pAL144) (*3*) produce high levels of anthocyanin. Pigment accumulation decreases slightly with increasing concentrations of steroid in lines constitutively expressing R (Figs. 1B and 2D).

Trichome production is also regulated in a hormone-dependent manner in R-GR seedlings. There is a dose-dependent increase in trichome number on the first true leaf to emerge after cotyledon expansion in the R-GR transformant (Fig. 1C). The trichome number and pattern of production are altered by steroid hormone treatment only in the R-GR transformant (Fig. 3A). This pattern of trichomes closely follows that induced by constitutive expression of 35S R alone (Fig. 3C). The hormone has little or no affect on wild-type or *ttg* mutant *Arabidopsis*, nor does it interact with R alone to alter trichome development (Fig. 3, B to D).

The untransformed *ttg* mutant produces an occasional trichome on rosette leaves (<0.1% of the seedlings). These trichomes are always at the tip or edge of the leaf blade. Similarly, the background R-induced trichome production in the absence of steroid hormone is visible (Fig. 3A) as trichomes produced along the leaf edge in the R-GR transformant. Approximately 25% of the uninduced seedlings from this R-GR transformant produce a few trichomes on rosette leaves. These spurious trichomes are at the tip or edge of the leaf blade only, never on the central surface of the blade.

The course of development of one leaf was followed with sequential scanning electron microscopy (SEM) images made from casts of molds produced with dental impression polymer, an elastic material that allows sequential replicas to be made of the same surface without tissue destruction (Fig. 4) (*19*). This plant was grown for 8 days in soil, then hormone was applied by total immersion of the plant in 1 μM Dex. Many but not all of the fully formed trichomes visible at day 9 were already visible at 24 hours. The same six representative cells are marked in each panel. Many trichomes are produced as clusters and groups of immediately adjacent cells. Nearly every cell on the edge of the younger leaf (Fig. 4B, lower left) is producing a trichome. This growth is never seen on wild-type leaves. The same clustering pattern of trichome production is observed in wild-type plants constitutively expressing high levels of R. The increased number and hence tighter spacing of trichomes on the R-GR leaf surface compared to that on wild-type leaves (Fig. 3D) indicates there is not a pattern of "pre-trichome" cells that are activated by R. Instead, it appears that any epidermal cell is capable of developing into a trichome, given the proper signal.

These results indicate that the GR steroid binding domain functions to inactivate R in whole *Arabidopsis* plants, repressing the activation of both anthocyanin and trichome target genes. This inactivation is reversed by Dex, resulting in phenotypes that closely parallel R expression alone from the same 35S promoter. The hormone has access to the entire *Arabidopsis* plant through the root system. Hormone concentrations as low as 1 nM elicit R-dependent phenotypes; near-maximal pigment production is achieved at 100 nM. The receptor-ligand affinity in *Arabidopsis* parallels those reported in mammalian cells (*20*).

This R-GR fusion allows facile manipulation of the repression and induction of trichome formation in the trichome-free *ttg* mutant. Experiments were performed to begin to define the spatial and temporal pattern of trichome initiation over the epidermis during leaf ontogeny. R-GR seeds were plated on hormone-free media for germination and development. At regular intervals after germination, seedlings were moved to plates containing hormones. The newly developing leaves were observed for their pattern of trichome formation. The reverse experiment was also performed, with seedlings moved from hormone-containing to hormone-free plates. It is possible to influence leaves whose epidermis is only partially differentiated (Fig. 3, E to G). In seedlings moved from hormone-free to hormone-containing media at day 6, the cotyledons are glabrous (hairless), as are the first and second true leaves, representing the normal state for the *ttg* mutant (Fig. 3E). The third leaf shows trichomes at its base only, whereas the fourth leaf has trichomes on approximately the bottom two-thirds of its surface. The fifth leaf (barely visible in the center of the rosette) and all subsequent leaves have trichomes on the entire surface.

When seedlings were moved from hormone-containing to hormone-free media, the reciprocal phenotype appeared (Fig.

3F). Early leaves are fully covered with trichomes, but later leaves, which have been caught during epidermal cell fate decision, have trichomes only at the tip and are glabrous at the base. Leaves completely without trichomes were not generally observed on these seedlings possibly because of residual hormone in the plant tissue. Hormone treatment can also be carried out under standard growth conditions (Fig. 3G).

This temporal pattern indicates that leaf epidermal cell fate determination begins at the tip and moves toward the base of each leaf as a wave during development. This tip-to-base wave is consistent with an inhibitor model of regular trichome positioning over a leaf surface. According to this model, a cell that has made the decision to produce a trichome influences surrounding epidermal cells, preventing them from becoming trichomes. This effect is similar to that in proposed models for *Drosophila* bristle development (*21*) or the spacing of leaf stomata (*22*). Either a diffusable factor or a physical force, which only the trichome initials produce, acts locally. When this factor or force becomes dilute or small enough at some distance from the trichome cell, another cell initiates a trichome and makes the factor. Individual epidermal cells enter a zone in which these decisions occur from the base and exit toward the tip of the leaf. Once the cell has gone past a certain stage in development or has exited this decision region, it can no longer be induced to become a trichome.

The Dex dose–responsive nature of trichome production presented here and the clustering of trichomes described in this and a previous report (*3*) indicate that trichome production is proportional to local

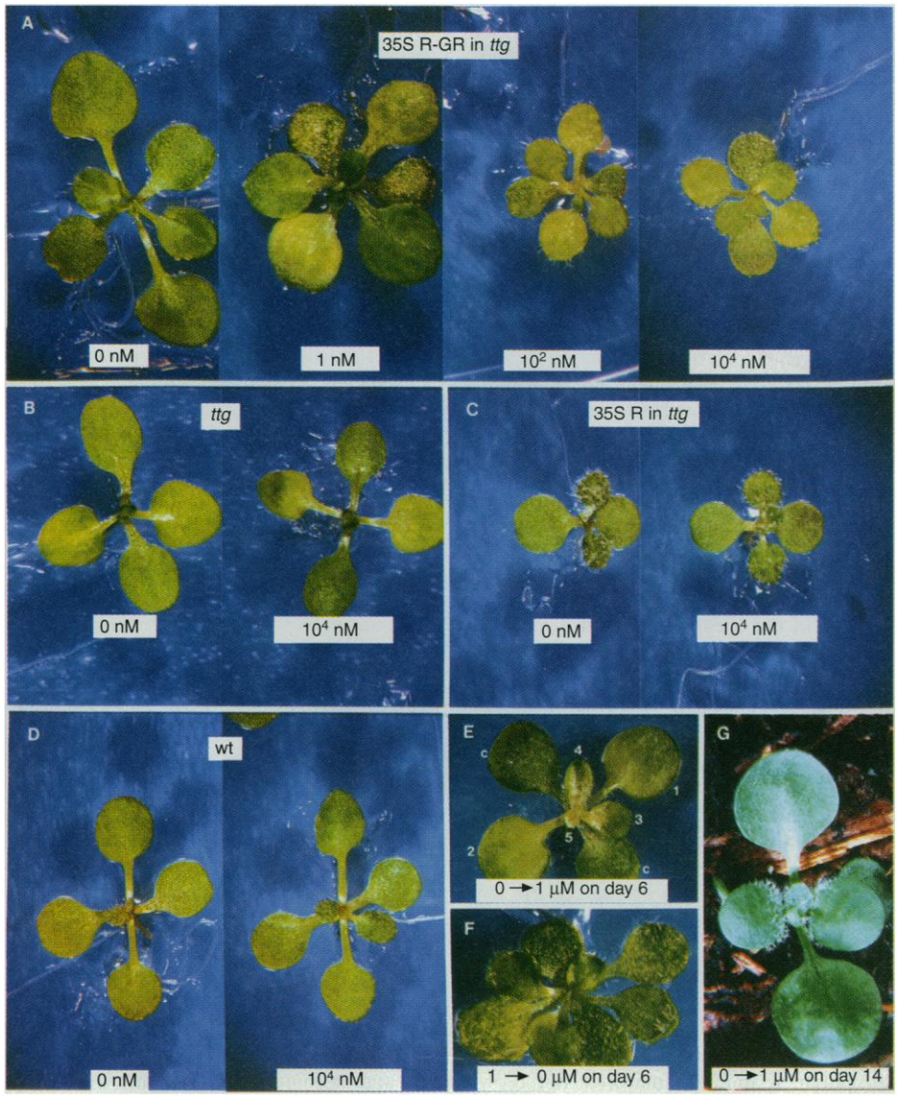

Fig. 3. Trichome induction in *Arabidopsis* plants. (**A**) The *ttg* mutation transformed with p35S R-GR grown on the Dex concentrations shown. (**B**) The *ttg* mutation transformed with pKYLX71. (**C**) The *ttg* mutation transformed with p35SR. (**D**) Wild-type RLD transformed with pKYLX71. (**E** to **G**) The *ttg* mutation transformed with p35SR-GR. (E) Seedling moved from 0 to 1 μM Dex 6 days after plating. Abbreviations follow: c, cotyledons; 1 to 5, first to fifth true leaves to develop. (F) Seedling moved from 1 to 0 μM Dex 6 days after plating. (G) Soil-grown seedling immersed in 1 μM Dex 14 days after planting.

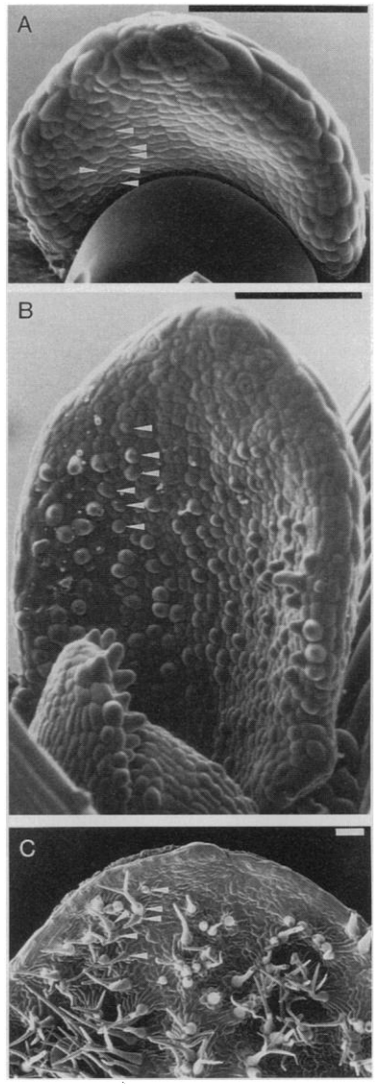

Fig. 4. Sequential SEM images of the same *ttg* 35SR-GR leaf during Dex-induced trichome initiation. Non-destructive molds and casts were made with dental epoxy and resin (*19*). (**A**) Leaf impression just before Dex treatment. (**B**) Leaf impression 24 hours after Dex treatment. (**C**) Leaf impression 9 days after Dex treatment. Seedlings were grown for 8 days in soil before treatment. Treatment was carried out by carefully lowering the pot and plant in 1 μM Dex until the entire plant was immersed. The seedling was left in this solution for approximately 2 hours and then removed, and the excess solution was allowed to drain. Similar water-treated seedlings showed no trichome induction. The bar represents 0.1 mm in each panel; arrowheads indicate the same six representative cells in each panel.

concentrations of active R in the plant. This conclusion is consistent with a model proposing that trichome initiation is governed by transcription factors such as R or TTG plus GL1, a myb homolog required for trichome initiation along with TTG (*23*), and that a trichome can exert a local inhibition that prevents later developing cells nearby from producing trichomes. When this inhibition is attenuated a few cells away, another trichome is initiated, which in turn produces a local inhibition. The data presented here are consistent with a model in which the cell fate decision to be or not to be a trichome hinges on a competition between positive regulators, such as R or TTG and GL1, and a local inhibition produced by a committed trichome cell. Alternatively, the local inhibition may work before the activation of R or TTG in the trichome determination pathway, inhibition that is bypassed by the ectopic expression of R.

Factors in addition to the expression patterns of GL1 or TTG dictate the regular pattern of trichome formation on the wild-type leaf surface. Hybridization in situ indicates that GL1 is expressed uniformly in the epidermis of leaf primordia before trichome initiation (*24*). Genetic mosaic analysis with GL1 indicates that it acts locally (over no more than a few cell diameters) rather than over a long distance and that it may be strictly cell autonomous (*25*). The *ttg* gene is probably expressed in all leaf epidermal cells before trichome initiation. This expression is suggested by the observation that anthocyanin production, which also requires TTG, can be seen in developing leaves before trichome initials are observed (*17*). In one described trichome spacing mutant, *Try* (*25*), trichomes occur in clusters. This mutant may be defective in the production of a repressor or in the signal transduction from the repressor to trichome initiation.

Although the full-length GR can activate transcription from a GR-responsive, element-containing promoter in tobacco protoplasts (*8*), no activation was seen in stably transformed *Arabidopsis* plants. The R-GR fusion described here may serve to stabilize the ligand binding domain of the GR. Fusion of the steroid binding domain of the GR to other developmental regulators, particularly transcription factors, can be a valuable research tool in plants and other multicellular organisms in which transformation can be used to probe developmental timing and the nature of positional information. There are many other steroid receptors that function in a manner similar to that of GR (*26*), and these may prove equally valuable as specific conditional regulators of plant development or gene expression.

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28. We thank Dawn Frame for preparation of replica leaf molds and casts; C. Schardl, A. Hunt, P. Godowski, S. Wessler, C. Koncz, and M. Koornneef for donations of plasmids and strains; and M. Campbell, K. Marrs, A. B. Lloyd, and P. Green for critical reading of the manuscript. Supported by National Institutes of Health grants GM 32422 (V.W.) and R37-H600198 (R.W.D.) and National Science Foundation grant DMB-910601 (R.W.D.).

13 June 1994; accepted 29 August 1994

Vancomycin Resistance: Structure of D-Alanine:D-Alanine Ligase at 2.3 Å Resolution

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The molecular structure of the D-alanine:D-alanine ligase of the *ddlB* gene of *Escherichia coli*, co-crystallized with an *S*,*R*-methylphosphinate and adenosine triphosphate, was determined by x-ray diffraction to a resolution of 2.3 angstroms. A catalytic mechanism for the ligation of two D-alanine substrates is proposed in which a helix dipole and a hydrogen-bonded triad of tyrosine, serine, and glutamic acid assist binding and deprotonation steps. From sequence comparison, it is proposed that a different triad exists in a recently discovered D-alanine:D-lactate ligase (VanA) present in vancomycin-resistant enterococci. A molecular mechanism for the altered specificity of VanA is suggested.

The bacterial cell wall and the enzymes that synthesize it are targets of many antibacterial agents, such as D-cycloserine, β-lactams (penicillins and cephalosporins), and glycopeptides (vancomycins). The construction of wall peptidoglycan requires a cross-linking of peptidyl moieties on adjacent glycan strands by transpeptidation between an amine group on one strand and the penultimate D-alanine of a D-Ala–D-Ala terminus on an adjacent strand. The transpeptidase or transpeptidases that catalyze this step are the target of β-lactam antibiotics (*1*, *2*). Vancomycin-type antibiotics, on the other hand, bind not to an enzyme but directly to the D-Ala–D-Ala terminus and inhibit cross-linking by the transpeptidase (*3*). In the face of β-lactamase production (*4*) and transpeptidase mutation (*1*, *2*), an attractive enzymic target for drug design is the D-alanine:D-alanine ligase (DD ligase) that produces one of the components of peptidoglycan (*5*).

Two isoforms of DD ligase from the *ddlA* and *ddlB* genes in *Escherichia coli* have been studied (*6*). The proposed mechanism for dipeptide formation begins with attack of

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