

Selection of Mutants in Complex, Undefined Biochemical Pathways: Application of Synthetic Lethal Selection to the Wound-induction Pathway in Plants.

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The preparation and characterization of mutants has been an invaluable tool in understanding complex cellular and biochemical pathways. The goal of our work is to make mutant plants that are blocked in biochemically undefined pathways. However, we do not want to screen for these mutants, but rather we have chosen to develop a molecular genetic system that would permit us to directly select for mutants in these inducible pathways. To accomplish this, we are applying to plants, methodologies that were pioneered in *Saccharomyces cerevisiae*. The development of such a system would permit the production of mutants in any complex biochemical pathway or signal transduction pathway for which a promoter expressing the desired phenotype were available.

This method of selection couples a powerful negative selectable marker with gene expression. When these plants are grown on fluorocytosine and the selectable marker (cytosine deaminase) is expressed, the native plant pyrimidine metabolic enzymes convert fluorocytosine into the toxic compound, FdUMP, which results in a thymine deficiency leading to cell death. Because plants naturally lack cytosine deaminase they grow normally in its presence. Therefore, expression of the cytosine deaminase results in a lethal phenotype.

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To construct a genetic system for the selection of mutant plants, we have outlined a series of discrete steps for the selection of these mutant plants.

Evaluate negative selection systems in plants

Fluoroorotic acid (FOA) selection

We have evaluated two methods of negative selection in plants. The first, using methods previously pioneered in, *S. cerevisiae*, was based upon fluoroorotic acid selection. FOA selection has been widely used in yeast research for the isolation and characterization of mutants (9, 21, 26, 27), in tobacco (40-43) and *Arabidopsis*. We spent several years evaluating this method of negative selection in plants. We have obtained and engineered negative selectable marker genes from *Dictyostelium discoideum* (94), tobacco (28) and *Arabidopsis* (32). We have demonstrated that FOA selection of mutants is feasible in both *N. tabacum* (40, 42) and *N. plumbaginifolia* (41, 43), and we have produced plants that do not express UMP synthase (40). However, these mutants are quite sick and do not lend themselves to the genetic manipulations required for the negative selection methods. Therefore, we have also investigated a gentler alternative method of selection.

Fluorocytosine (FC) Selection:

A less injurious method of selection also utilizes the pyrimidine biosynthetic pathway, but with fluorocytosine instead of fluoroorotic acid. Because plants lack cytosine deaminase, fluorocytosine is not toxic to wild type plants. We have grown plants on media containing 2 mg/ml of fluorocytosine without effect. Fluorocytosine is taken up into the cells by the pyrimidine permease transporter. Once inside the cell, if the enzyme cytosine deaminase (CDase) is present, fluorocytosine is converted into fluorouracil. Fluorouracil is eventually metabolized into 5-fluorodeoxy UMP (FdUMP) which is a suicide inhibitor of thymidylate synthase in both microbes (21) and plants (42). This results in a thymine starvation that kills cells (10, 30).

While most bacteria (15, 38, 39) and many fungi (18, 19) produce CDase, plants do not have CDase (13). Cells which do not express CDase are completely insensitive to fluorocytosine because fluorocytosine is not metabolized in cells which lack this enzyme. This link between expression of CDase and sensitivity to FC has been demonstrated for bacteria (7), yeast (18, 20), mammals (18, 25) and plants such as *Nicotiana tabacum* (44), *Arabidopsis thaliana* (36), *Nicotiana plumbaginifolia*, and *Lycopersicon esculentum*. Further, expression of CDase in cells of species lacking this enzyme results in a lethal sensitivity to FC. Initial demonstrations of

this were performed with mammalian cells (73), but these studies have now been extended into plant cells (44). Because these wild type plants are insensitive to fluorocytosine, they can provide the basis for a very strong negative selection system.

Identify and obtain functional gene fragments

The CDase gene from *E. coli* (44) was kindly supplied to us by Dr. Jens Stougaard. From this gene, we have prepared a cassette for the *codA* gene as well as promoterless plant transformation vectors (Zhou and Thornburg, unpublished)

Promoters to drive codA

We have chosen a pair of unrelated genes, the Nitrilase 1 gene (*Nit1*) from thale cress (*Arabidopsis thaliana*) and Proteinase Inhibitor II gene (*pin2*) from potato (*Solanum tuberosum*) to conduct this work. The Nitrilase I gene was chosen because it is constitutively expressed in plants (3). Constitutive expression should permit the easiest mode of selection using the system outlined below. The constitutive promoter was initially used to verify the negative selection. The Proteinase Inhibitor II gene was chosen because it is wound-inducible in plants (45) and has been the primary focus of the PI's research for many years (22, 23, 24, 33, 34, 45, 46, 47, 48, 53).

Constitutive expression (Nitrilase 1)

Nitrilase cDNAs (3, 4, 5) and genes (3) have been cloned from *Arabidopsis*. The cDNAs show strong homology to the bacterial nitrilase genes (4). Subcellular localization of *Nit1* indicates that *Nit1* is soluble and is expressed throughout development (5). Gene analysis indicates that there is a small gene family with 4 gene members, three of which (*Nit1*, *Nit2*, and *Nit3*) are clustered on chromosome III and *Nit4* resides alone on chromosome V (3). Northern analysis indicates that this gene family is differentially expressed: *Nit1* is constitutively expressed in the leaves with lower levels expressed in siliques, *Nit2* is expressed principally in siliques, *Nit3* is expressed predominantly in the roots, and *Nit4* is expressed in siliques, stems, and leaves (3).

The Nitrilase 1 gene from *Arabidopsis thaliana* was originally isolated by Dr. Bonnie Bartel (3). She very kindly provided us with the clones for Nitrilase 1 and Nitrilase 2. We have completely sequenced these genes [*Nit1*, (55); *Nit2*, (56)] and isolated the promoter fragments from each for use in these studies.

The *Nit1* gene precisely fits the type of expression that we desired for our constitutive

promoter candidate. At day 3 the promoter is expressed in the cotyledons, hypocotyl and root tips, at day 12 it is expressed in the leaves and root tips, and in mature plants it is expressed throughout the plant.

Wound Inducible Expression (Proteinase Inhibitor II)

The long term goals of my laboratory are to understand in molecular terms, how plants respond to insect attack. The plant response is extremely complex, and includes complex long range signaling pathways, signal transduction, hormones, nuclear factors, and probably other unknown activities. We have developed the potato proteinase inhibitor II (*pin2*) reporter gene system for use in characterizing this response (2, 22, 33, 34, 45, 46, 47, 48, 53). Through the work of many investigators, the *pin2* gene system is, arguably, the best characterized inducible gene system in plants. It is one of the few genes that can be induced at long ranges from the site of injury.

The Proteinase Inhibitor II gene was originally isolated from *Solanum tuberosum* by the PI (45). The promoter from this gene has been well studied (22, 23, 24, 33, 34, 45, 46, 47, 48, 53) and its expression has been examined monocots (53, 54), herbaceous dicots (22, 46) as well as woody dicots (23, 24). Further, this promoter has been used to express a number of genes in transgenic plants [chloramphenicol acetyltransferase, (45); -glucuronidase, (34); bacteriophage T7 RNA polymerase, (unpublished); UMP synthase, (submitted); and cytosine deaminase, this work].

The current hypothesis detailing the mechanism of induction of the *pin2* gene system is as follows. Systemin appears to be the long range signal molecule (35). It is thought to interact with an undefined receptor at the cell surface to trigger the activation cascade. Various short range signals [plant cell wall fragments (11, 12, 37) and fungal cell wall fragments (49)] may also activate the cascade by interacting with other receptors. None of these proposed receptors have been identified, nor has the signal transduction machinery been defined. But the current thought is that the signal received at the cell surface triggers release of 18:3 linolenic acid in the chloroplast (11). A native lipoxygenase which is either constitutively expressed (8) or induced (11) oxidizes the 18:3 linolenic acid into 18:2 oxo-PDA (50). The 18:2 oxo-PDA is then metabolized into jasmonic acid (51, 52). The conversion of 18:3 linolenic acid into 18:2 oxo-PDA is thought to be catalyzed by an enzyme similar to a prostaglandin synthase. Indeed, inhibitors of prostaglandin synthesis such as acetylsalicylic acid (14), and Ibuprofen (31) block the production of jasmonate. Once jasmonic acid has been produced, it induces *pin2* and other wound-inducible genes (8, 16), presumably through a jasmonate-binding protein/trans-acting

factor. Again, these jasmonate binding proteins have not yet been identified.

Even though this may be the best characterized inducible plant gene system, our detailed knowledge of this signal transduction pathway is lacking compared to what is known for many animal and yeast gene systems. To try to examine this black box in more detail, we are developing a long term molecular genetic approach to study this wound-inducible pathway *Arabidopsis*.

Construct reverse selectable genes with these promoters

We have utilized both the *Nit1* promoter and the *pin2* promoter to prepare chimeric gene constructions. Each of these constructs was first prepared in a pUC vector and then transferred into one of our own plant transformation vectors. The construct containing the *Nit1* promoter linked to the cytosine deaminase gene is pRT354. The construct containing the *pin2* promoter linked to the cytosine deaminase gene is pRT349.

The constructs were then characterized. For each construct, we routinely verify the presence of all DNA fragments by Southern blot analysis. First, all fragments are mapped to the appropriate location of the construct, then we sequenced through each of the gene fusions to confirm that the gene pieces were correctly positioned.

Transform the plants with the *codA* constructs.

Once we have confirmed the structure of the individual constructs, we then prepare transgenic plants. Both of these constructs, pRT354 and pRT349 were transformed into *Agrobacterium tumefaciens* GV3101pMP90 by electroporation. The *A. thaliana* cv Columbia plants were transformed by the vacuum infiltration method of Bechtold, et al., (6). In this procedure plants with multiple secondary bolts are vacuum infiltrated with *Agrobacterium tumefaciens* harboring the *codA* constructs.

After several weeks, when the siliques had developed and were very dry, seed was harvested, pooled, sterilized and plated on media containing 50 ug/ml Kanamycin. After vernalization and subsequent growth, a few plants (<0.05 to 0.1% of those plated) survived on kanamycin. We have produced 28 Tr354 plants transformed with *Nit1-codA* and 6 Tr349 plants transformed with *pin2-codA*.

Evaluate Expression of the *codA* constructs in plants.

We have self-pollinated these plants to produce a sufficient quantity of plant material and seeds to analyze the expression of the transgene *in planta*. Both the Tr354 plants and the

Tr349 plants are currently in the F7 generation. We have evaluated the expression of each of the transgenes in the transgenic *Arabidopsis*.

Initially, we utilized enzyme assays to verify the presence of the transgenes in our transgenic plants. We assay cytosine deaminase by the method of Andersen et al. (1). This is a thin layer chromatographic method that utilizes a separation of radiolabeled cytosine and uracil.

The *Nit1-codA* plants show good levels of expression of cytosine deaminase. We have tested all 28 plants transformed with the *Nit1-codA* construct and every plant shows a similar constitutive, hi level of cytosine deaminase expression.

For the *pin2-codA* plants, the cytosine deaminase was expressed in both tobacco and *Arabidopsis* in a wound-inducible manner. We also demonstrated that the expression of *pin2-codA* in *Arabidopsis* is similar to the expression of the wild-type gene in solanaceous plants. We observed induction by wounding, methyl jasmonate, sucrose, and high levels of ABA.

Demonstrate fluorocytosine selection

Our initial objective was to demonstrate the efficacy of this fluorocytosine selection. Because selection is caused by the conversion of fluorocytosine into fluorouracil, we initially demonstrated that fluorocytosine was not toxic to wild-type seedlings while fluorouracil was toxic. When wild-type seedlings were plated on media containing fluorocytosine, there was no effect on plant survival, even at very high concentrations of fluorocytosine. In contrast, fluorouracil, was very toxic to seedlings.

Next, we wanted to demonstrate that expression of *codA* in transgenic plants resulted in a lethal phenotype when plated on fluorocytosine. The *Nit1-codA* construct is constitutively expressed in Tr354 seedlings. Therefore, we plated seed from three separate Tr354 plants on media containing varying levels of fluorocytosine. Each of these plants failed to grow on media containing fluorocytosine at concentrations above 500 ug/ml. Again the wild type, untransformed *Arabidopsis* seedlings were unaffected by the presence of fluorocytosine. From these results we concluded that expression of the *codA* gene results in a lethal phenotype in the presence of fluorocytosine.

Selection of mutants in undefined pathways

Plants were made homozygous for the transgene by screening subsequent selfed T₂ and T₃ generations for kanamycin resistance. The T₂ seed were grown and scored for sensitivity to kanamycin. Those families that showed 75% survival on kanamycin were interpreted as being hemizygous for the transgene. Those families that show 100% survival on kanamycin were

interpreted as being homozygous for the transgene. Each of the six initial transformants were made homozygous by this procedure. Subsequently, the number of copies of each construct were determined by southern blot analysis. Once made homozygous, the plants were grown in bulk to supply a large quantity of seed for the remainder of these studies.

Because natural mutations occur with such low frequencies, we decided to mutagenize the seeds before selection to have a reasonable chance to obtain our desired mutants. Mutagenesis of seeds was conducted according to the methods of Guzmán and Ecker (17). We mutagenized the seed at a rate that gave 37% survival of the M₁ seeds, because this rate produces the optimum yield of mutations (29). Because the plants arising from the M₁ seed are chimeras of genetically different lineages, we self-pollinated these plants and limited our screening to the M₂ generation.

For the production of pooled M₂ seed, the M₁ mutagenized seed will be planted in flats with about 1,000 plants per flat in growth chambers. The initial bolts were removed from these plants to produce multiple secondary bolts, thereby increasing seed yield. Upon maturity, the seed were harvested for the selection studies.

Approximately 75,000 to 100,000 M₂ mutagenized seed from the transgenic plants were surface sterilized, plated on Murashige-Skoog selection media, vernalized for two days, and incubated at 23°C with 16h days and 8h nights (17). After two weeks, approximately 30 plants survived the selection. These plants were grown to maturity and seed harvested from each plant. The progeny of these plants were regrown on selection media to provide a second round of selection. Four mutant plants survived this second round of selection. These plants were self-pollinated to provide sufficient levels of seed for future biochemical and genetic studies. Currently, these plants are in the T7 generation and the phenotype remains stable.

Characterization of the mutants

Progeny of those plants that were selected on fluorocytosine and that survived a second round of selection on fluorocytosine will be analyzed both biochemically and genetically. Initially, we analyzed the selected plants for their ability to express the *pin2-codA* transgene.

There are three potential classes of mutants that will result in survival on fluorocytosine: 1) those mutants that affect general pyrimidine metabolism but are unrelated to the *pin2-codA* transgene, 2) those *cis* mutants that alter expression of the *pin2-codA* gene by inactivation of that specific gene, and 3) those *trans* mutants that alter expression of the *pin2-codA* gene by inactivation of a gene other than the *pin2-codA* gene.

Because the transgenic plants contain multiple copies of the *pin2-codA* construct, the likelihood that multiple mutations occur in each of the multiple gene copies is so low that the class 2 (*cis*) mutants can be disregarded. Therefore, the mutants produced from this screen should either be class 1 or class 3 mutants.

These two classes can be readily discerned. Class 1 mutants will show normal, wound-induction of cytosine deaminase (the same as the initial transformants). On the contrary, class 3 mutants will show reduced levels of cytosine deaminase. We have examined each of the four mutant plants. In every case, the cytosine deaminase is completely non-inducible, thus each of these mutants are class 3, *trans*-acting mutants that affect expression of the *pin2-codA* transgene.

Further, we have examined each of these four mutants for expression of the *pin2-codA* transgene in response to a variety of induction protocols including mechanical wounding, methyl jasmonate induction, 3% sucrose induction, and induction by high levels of ABA. While each of these methods induces the *pin2-codA* construct in the parental transgenic lines, none of the four isolated mutants can be induced by any of these methods. Thus, each of these mutants has lost the wound-inducible phenotype as well as induction by biochemical inducers.

We are currently preparing genetic crosses with each of the mutants for genetic studies. The goal of these genetic analyses is three fold. First we will verify the dominant/recessive nature of the mutation. Second, we will place each of these mutations into allele complementation groups. This will provide us with a measure of the complexity of the system of genes regulation of wound-inducibility in plants. Third, we will determine the chromosomal location of the mutation. This will set the stage for map based cloning of these mutations.

Summary

The Nitrilase 1 studies were included to insure that the selection would work. Nitrilase 1 is expressed constitutively in young seedlings for at least two weeks after germination (3). This was ideal for these selection studies and verified that the selection scheme would indeed work.

The proteinase inhibitor II studies were designed to determine whether we can use this negative selection system to produce mutants in a more complex undefined biochemical pathway, that of wound-induction of a plant gene. We anticipated that a wide variety of mutants would be obtained by this negative selection scheme. We know that hormones, cell wall components, lipid derivatives, and signal polypeptides are all involved in the induction of the *pin2* gene system. We further anticipated that there were additional factors which could be involved in this

process, including: receptors, signal transduction molecules, *trans*-acting factors and perhaps molecules of other unknown types. We expect to find a large number of different phenotypic classes for the mutants which we isolate from our screens. We would especially be interested in obtaining mutants that are blocked in any of the known steps in wound-induction, as these would permit us to directly test the validity of the current hypothesis of wound-induction. Thus, the mutants that we produce in these studies should encompass all stages of gene expression from receptor interactions, to signal transduction, to transcription and to post-translational modifications. T

Further, based upon mutant analysis of simple systems, such as nitrate assimilation in *Aspergillus nidulans* in which at least 15 complementation groups exist for blockage of nitrate assimilation, only two of which are the structural genes (12) it is clear that a large number of regulatory mutations can be obtained. Initially, our desire is to gain some understanding of the number of different phenotypic classes of mutants which we will identify with our screens. Complementation tests will provide the simplest way of establishing whether mutants with similar phenotypes have arisen by mutation in the same or different genes.

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