Published in final edited form as:

J Biotechnol. 2016 November 20; 238: 9–14. doi:10.1016/j.jbiotec.2016.09.007.

Target-directed discovery and production of pharmaceuticals in transgenic mutant plant cells

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Abstract

Plants are a source of complex bioactive compounds, with value as pharmaceuticals, or leads for synthetic modification. Many of these secondary metabolites have evolved as defenses against competing organisms, and their pharmaceutical value is “accidental”, resulting from homology between target proteins in these competitors, and human molecular therapeutic targets. Here we show that it is possible to use mutation and selection of plant cells to re-direct their “evolution” toward metabolites that interact with the therapeutic target proteins themselves. This is achieved by expressing the human target protein in plant cells, and selecting mutants for survival based on the interaction of their metabolome with this target. This report describes the successful evolution of hairy root cultures of a *Lobelia* species toward increased biosynthesis of metabolites that inhibit the human dopamine transporter protein. Many of the resulting selected mutants are overproducing the active metabolite found in the wild-type plant, but others overproduce active metabolites that are not readily detectable in non-mutants. This technology can access the whole genomic capability of a plant species to biosynthesize metabolites with a specific target. It has potential value as a novel platform for plant drug discovery and production, or as a means of optimizing the therapeutic value of medicinal plant extracts.

Keywords

Lobelia cardinalis; human dopamine transporter protein (hDAT); activation tagging mutagenesis (ATM); hairy root cultures; 1,2,3,6-tetrahydropyridine (MPTP)

1. Introduction

The pharmaceutical industry is increasingly abandoning plants as a source of drugs (see Littleton 2007). One reason is that many bioactive plant metabolites are too complex for convenient chemical synthesis, making them difficult to optimize for interaction with the molecular target. Another, is that production of such compounds requires purification from

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the variable and miniscule amounts in intact plants. A solution to the latter (pioneered by Phyton for taxanes) is to use cultured plant cells as production systems (Howat et al 2014). Here we show that mutant plant cells in culture can be used both as optimized production systems, and as sources of metabolites with optimized activity at the target. The concept is simple. First a plant species is identified that contains complex metabolites with activity at a molecular target protein. Then transgenic plant cells are created that stably express this target protein. Mutants of these transgenic cells are then selected under conditions that favor survival of individuals containing metabolites with the desired interaction with the target. The surviving sub-population is enriched in mutants containing metabolites with the appropriate pharmacological activity. These may be metabolites that are present in the wild-type plant or, if a mutation alters a relevant biosynthetic pathway, then “novel” active metabolites with enhanced activity at the target may be generated (Rogers et al 2003). Target proteins have previously been expressed in plant cells as screens (Littleton 2007, Doukhanina et al 2007, Zhao et al 2012, Gunjan et al 2013), but this is the first report in which survival of mutant plant cells expressing a foreign target protein has been used to direct secondary metabolism toward a specific pharmacological phenotype. Proof of concept uses *Lobelia cardinalis*, which contains lobinaline, a novel inhibitor of the human dopamine transporter (DAT) (Littleton et al 2004, Brown et al 2016). Metabolites with this activity have evolved in plants to inhibit similar transporter proteins in the brains of herbivorous insects to deter feeding (Chen et al 2006)). This homology makes them valuable as lead compounds in degeneration of dopaminergic neurons in the human brain (Rudnick et al 2014, Segura-Aguilar & Kostrzewa 2015). However, lobinaline, is a complex binitrogenous decahydroquinoline alkaloid (Manske 1938, Robison & Pierson 1964) with 5 chiral centers. Chemical synthesis is difficult, and lobinaline is present in low yields in the wild type plant. Lobinaline is therefore a poor candidate as a conventional lead for DAT inhibitors, but an excellent example for target-directed evolution of biosynthesis (see Figure 4).

2. Materials and methods

2.1 Expression of the functional target protein

First, full-length cDNA of the human dopamine transporter (*hDAT*) was introduced into hairy root cultures of *L. cardinalis* by *Agrobacterium rhizogenes*-mediated transformation (Tsugawa et al 2004). The *hDAT* gene was present by PCR and its expression was demonstrated by gel-based electrophoresis. However for this selection procedure it is essential that the expressed protein should be functional, and retain the pharmacology of the native protein in mammalian systems. Therefore, the hDAT protein was shown to be expressed by specific binding of the DAT radioligand [³H]GBR12935 as for mammalian membranes (Reith & Selmeci 1992) (Figure 1). The transgenic *hDAT* plant cells showed significantly increased uptake of [³H]Dopamine (Williams & Steketee 2004), which was prevented by the DAT inhibitor GBR12909 (Rothman et al 2008) (Figure 2). Expression of the unmodified cDNA for the hDAT therefore appears to produce transgenic plant cells in which the functional hDAT is present in the plasma membrane”. In addition, neurotoxins that are concentrated intracellularly by activity of the DAT (Segura-Aguilar & Kostrzewa 2015) were more cytotoxic to transgenic *hDAT* plant cells than controls, and these were rescued by GBR12909 (see data for 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine, MPTP, in

Figure 3). Exposure of transgenic *hDAT* plant cells to any of these neurotoxins should therefore favor survival of mutants overproducing endogenous inhibitors of the DAT (see Figure 4). 100uM MPP+, the ionic active moiety of MPTP, is rapidly lethal to non-mutant transgenic *hDAT* hairy roots and was chosen as the selection pressure.

2.2 Mutation, selection and analysis

Activation tagging mutagenesis (ATM) by *A. rhizogenes* (Fritze & Walden 1995) was used to insert viral “enhancer sequences” randomly into the genome of cultures derived from the stable transgenic *hDAT* line. This should cause stable activation of one or two plant genes at random in each mutant “secondary” hairy root derived from each transgenic (*hDAT*) “primary” hairy root. In normal medium, a single primary hairy root gives rise to ~20 secondary hairy roots, but when transferred to 100uM MPP+ only ~1/300 transgenic secondary hairy roots develop, whereas all primary transgenic *hDAT* hairy roots die (see Figure 5). Toxin-resistant hairy roots were maintained in MPP+ for four months to ensure that the resistant phenotype was stable. The surviving cultures were then transferred to medium containing no MPP+ for two months to ensure that (a) the resulting pharmacological phenotype was stable (b) no MPP+ remained to confound pharmacological analysis (confirmed by GC/MS, see below). All toxin-resistant individuals (109 hairy roots) were sub-cultured, and extracts were analyzed for DAT inhibition (Williams & Steketee 2004) and lobinaline content in comparison with non-selected transgenic secondary hairy roots, and wild-type and transgenic *hDAT* primary hairy roots.

3. Results and Discussion

3.1 Pharmacological analysis

DAT inhibitory activity did not differ between the wild-type and transgenic *hDAT* primary hairy root cultures (Figure 6). Extracts from the non-selected secondary hairy roots showed a non-significant increase compared to the control populations, but extracts from the MPP+-resistant mutant population showed a markedly increased mean, and a much greater frequency of individuals with high levels of DAT inhibitory activity than any other population (Figure 6). Indeed 11 MPP+-resistant cultures showed such a high level of activity that they could not be accommodated on this scale (see Figure 6 legend). Overall, 58/109 individual toxin-resistant transgenic mutant cultures contained activity more than 3 SDs above the wild-type control mean. These changes probably result from stable mutations because none of the several thousand hairy roots that were not subjected to mutagenesis ever survived MPP+. Also, the DAT inhibitory phenotype persisted after 2 months in normal medium, when an elicited response should have subsided (Wang & Wu 2016). Silencing of the *hDAT* transgene is a possible alternative mechanism for MPP+-resistance, but the *hDAT* has remained functional in the transgenic line used for selection since this was generated 4 years ago. Thus, a single round of mutation and selection has driven the pharmacological phenotype of this species toward increased activity at the foreign target protein.

3.2 Chemical analysis for lobinaline

GC/MS showed that the expression of the *hDAT* in primary hairy roots did not significantly affect the mean lobinaline content, or its distribution (Figure 7). The non-selected secondary

hairy roots showed a small but significant increase in mean lobinaline content, as did the MPP+-resistant population. Although these two mutant populations did not differ in their means, the MPP+-resistant population showed a higher proportion of “lobinaline overproducers” (see legend to Figure 3) up to a maximum of ~20× the wild-type control mean in one mutant. These mutants continue to overproduce lobinaline in liquid culture, suggesting that they may be of value as production systems. However, only 16 mutants in this population contained sufficient lobinaline to explain the DAT inhibitory activity observed in the extract from the same culture. In addition, comparison of DAT inhibitory activity (Figure 2) with lobinaline content (Figure 3) shows a completely different distribution, and regression analysis shows no correlation between these measures. The MPP+-resistant population must therefore contain mutants in which increased DAT inhibition is caused by metabolites other than lobinaline.

3.3 Chemical analysis for other protective metabolites

GC/MS identified 17 mutants with significantly increased concentrations of unsaturated fatty acids, which inhibit the DAT (Appell et al 2003). However, 25 mutants showed no increase in known DAT inhibitors, but contained “novel” GC peaks that are not observed in controls. Some probably represent lobinaline derivatives because they have similar retention times and molecular weights. Others contain complex alkaloids, with similarity to known synthetic DAT inhibitors (e.g. Kunstmann et al 1984), but the MS match is only partial. Methanol extracts from all these cultures were separated by preparative HPLC (Kesting et al 2009). Nine HPLC peaks were present that were not readily detectable in controls, and these were collected and assayed for DAT inhibition. All showed significant concentration-dependent inhibitory activity. This suggests the biosynthesis of novel metabolites with the desired effect on the foreign target protein. GC/MS analysis of the 51 resistant cultures that do not show increased DAT inhibitory activity indicates that many are over-producing metabolites that inhibit MPP+-induced oxidative damage to mitochondria (Cassarino et al 1999). These include squalene (Kabuto et al 2013) and 2,4-Di-tert-butylpheno (Choi et al 2013). Many of these cultures also have novel GC/MS peaks, which may represent novel neuroprotective metabolites (Xu & Moller 2011)

4. Conclusion

In these proof-of-concept studies we show that a single round of mutation and selection can rapidly “evolve” plant cells toward a specific pharmacological phenotype based on interactions with a foreign target protein. The same technology could be applied to agrochemical and nutritional targets, with major implications for agriculture, but our focus is on the pharmaceutical industry. Here the technology could be developed for plant drug discovery and production, as well as to optimize biosynthesis in medicinal plants to enhance therapeutic activity in traditional medicines. It should be possible to protect intellectual property in all of these products because they are derived from unique genetic mutations. If we can obtain proof of application, the technology could make plants once again a major global source of medicines, a position they have occupied throughout most of human history.

Acknowledgments

This research was supported by SBIR R44AA018226 “Selection-driven plant metabolites for treatment of CNS diseases” awarded under the Roadmap initiative “Novel tools for brain and behavior” to Naprogenix Inc (PI JML) from the National Institute for Alcohol Abuse and Alcoholism. The authors greatly acknowledge the technical expertise provided by Mr. Jatinder Sami, and Ms Grace Burgess-Poole. Conflict of interest: DTR and JML are employees of Naprogenix Inc and JML also owns stock in the company.

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Highlights

- We discovered a novel inhibitor of dopamine transport in *Lobelia cardinalis*
- We functionally expressed the Human dopamine transporter in *L. cardinalis* plant cells
- Next a Gain-of-function mutant transgenic population was generated from these cells, in presence of a neurotoxin accumulated by the transporter.
- The resulting toxin-resistant population was highly enriched in mutants with increased inhibitory activity on the transporter
- Some toxin-resistant mutants overproduce the known active metabolite from *wild-type*.
- Many others contain other active metabolites including some not readily detectable in the wild-type plant

Figure 1. Binding of [³H]GBR12935 to membranes derived from hairy root cultures of *Lobelia cardinalis*

The upper curve shows saturable specific, one-site binding of [³H]GBR12935, a selective radioligand for the DAT, to membranes from transgenic *hDAT* hairy roots. Binding parameters were K_d 7.33 nM and B_{max} = 1.04±0.9 pmol/mg protein (n=4). Non transgenic hairy roots (lower curve) showed no specific binding. Each data point represents mean ± standard error of the mean (SEM) of 4 replicates and the curve represents best fit to a one-site binding model using Graphpad nonlinear regression software.

Figure 2. Uptake of [³H]Dopamine into hairy root cultures of *L. cardinalis*

The first two bars compare [³H]Dopamine uptake into wildtype hairy roots (blue bars) with that into transgenic hairy roots (red bars). Cultures were incubated with [³H]Dopamine for 30min at 37°C, non-specific uptake was determined at 0°C. For each replicate, the radioactivity taken up by 10 roots was pooled and specific uptake calculated by subtracting non-specific uptake from total uptake values. Each bar represents four averaged replicates and data is expressed as mean±SEM DPM/mg tissue. The transgenic *hDAT* hairy roots showed significantly greater uptake of [³H]Dopamine ($5.88 \pm 0.54^{^^}$, One-way ANOVA, Bonferroni post hoc, $P < 0.001$) than wildtype (2.79 ± 0.31). Pretreatment with the selective inhibitor of the DAT, GBR12909 (100μM, +++, One-way ANOVA, Bonferroni post hoc, $P < 0.001$), prevented the increased uptake of [³H]Dopamine into transgenic *hDAT* hairy roots, as did substitution of Na⁺-containing buffer with Na⁺-free buffer (***, One-way ANOVA, Bonferroni post hoc, $P < 0.001$) (the function of the hDAT requires the presence of Na⁺), relative to vehicle-treated controls expressing hDAT (blue bar, VEH).

Figure 3. Increased toxicity induced by dopaminergic neurotoxins in transgenic (*hDAT*) hairy roots of *L. cardinalis*

Toxicity in hairy roots was induced by addition of MPTP, MPP+ or 6-hydroxydopamine to the culture medium for 24h at concentrations that just failed to induce toxicity in wild-type hairy roots. Trypan blue staining was used to assess toxicity. Similar data were obtained for all these neurotoxins, in that toxicity was significantly increased in the transgenic hairy roots, relative to wild-type control. The data shown are means $\pm$ SEM of cell viability of *hDAT* transgenic hairy roots expressed as a percentage of wild type control, where $n=6$. 24h MPTP exposure caused toxicity in ~50% of cells in transgenic *hDAT* hairy roots (One way

ANOVA followed by Bonferroni post hoc, $p < 0.001^{***}$). This toxicity was reduced by the selective DAT inhibitor GBR12909 at 10 μ M concentrations ($p < 0.01$, ** relative to vehicle treated groups) and MPTP toxicity was reversed by 100 μ M GBR12909 ($p < 0.01^{++}$, relative to MPTP treatment without GBR12909 pretreatment). These data indicate that cytotoxicity is increased by activity of the DAT, and that overproduction of DAT inhibitors should “rescue” transgenic (*hDAT*) hairy roots. However, MPTP requires intracellular metabolism to the active moiety, MPP $^{+}$, so the cytotoxicity of MPP $^{+}$ is more directly related to transport by the DAT. MPP $^{+}$ (100 μ M) was therefore chosen for selection of mutant transgenic (*hDAT*) hairy roots.

Figure 4. The selection procedure for mutant transgenic *hDAT* hairy root cultures generated in **100 μ M MPP+**

Expression of the functional *hDAT* (transgenic protein-TPG) in hairy root cultures makes them susceptible to toxicity induced by MPP+, which is concentrated intracellularly by the *hDAT*. Therefore mutants (M) that overproduce inhibitors of the hDAT should have a survival advantage when exposed to MPP+. Although many mutants may survive by other mechanisms, the surviving population should therefore be “enriched” in individual mutants containing metabolites that inhibit the target protein.

Figure 5. Representative transgenic *hDAT* hairy root cultures after activation tagging mutagenesis (ATM) in medium containing 100 μ M MPP+

This photograph shows about 120 primary hairy roots that have died under selection in 100 μ M MPP+ with 2 mutant secondary hairy roots emerging from dead primary hairy roots, and developing normally (2nd and 3rd plates, upper row). In the absence of MPP+ about 2,000 secondary hairy roots would have developed from these primary hairy roots after ATM.

Figure 6. Frequency distribution of DAT inhibitory activity from individuals in different sub-populations of hairy root cultures of *L. cardinalis*

Methanolic extracts from individual cultures were dried and re-suspended in DMSO/buffer before assay for inhibition of [3 H]-Dopamine uptake into rat striatal synaptosomes. DAT inhibitory activity is expressed as $\log_{10}$ arbitrary units. For comparison with the data from Figure 3 these values were corrected to “lobinaline equivalents” based on lobinaline concentrations measured in methanol extracts of wild-type cultures. The means $\pm$ standard error of the mean (SEM) of each population (68–100 hairy roots in each) were then compared using one-way ANOVA followed by Bonferroni’s post-hoc analysis. The wildtype control (blue squares, 40.6 ± 4.0 $\mu\text{g/ml}$), the transgenic *hDAT* cultures (red squares 32.8 ± 2.7 $\mu\text{g/ml}$) and the non-selected transgenic *hDAT* mutant populations (green triangles 48.2 ± 3.5 $\mu\text{g/ml}$) did not differ significantly from each other ($P > 0.05$). However, the MPP $^{+}$ -resistant transgenic *hDAT* mutants (yellow triangles 282.1 ± 52.5 $\mu\text{g/ml}$) showed significantly ($P < 0.0001$) greater DAT inhibition than the other populations. This data and analysis does not include 11 MPP $^{+}$ -resistant mutants that completely prevented [3 H]Dopamine uptake, and could not be accommodated on this scale.

Figure 7. Frequency distribution of lobinaline concentrations in extracts from individuals in different sub-populations of hairy root cultures of *L. cardinalis*

The Figure shows lobinaline concentration, determined by GC/MS in methanol extracts from individual cultures in the same populations shown in Figure 2. The same data presentation and statistical analysis was performed as for Figure 2. Means $\pm$ SEM for lobinaline concentration are wildtype control (blue squares); 41.3 ± 1.9 $\mu\text{g/ml}$; transgenic *hDAT* cultures (red squares) 38.3 ± 1.8 $\mu\text{g/ml}$; non-selected transgenic *hDAT* ATM mutants (green triangles) 56.5 ± 1.8 $\mu\text{g/ml}$; MPP+-resistant transgenic *hDAT* mutants 59.2 ± 2.5 $\mu\text{g/ml}$. One-way ANOVA followed by Bonferroni's post-hoc analysis showed that extracts from the two mutant populations had a significantly greater lobinaline content from the control populations ($P < 0.001$). The non-selected and MPP+-resistant mutants did not differ significantly from each other in means $\pm$ SEM, but the MPP+-resistant population included a higher proportion of individuals with a lobinaline concentration more than 3 SD above the control mean (37.9% vs 18.1%).