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Applications of Genetically-Encoded Biosensors for the Construction and Control of Biosynthetic Pathways

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Abstract

Cells are filled with biosensors, molecular systems that measure the state of the cell and respond by regulating host processes. In much the same way that an engineer would monitor a chemical reactor, the cell uses these sensors to monitor changing intracellular environments and produce consistent behavior despite the variable environment. While natural systems derive a clear benefit from pathway regulation, past research efforts in engineering cellular metabolism have focused on introducing new pathways and removing existing pathway regulation. Synthetic biology is a rapidly growing field that focuses on the development of new tools that support the design, construction, and optimization of biological systems. Recent advances have been made in the design of genetically-encoded biosensors and the application of this class of molecular tools for optimizing and regulating heterologous pathways. Biosensors to cellular metabolites can be taken directly from natural systems, engineered from natural sensors, or constructed entirely *in vitro*. When linked to reporters, such as antibiotic resistance markers, these metabolite sensors can be used to report on pathway productivity, allowing high-throughput screening for pathway optimization. Future directions will focus on the application of biosensors to introduce feedback control into metabolic pathways, providing dynamic control strategies to increase the efficient use of cellular resources and pathway reliability.

1. Introduction

Metabolic engineering broadly encompasses the engineering of biological systems that process chemicals and materials. Several recent examples highlight the exciting potential of engineered biosynthetic pathways in microbial hosts to provide renewable synthesis strategies for commodity chemicals and alternate sources for pharmaceuticals (Atsumi et al., 2008; Ma et al., 2009; Ro et al., 2006; Szczebara et al., 2003). However, current approaches require a significant investment of time and resources for each individual pathway, limiting the number of compounds to which these strategies can be applied and thus the scalability of

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biosynthetic approaches. Synthetic biology is a growing field that aims to make the engineering of biology faster and more predictable (Endy, 2005; Smolke and Silver, 2011). Core activities in the field have focused on the development of new tools and technologies that support the design, construction, and optimization of complex biological systems. As engineered microbial biosynthesis platforms have the most immediate practical applications in terms of development of industrial products, it is not surprising that many of the advances in tool development have been directed to metabolic pathway engineering. Here, we review recent work on genetically encoded sensors, an important class of tools for metabolic engineering design and optimization.

Genetically-encoded cellular sensors are valuable tools in the field of metabolic engineering. The ability of these genetic devices to sense and respond to changing levels of small molecules within a host cell enables researchers to monitor and optimize native and introduced metabolic pathways. For example, when engineering a yeast strain to produce a pharmaceutical drug or biofuel, protein- and RNA-based sensors may be used to inform the engineering process by detecting pathway improvements or identifying substrate limitations and bottlenecks in under-performing strains. Sensors may also be used to minimize cellular stress by balancing flux through the engineered pathway and by regulating just-in-time synthesis at individual pathway steps. Ultimately, sensors will provide for enhanced production of valuable metabolites through the construction of closed loop control systems with complexity equivalent to that found in native metabolic pathways.

In this review we define a sensor as a genetically-encoded RNA or protein with a minimum of two functional regions, an input component and an output component, that act to detect the presence of a small molecule and report its concentration (Fig. 1). Sensing is initiated when the target small molecule (e.g., substrate, product, or cofactor) interacts with the input component. In an RNA sensor this region is termed the aptamer and in a protein sensor it is the ligand-binding domain. The reversible binding of the small molecule to the input component is mediated by weak interactions between the nucleotides or amino acid residues within this region of the RNA or protein sensor, respectively, and results in a change in the conformation of the input component that is transmitted to the output component. The output component is a regulatory element that mediates processes such as mRNA or protein stability, the initiation of transcription or translation, or the post-translational control of protein activity. The overall result of the binding event at the input component and change in activity of the output component can take many forms, from the control of a reporter gene to regulation of an enzyme's activity in an engineered metabolic pathway.

2. RNA Sensors

2.1. Natural RNA sensing-regulatory elements

Natural biological systems can use RNA-based sensors to detect intracellular metabolite levels. Riboswitches are a class of RNA-based sensing-regulatory elements that couple an RNA sensing function, encoded in an aptamer, to a gene-regulatory function. Riboswitches that respond to core cellular metabolites and cofactors have been discovered in both prokaryotes and eukaryotes (Montange and Batey, 2008). The binding of the metabolite ligand at the aptamer domain directs a structural change in the riboswitch, which ultimately modulates the activity of the gene-regulatory domain. Natural biological systems are presumed to use riboswitches to link changes in metabolite concentrations to changes in the levels (and thus activities) of enzymes in related biosynthesis pathways (Mandal and Breaker, 2004). These natural RNA-based sensing systems have highlighted the sensitivity and specificity with which RNA can detect intracellular ligands and have fueled the development of engineered counterparts that can be applied to the detection of user-specified small molecules.

2.2. Engineered RNA sensing-regulatory devices

RNA aptamers are typically used in cellular sensors to translate molecular binding information into a measurable readout, such as fluorescence (Lynch and Gallivan, 2009; Weigand et al., 2008; Wieland et al., 2009; Win and Smolke, 2007), enzyme activity (Lynch et al., 2007; Topp and Gallivan, 2008), or cell viability (Dixon et al., 2010; Win and Smolke, 2007). As such, RNA-based cellular sensors couple RNA aptamers, both natural and synthetic, with RNA-based gene-regulatory components to construct sensing-regulatory devices. Researchers have developed diverse strategies for building such RNA devices that differ in the strategy taken to link the sensing and regulatory components and the mechanisms of action of the integrated gene-regulatory component (Win et al., 2009). The choice of linkage has direct implications for how readily the system can be tailored to detect new metabolites of interest, whereas the mode of action has implications for the cellular chassis in which the device can be implemented.

In one strategy, RNA devices are constructed by directly linking the RNA aptamer and gene-regulatory component, such that the regulatory effect is imparted by the conformational change upon ligand binding to the aptamer. As one example, a stem in the group I self-splicing intron from a thymidylate synthase gene, which is essential for cell viability, was replaced by the theophylline aptamer (Thompson et al., 2002). The device demonstrated theophylline-dependent growth in *Escherichia coli* when coupled to its native gene. RNA aptamers have also been directly linked to an RNA-based transcriptional activator, a 5′ splice site, and an RNase III hairpin to construct sensing-regulatory devices in *Saccharomyces cerevisiae* (Babiskin and Smolke, 2011; Buskirk et al., 2004a; Weigand and Suess, 2007).

In a second strategy, RNA devices are constructed by coupling the RNA aptamer to the gene-regulatory component through a linker sequence that participates in a conformational change in the molecule and affects the active state of the gene-regulatory component (Fig. 2A). In one example, the theophylline aptamer was linked to a ribosomal binding site (RBS) through a linker sequence to construct a device that functions in *E. coli* by modulating access of the RBS via strand displacement and ligand binding events associated with the aptamer component (Desai and Gallivan, 2004). Other similar RBS-based devices have been constructed by incorporating linkers that act through different mechanisms or using additional gene-regulatory components, such as a ribozyme, to regulate ribosomal access (Ogawa and Maeda, 2008; Suess et al., 2004; Wieland and Hartig, 2008). Because sequences in the aptamer and gene-regulatory domains typically interact with each other in device conformations built through this design strategy, tailoring of device function by the incorporation of new aptamer sequences generally requires a redesign of the entire device. Therefore, screening and selection strategies have been applied to identify functional linker sequences through colorimetric, flow cytometry, and cell motility assays to offer greater flexibility in tailoring device activity for specific applications (Lynch et al., 2007; Lynch and Gallivan, 2009; Topp and Gallivan, 2008). These screening and selection strategies have also been applied to optimize linker sequences between an aptamer and natural riboswitch element to generate devices that exhibit logic operations or to modify the information processing function encoded in a natural riboswitch (Nomura and Yokobayashi, 2007; Sharma et al., 2008).

As a third design strategy, functional composition frameworks have been proposed that allow for modularly assembling RNA devices from aptamer, linker, and gene-regulatory domains in *S. cerevisiae* (Win and Smolke, 2007). In one example, ribozyme-based devices were constructed by linking an aptamer to a ribozyme through a linker sequence designed to insulate the aptamer and gene-regulatory domains and standardize the communication interface between these domains (Fig. 2B). This design strategy allowed for the rational

design and tuning of encoded device functions by altering the sequence of the linker domain and the encoded strand displacement event. The modular composition framework of the ribozyme-based device further supported independent swapping of the sequence in the aptamer domain and thus tailoring of the encoded sensing function without complex device redesign. This RNA device platform was applied to the noninvasive detection of xanthine biosynthesis in a yeast host (Win and Smolke, 2007). In addition, this plug-and-play strategy allowed modular assembly of multiple aptamer, linker, and ribozyme domains to construct devices that exhibit higher-order information processing functions, including logic operations, signal filters, and programmed cooperativity (Win and Smolke, 2008).

2.3. Generation of new RNA sensing functions

A unique advantage of RNA-based sensing systems is the potential to tailor the sensing function to detect a diverse array of small molecules through the *de novo* generation of RNA aptamers (Ellington and Szostak, 1990; Tuerk and Gold, 1990). *In vitro* selection strategies have been applied to generate RNA aptamers to many metabolite ligands, including alkaloids, amino acids, and oligosaccharides (Hermann and Patel, 2000). Partitioning methods based on affinity chromatography are generally used with small molecule ligands, where the target small molecule is conjugated to a solid support through an appropriate functional group. The initial RNA library (~10^{14}–10^{15} molecules) is incubated with the immobilized ligand, and bound sequences are isolated, amplified through RT-PCR, and used as the input library for the next selection round. Iterative rounds of selection with increasing stringencies are applied to enrich the population for rare sequences with high affinity to the target ligand. By incorporating appropriate counterselection cycles, aptamers have been selected *in vitro* to discriminate between structurally similar metabolites. In one example, an aptamer selected to theophylline exhibits a 10,000 fold lower affinity for caffeine, which differs from theophylline by a single methyl group (Jenison et al., 1994).

One key challenge to the generation of RNA aptamers to detect new metabolite ligands is the limited scalability of current *in vitro* selection methods. In particular, current strategies require that the small molecule ligand have functional groups exhibiting chemistries that are appropriate for conjugation to solid supports. Often such functional groups are not readily available, necessitating the development of chemical modification strategies to append appropriate functional groups to each metabolite ligand. The need for coupling strategies have added disadvantages associated with (i) longer, more cumbersome, less efficient partitioning processes based on column chromatography; (ii) presentation of the ligand in an environment that does not closely mimic that of the cellular environment (i.e., ligand in solution versus ligand coupled to solid support); and (iii) removal of functional groups through chemical coupling that could play important roles in interacting with the RNA sequence to increase binding affinities. Improved solution-based partitioning methods, such as capillary electrophoresis, have been developed for the selection of aptamers to protein ligands (Berezovski et al., 2006). However, these methods are often based on the large size of the protein ligand and are not applicable to small molecule ligands. Therefore, the development of rapid, solution-based partitioning methods for small molecule ligands is critical to scaling aptamer selection to diverse metabolites. In addition, the application of RNA-based sensors in metabolic pathway engineering may require the generation of many RNA aptamers that can distinguish between structurally similar metabolites. As the early cycles in a selection focus on enriching the small number of functional sequences in highly diverse libraries, there may be opportunities to gain efficiencies in selection strategies through library design, where a smaller library can be generated for a family of metabolites based on randomization of fewer nucleotides in a parent aptamer sequence. In one example, an aptamer to arginine was generated in four rounds of selection starting from a library based on randomization of a parent citrulline aptamer (Famulok, 1994). In another example,

mutations were introduced into the aptamer domain of a natural adenine-sensing riboswitch and the resulting library was screened against 80 structurally similar analogues, allowing the identification of two new aptamers that respond to non-natural ligands (Dixon et al., 2010).

A second challenge in the generation of RNA aptamers to detect new metabolite ligands is that RNA aptamers selected *in vitro* may not function when moved *in vivo*. To address this challenge, researchers have developed approaches that combine *in vitro* selection and *in vivo* screening, by performing fewer rounds of *in vitro* selection on a large aptamer library and transforming the resulting enriched aptamer library into a cell host for a secondary screen (Sinha et al., 2010; Weigand et al., 2008). In one recent example, an enriched aptamer library to atrazine generated through *in vitro* selection was linked to an RBS through a randomized linker region to generate an RBS-based device library in *E. coli* (Sinha et al., 2010). The device library was coupled to the *cheZ* gene, which controls cell motility, and a cell-based motility assay was used to identify functional devices responsive to atrazine. By linking the sensing function encoded in an aptamer to a gene-regulatory function within the context of a device platform, the resultant device can be readily coupled to different target genes without significant device redesign.

3. Protein Sensors

Like RNA sensors, protein sensors couple the binding of a target small molecule at an input domain to a change in the level of activity of an output domain. However, while RNA sensors act to regulate the expression levels of functional proteins, the design of protein sensors also extends to the post-translational control of enzymes and their activity. This mechanistic scope is reflected in the three outputs commonly designed in protein sensors: transcriptional activation, enzymatic activity, and fluorescence.

3.1. Transcriptional activators as sensors

3.1.1. Transcription factor sensors—The allosteric regulation of transcription factors by small molecules is a sensing mechanism that is ubiquitous in nature and is readily adaptable for use in engineered cells. Sensing is achieved by coupling ligand-dependent activation of a transcription factor to any output that is accessible through transcriptional regulation under the control of the cognate promoter element (Fig. 3A). At present, the key limitation to this method of sensor design is the limited number of known transcription factors responsive to relevant small molecules, such as the precursors and intermediate metabolites of industrial chemicals, pharmaceuticals, and biofuels.

New sensing functions can be evolved in transcription factors identified from natural systems to expand the utility of transcription factor-based sensing in metabolic engineering applications. One example is LuxR is a quorum-sensing regulator from *Vibrio fischeri* that is activated by a specific acyl-homoserine lactone (acyl-HSL) ligand and induces transcription of a luminescence operon from the Lux promoter. New sensing functions have been engineered in LuxR by generating a library of LuxR variants and selecting mutants responsive to acyl-HSLs that are not naturally recognized by this regulatory protein (Collins et al., 2005). Activation of LuxR by new acyl-HSLs was detected in *E. coli* by driving expression of a GFP reporter gene from the Lux promoter element. While new functionality was successfully evolved, it was determined that the variants exhibited promiscuous ligand responsiveness to other related acyl-HSLs. Additional rounds of mutagenesis coupled to an antibiotic survival-based selection strategy were applied to re-specialize the promiscuous LuxR mutants to individual acyl-HSL ligands (Collins et al., 2006).

The evolution of LuxR demonstrates that laboratory evolution strategies are suitable for introducing new ligand specificities but can also increase the promiscuity of the resulting

mutant ligand binding pockets and are most effective at generating sensing functions to molecules that are structurally similar to the original ligand. Despite these challenges, transcription factor sensors have shown promise in metabolic engineering applications including the production and sensing of industrially- and environmentally-relevant aromatic hydrocarbons (Mohn et al., 2006; van Sint Fiet et al., 2006). In another example involving a precursor for the commercial production of isoprenoids in microbes, the arabinose-responsive transcription factor AraC was engineered to bind mevalonate (Tang and Cirino, 2011).

3.1.2. Yeast three-hybrid sensors—A second type of transcription factor sensor uses a ligand-dependent interaction between two proteins to produce a transcriptional output. This sensing strategy is based on the traditional yeast three-hybrid system, an extension of the two-hybrid assay to include small molecule-dependent protein-protein interactions (Fig. 3B). In the yeast three-hybrid system, the two domains of the Gal4 transcription factor, a DNA-binding domain and an activating domain, are fused to a bait protein and a library of prey proteins, respectively, such that in the presence of a given small molecule the protein-protein interaction between bait and prey reconstitutes the transcriptional activator and drives expression of a reporter gene. This system is readily extended to measure levels of a metabolite by replacing the bait and prey with two known proteins whose binding depends on the small molecule of interest.

Proof-of-concept for the three-hybrid sensor design was demonstrated for retinoic acid and a closely-related synthetic molecule using retinoid X receptor α (RXR), which binds retinoic acid, as “bait” with its transcriptional coactivator ACTR as “prey” (Schwimmer et al., 2004). While this system was principally developed for the screening of RXR variants that bind a synthetic retinoid-like compound, it could conceivably be used to measure concentrations of retinoic acid or the synthetic molecule. However, the major limitation of the yeast three-hybrid sensor design is that it can only be applied to the detection of small molecules for which bait-prey protein partners are available, primarily hormone receptors and other cell signaling components. Therefore, despite its obvious sensing capabilities, this class of sensors will have limited utility in metabolically engineered systems except in rare instances.

3.1.3. Chemical complementation sensors—Chemical complementation is a third type of transcription factor sensor that overcomes the requirement of the yeast three-hybrid system for ligand-dependent bait-prey interactions. Chemical complementation avoids this problem by synthesizing novel small molecules that fuse the ligands of two unrelated proteins. The presence of intact divalent ligand leads to dimerization and transcription (Fig. 3C). For example, a fusion of methotrexate (Mtx) and dexamethosome (Dex) can dimerize dihydrofolate reductase (DHFR, which binds Mtx) and the glucocorticoid receptor (GR, which binds Dex) (Lin et al., 2000). By fusing the DHFR and GR domains to appropriate transcriptional activator domains, the synthetic Mtx-Dex small molecule controls dimerization and thus transcriptional activation. This system can be used to sense enzymatic activity by including a small molecule enzyme substrate as a bridging molecule in the synthetic Mtx-Dex ligand. The amount of Mtx-substrate-Dex is therefore dependent on the activity of the enzyme on the bridging substrate, and the level of enzyme activity is reported by the amount of uncleaved Mtx-substrate-Dtx available to mediate chemical dimerization of the transcriptional activator and drive expression of a reporter gene. Chemical complementation was first demonstrated with cephalosporin and a β-lactamase enzyme that cleaves the β-lactam bond in this antibiotic to split the Mtx-substrate-Dex divalent ligand into two molecules (Baker et al., 2002; Sengupta et al., 2004). In addition to bond cleavage mechanisms, chemical complementation can be applied to the detection of bond formation by utilizing an enzyme that condenses two small molecule substrates and covalently linking

each substrate to Mtx or Dex. For example, a chemical complementation bond formation detection system was demonstrated for a Cel7B mutant with glycosynthase activity, which was capable of dimerizing Mtx and Dex linked to disaccharides (Lin et al., 2004).

These first applications demonstrate that chemical complementation enables sensing of metabolically active small molecules within a synthetic ligand. However, the ligand must be synthesized *in vitro* and supplied exogenously to the cell. Furthermore, the ligand must be able to cross the plasma membrane and nuclear envelope to provide the third component of a transcriptional activator complex. In systems where it can be applied, chemical complementation will be a valuable sensing tool in the screening of suitable enzyme activities.

3.2. Protein activity-based sensors

3.2.1. Combined domain sensors—The second class of protein sensors, protein activity-based sensors, act independently of transcriptional regulation by directly linking the activity of a screenable or selectable reporter to the binding of a small molecule. Nature contains many examples of sensing by allosteric regulation of protein activity. To be useful for metabolic engineering applications, sensors must bind a ligand relevant to the engineered pathway and transmit this event to a change in the activity of a protein useful for reporting, screening, or regulating other pathway components.

Sensors with desired input and output functions can be generated by combining two independent proteins, or protein domains, in such a way that binding of the small molecule ligand to the input component induces a conformational change that alters the enzymatic activity of the output component (Fig 3D). This strategy has been used to develop maltose sensors that report maltose binding as an increase in β-lactmase (BLA) activity (Guntas et al., 2005; Guntas et al., 2004; Guntas and Ostermeier, 2004). The maltose binding domain is provided by maltose binding protein (MBP), one of many bacterial periplasmic binding proteins (bPBPs) that bind nutrients, including sugars, ions, and peptides. PBPs have two domains joined by a hinge region, where ligand binding to the surface between these domains directs a hinge twist conformational change in the protein. The ligand binding activity of MBP and selectable activity of BLA were combined into chimeric MBP-BLA proteins by randomly or specifically inserting BLA into MBP. In these sensors, maltose binding to MBP induced a conformational change in the active sensors that allosterically regulated β-lactamase activity and led to increased cell survival on β-cyclodextrin, thus reporting on the level of maltose in *E. coli*.

A key observation gained from the construction of the MBP-BLA sensors has been the importance of the method by which the input and output domains are combined. In particular, circular permutation of BLA and insertion into MBP at randomly- or rationally-selected sites was used to identify permissive sites that fuse the two functional domains, retain domain function, and establish dependency of the activity of the input domain (MBP) on the activity of the output domain (BLA) (Edwards et al., 2008; Guntas et al., 2005; Guntas et al., 2004; Guntas and Ostermeier, 2004). The sensing capabilities of these sensors were developed further to allow the detection of new small molecules by mutating the ligand-binding pocket or changing the ligand-binding domain. For example, rational design strategies were applied to the MBP domain to develop an MBP-BLA sensor that detected sucrose, and a cytochrome b heme-binding domain was inserted within BLA to negatively regulate antibiotic resistance in the presence of heme (Edwards et al., 2008; Guntas et al., 2005). Given the range of available binding domains from bPBPs and other proteins, the capability to introduce mutations that provide for new ligand binding specificities, and methods for permissibly combining these input domains into potentially any output enzyme,

there are conceivably few limitations to the application of this class of sensors to the field of metabolic engineering.

3.2.2. Intein-based sensors—A second type of protein activity-based sensor uses inteins, which are sequences in a polypeptide that are excised following translation to generate an active mature protein. Small molecule-dependent intein splicing systems have been developed by inserting a ligand-binding domain within the N- and C-terminal regions of an intein and then inserting this split-intein sequence into a reporter protein (Fig. 3E). Binding of the small molecule can either promote or inhibit splicing by influencing the ability of the two intein domains to come together in a conformation that stimulates splicing. As such, the level of active spliced protein can be used as a readout of small molecule ligand concentration.

In one example, hormone analog-dependent splicing was engineered into the RecA intein from *Mycobacterium tuberculosis* by inserting a human estrogen receptor binding domain (ER) between the N- and C-termini (Buskirk et al., 2004b). Directed evolution of the intein-ER fusion was performed in yeast in the context of several reporter proteins to select for a variant with ligand-induced splicing. In contrast, reduced intein excision in the presence of the same hormone analogue was observed for an ER sensor derived from a modified RecA intein, demonstrating that inteins can be applied to both the activation and inhibition of proteins (Skretas and Wood, 2005). Although the only examples of engineered ligand-responsive inteins developed thus far are hormone-responsive and incorporate receptor binding domains, it is plausible that this same design principle could be used to incorporate binding domains for metabolites. Furthermore, once a metabolite-binding intein is developed it could potentially be inserted into any polypeptide to control processing to an active protein in response to the small molecule ligand. Thus, inteins can be used as small molecule sensors that act post-translationally to control the expression of pathway enzymes within a host cell.

3.3. Fluorescent sensors

The third class of protein sensors uses the spatial dependence of fluorescence resonance energy transfer (FRET) between a donor and acceptor fluorescent protein pair to detect a conformational change induced by binding of a small molecule. FRET occurs between two fluorescent proteins with different emission and excitation wavelengths such as cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). In this FRET pair, when CFP is excited with short wavelength radiation it emits light at a longer wavelength that is suitable to excite YFP, and the FRET interaction is detected as long wavelength emission from YFP. The two proteins must be within a few nanometers from each other for this energy transfer to occur. FRET is therefore ideal for sensor applications, as it allows a small conformational change caused by ligand binding to be detected as a change in the ratio of CFP and YFP fluorescence.

Small molecule FRET-based sensors are engineered by fusing a ligand-binding domain between the two fluorescent proteins in such a way that ligand binding induces a conformational change that alters the distance between the FRET pairs (Fig. 3F). Due to their mechanism of ligand binding, bPBPs are particularly well suited to the development of FRET sensors and a common design is to fuse the fluorescent protein pair to the bPBP with one to each termini. Type I bPBPs, such as glucose/galactose binding protein, undergo a hinge-twist conformational change upon ligand binding that pushes the termini further apart, thus lowering the FRET signal in response to small molecule binding events (Fehr et al., 2003). In contrast, ligand binding to type II bBPBs, of which MBP is a member, causes a different type of rearrangement in which the termini are bought closer together. Therefore,

when type II bPBPs are used in FRET sensors, small molecules are detected as increases in FRET (Fehr et al., 2002).

Intracellular FRET sensors based on the insertion of different bPBPs have been developed for a number of nutrients, including maltose, glucose, galactose, ribose, arabinose, and several amino acids (Chaudhuri et al., 2011; Fehr et al., 2002; Fehr et al., 2003; Kaper et al., 2008; Lager et al., 2003; Okada et al., 2009). These fluorescent sensors have been used to visualize and quantify small molecules in microbes, mammalian cells, and whole plants. Improvements in FRET sensor design have focused on modifying the activity of the ligand-binding component. For example, mutations are commonly introduced to lower the binding affinities to levels suitable for accurate quantification at physiologically-relevant metabolite levels (Fehr et al., 2002; Fehr et al., 2003; Kaper et al., 2008; Lager et al., 2003). The structure of the whole sensor protein has also been optimized to reduce the linker lengths between the fluorescent proteins and the bPBP to remove flexibility that might attenuate the transmission of a conformational change upon binding of the ligand (Deuschle et al., 2005; Kaper et al., 2008; Okada et al., 2009). In addition, modified sensor architectures have been examined to identify permissive internal binding sites as alternatives to fusing the fluorescent proteins and binding domain through the termini end-on-end (Deuschle et al., 2005). Although FRET sensors have so far only been applied to address biological questions in natural systems, their ability to report on metabolite concentrations through a CFP/YFP fluorescence ratio will make them a valuable diagnostic tool for metabolic systems.

3.4. Cell sensors

In addition to protein- and RNA-based sensors, whole cell sensors based on microbial auxotrophy have been used to report the concentration of growth-limiting small molecules. For example, a whole cell mevalonate sensor was generated by engineering mevalonate-dependent growth in *E. coli* (Pfleger et al., 2007). By knocking out the native non-mevalonate isoprenoid pathway and introducing a heterologous operon for the utilization of mevalonate together with an independent GFP reporter gene, the level of growth-supporting mevalonate in the media was monitored through cell density as reported by GFP fluorescence. Furthermore, a recently reported computational design strategy for generating auxotrophic *E. coli* mutants may expand the number of available cell sensors to as many as 53 small molecules (Tepper and Shlomi, 2011). As one of the goals of metabolic engineering is to produce new molecules from substrates supplied directly by the host cell metabolism, whole cell sensors will be valuable tools for optimizing this connection between the native primary metabolism of the host cell and the introduced heterologous pathways.

4. Sensors Linked to Reporters

RNA and protein sensors can be linked to reporters such as antibiotic resistance genes, colorimetric assayable enzymes and auxotrophic growth markers. For this application, the advantage of many of the sensors described above is that they are modular in terms of their input and output functions. For example, a transcription factor can readily link ligand binding to expression of a convenient reporter or a selectable marker. In other examples, such as allosteric control of enzyme activity, the linkage of sensor and reporter is difficult or impossible to modify and the choice of screen or selection is fixed by the sensor. The combination of sensor and reporter allows high throughput *in vivo* screening or selection for pathway construction and optimization. This optimization could happen at the level of a single step, e.g. through enzyme engineering, or for an entire pathway, such as balancing the expression level of several pathway enzymes.

Depending on the optimization strategy, the change in production may vary dramatically; some modifications might be expected to provide increases of 40%–80% (Leonard et al., 2010; Santos and Stephanopoulos, 2008) while others might produce increases of 5–10 fold (Lin et al., 2004; Pfleger et al., 2006). The combination of sensor and reporter must be able to distinguish changes in pathway production of the magnitude produced by the modifications. An optimization strategy that produces sequential small increases in activity requires a more precise sensor than one which produces large increases in activity. For thresholded sensors, including many antibiotic resistance markers, the range of pathway production must cross the threshold boundary to be detected. If the initial pathway output is below the threshold and all improved variants remain below the threshold, beneficial modifications may go unnoticed. Similarly, if the initial production is above the threshold, the sensor can only be used to remove variants that are less active than the starting point. A tunable threshold avoids this problem by allowing the threshold to gradually increase and select for continual improvement in output.

In one example, Desai and Gallivan used as their sensor an RNA device that responds to a small molecule, theophylline, and controls protein translation (Desai and Gallivan, 2004). When the RNA sensor was used to regulate expression of a chloramphenicol acetyl transferase reporter, growth under chloramphenicol selection was dependent on the presence of theophylline, and the chloramphenicol concentration was used to set the threshold by tuning the sensitivity of the system to theophylline. The selection strategy was applied to distinguish between active and inactive sensors at an initial frequency of 10^{-6}. After one round of selection with chloramphenicol and theophylline, 100% of the resulting colonies contained the active (theophylline-responsive) RNA device. This selection strategy could be applied to evolve an enzyme that produced theophylline from a substrate, such as caffeine, that itself did not activate the device. RNA devices offer great flexibility, as new aptamer binding domains can be generated *de novo* through a variety of methods and used to construct sensors to novel ligands. However, the dynamic range of RNA devices can limit the enrichment of the resulting screen. In this example, the sensor output increased roughly 5-fold upon addition of theophylline. Therefore, an optimization strategy that produces small changes in activity might not produce a sufficient response to effectively enrich for improved mutants using RNA-based sensors.

Natural transcription factors have also been adapted to serve as sensors, where the expression of a reporter from the associated promoter links sensor and reporter. In one example, a mutant version of a transcription factor that responds to benzoic acid was used to control *lacZ* and the tetracycline efflux pump *tetA* (van Sint Fiet et al., 2006). When the sensor was linked to *tetA*, growth under tetracycline selection was dependent on the presence of benzoic acid. Addition of a benzaldehyde dehydrogenase (XylC), which converts benzaldehyde to benzoic acid, allowed benzaldehyde-dependent growth of cells containing XylC. The selection strategy was tested against a mock library of wild type cells spiked with cells containing XylC at an initial frequency of 10^{-6}. After one round of selection with tetracycline and benzaldehyde, 70–90% of the resulting colonies contained XylC. Similarly, a mutant version of the XylR transcription factor that responds to 1,2,4-trichlorobenzene (TCB), a degradation product of the insecticide lindane, was used to control the expression of *lacZ* (Mohn et al., 2006). When the sensor was coexpressed with a lindane-degrading γ-HCH dehydrochlorinase, LacZ expression was induced by the addition of lindane. These transcription-factor based screens exhibit a large dynamic range, allowing high enrichments in a given round of screening. However, they are limited by their reliance on identifying or re-engineering natural transcription factors. While substrate specificity can be modified (Collins et al., 2006), such modifications are non-trivial and the range of acceptable substrates is limited.

Chemical complementation, a variation of the yeast three hybrid assay, has been used as a sensor platform for several different applications. In one example, a sensor was constructed using a cephem antibiotic, where cleavage of the antibiotic by a beta-lactamase would lead to a decrease in transcription from a promoter containing a LexA recognition site. This sensor was linked to *lacZ* expression and used to screen a mock library containing 5% active lactamase. After one round of screening, 80% of the resulting library had an active enzyme (Baker et al., 2002). In another example, the sensor was modified to detect the formation of a glycosidic bond. This sensor was linked to expression of *LEU2*, such that growth of a leucine auxotroph in the absence of leucine was dependent on bond formation and Leu2p expression. The glycosynthase that catalyzes the bond forming reaction was randomized at one residue, and the resulting library screened for growth in the absence of leucine. A mutant was identified that showed 5-fold increased activity relative to parent (Lin et al., 2004). As a final example, the same platform was used to detect the cleavage of a tetrasaccharide substrate by an endogluconase. The sensor was used to regulate the expression of *URA3*, where cleavage of the tetrasaccharide led to decreased Ura3p expression. Shuffling of three endogluconase domains and screening the resulting library of 10^8 mutants for growth on 5-fluoroorotic acid led to the identification of a mutant with a 5.7-fold improvement in catalytic rate relative to the best parental enzyme (Peralta-Yahya et al., 2008). These examples demonstrate that the chemical complementation system is modular. However, it is limited to reactions in which a substrate is cleaved or two substrates are covalently linked together. In addition, the enzyme must be able to tolerate extra groups attached to the substrate(s), and the methotrexate-substrate-dexamethosome compound must be synthesized for each new substrate. When these restrictions are met, the resulting screens and selections are quite powerful.

At the level of pathway optimization, a microbial biosensor (Pfleger et al., 2007) was used to optimize the relative expression of a series of genes in the early steps of the biosynthetic pathway for artemisinin (Pfleger et al., 2006). The biosensor strain was auxotrophic for mevalonate, an intermediate in artemisinin production. A library was constructed in which the genes for mevalonate production were flanked by a range of post-transcriptional control elements. Screening was performed by taking spent supernatant from cultures of the mevalonate-expressing strains and applying it to cultures of the biosensing strain. Higher growth of the biosensing strain indicated that the expression strain was producing more mevalonate. This screen resulted in the identification of several variants with a 7-fold increase in mevalonate production. However, the method is limited to compounds for which an auxotroph can be identified or constructed. As a result, the applications are generally restricted to optimizing the availability of precursors, such as mevalonate, rather than products such as artemisinin.

Sensors linked to reporters are just beginning to find applications in enzyme and pathway optimization, but their promise is clear. The first principle of directed evolution is ‘you get what you screen for.’ If the goal is to optimize activity *in vivo*, a screen performed under similar conditions *in vivo* will provide the most accurate enrichment for the desired activity. Direct measurement of metabolites, such as through liquid or gas chromatography, limit the throughput of the screen and therefore the size of the library that can be considered. High throughput screens, using biosensors linked to reporters, will allow the analysis of large libraries of enzyme or pathway variants under relevant conditions.

5. Sensors Linked to Actuators

In addition to linking sensors to reporters to optimize a pathway, they can be linked to actuators to dynamically regulate the pathway. In this context, an actuator refers to a molecule that affects the pathway being regulated. Regulating expression is perhaps the

simplest example of an actuation mechanism. Post-translational actuation mechanisms, such as allosteric enzymes (Guntas et al., 2005), inteins (Buskirk et al., 2004b), localization (Czlapinski et al., 2008), or degradation (Davis et al., 2009), could enable regulation of complex system behaviors on timescales faster than transcription and translation.

The combination of sensor and actuator forms a controller. Controllers may be divided into two broad categories, open loop and closed loop, based on the ligand being sensed. An open loop controller responds to a ligand that is distinct from the pathway being controlled; an inducible promoter may be considered an example of open loop control. Closed loop controllers measure the current status of the pathway and respond accordingly. For a closed loop controller, the details of the linkage between sensor and actuator define the control law for the circuit. In a simple negative feedback loop, the sensor might respond to the product of an enzymatic reaction and directly regulate the expression of the associated enzyme; the enzyme serves as the actuator, and the feedback is roughly proportional. Sensors linked to actuators require a great deal of design flexibility to ensure proper operation. In contrast to electronic controllers, the set point and control law of a metabolic controller are fixed by biochemical parameters of the component elements. If the metabolic engineer lacks sufficient tools to manipulate these parameters, the controller is unlikely to work reliably.

5.1. Open loop control

In a simple model, a production run may be divided into two portions: a growth phase, in which cells replicate and accumulate biomass, and a production phase, in which the cells synthesize the desired compound. Expression of the engineered pathway during the growth phase may be deleterious, if the pathway starves the cells of needed nutrients. As described before, inducible promoters may be considered examples of open loop control, where the addition of the inducer molecule during the production phase ensures expression of the engineered pathway only in the desired phase. However, the expense of inducers prevents their use in many metabolic engineering applications. Promoters that respond to native signals can avoid this limitation and effectively autoregulate pathway expression.

Oxygen limitation is a common indication that a culture has reached the end of its growth phase. Therefore, promoters responsive to oxygen levels would allow autonomous induction of a pathway once the growth phase finishes. This approach has been studied in *E. coli* (Khosla et al., 1990) and *S. cerevisiae* (Nevoigt et al., 2007). The *E. coli* system relies on a heterologous promoter from the obligate aerobe *Vitreoscilla* bacterium that can be transported directly into *E. coli* to induce gene expression in the absence of oxygen. In *S. cerevisiae*, researchers started with a native oxygen-responsive promoter, the *DAN1* promoter, and evolved it to respond to physiological levels of oxygen limitation.

Bacteria offer another strategy for density-dependent pathway expression through the use of quorum sensing systems. In one example, a computational model was used to study the application of quorum sensing to regulate a metabolic pathway (Anesiadis et al., 2008). In the model, a quorum sensing module measured population density. This sensing module was connected to a genetic toggle switch (Gardner et al., 2000) that controlled expression of a metabolic pathway. The toggle switch would initially be set to allow efficient cell growth and limited productivity. When the cell density reached a sufficient level, the toggle would switch and the cells would change to favor pathway productivity at the expense of growth. The authors considered the case of ethanol production, where initially phosphoacetyltransferase (*pta*) was expressed, directing excess acetyl-CoA into acetate. Once the toggle was switched, *pta* expression was shut off, and acetyl-CoA was preferentially converted into ethanol. The model demonstrated that dynamic control increased the pathway productivity at a cost to overall yield. A similar system was constructed in *E. coli* using the native quorum sensing system to drive expression of the T7

polymerase (Tsao et al., 2010). Protein expression from a T7 promoter is induced only when accumulation of the quorum sensing molecule indicates that the culture has transitioned to the stationary phase. These efforts are promising, but have not yet been demonstrated on a relevant metabolic pathway.

Open loop controllers allow automatic regulation of pathway expression in response to cell density or related factors such as oxygen limitation. These controllers replace costly inducers and therefore scale well to production scales. Tuning efforts, such as those applied to the *DAN1* promoter, will broaden the scope of applications by allowing the metabolic engineer to choose the density at which production is induced.

5.2. Closed loop control

In contrast to open loop control, closed loop controllers directly monitor the relevant pathway and respond to disturbances in the pathway. In a classic example of engineering closed loop metabolic control, a transcription factor-based sensor was used to detect excess flux through the glycolytic pathway (Farmer and Liao, 2000). Specifically, a natural *E. coli* transcription factor that functions in the regulation of nitrogen metabolism, NRI, was used as a sensor for acetyl phosphate by deleting the natural activator of NRI (Fig. 4A and 4B). The sensor linked increases in acetyl phosphate concentration to increases in transcription from its cognate promoter, *glnAp2*. The promoter was used to regulate genes that divert the glycolytic flux away from acetyl phosphate to an engineered lycopene biosynthetic pathway, producing a closed loop control system designed to maintain acetyl phosphate levels at a set value. Two genes in the lycopene pathway were placed under the control of either a strong constitutive promoter or the acetyl phosphate-responsive *glnAp2* promoter. Expression from the strong constitutive promoter led to growth arrest and low production of lycopene. When the genes were expressed in an acetyl phosphate-dependent manner, the cells grew normally and produced high titers of lycopene. The controller functioned similarly to the open loop controllers described previously and allowed the cells to switch on the pathway only after the cell density had reached a critical level. However, this controller also demonstrates several disadvantages of relying on natural transcription factors. The system can only respond to acetyl phosphate, and shifting to regulate the pathway based on another metabolite would be difficult. Additionally, the control law is difficult to modify; the set point of the controller is largely fixed by the relationship between acetyl phosphate concentration and NRI phosphorylation. Varying regulatory elements, such as the RBS (Salis et al., 2009), may allow some tuning of the control response by changing the strength of the linkage between sensor and actuator, but much of the system response is fixed by the choice of components.

Due to the difficulties involved in constructing synthetic controllers, recent work has focused on theoretical analyses of metabolic controllers. One recent example considered the effects of closed loop control of efflux pump expression in a biofuels-producing strain (Dunlop et al., 2010). Efflux pumps can reduce the toxicity of biofuel molecules by exporting the molecules out of the host cell, but their overexpression can also be toxic. A model that combined the two toxicity effects was developed and applied to four control strategies, including strategies for controlling expression in a biofuel-dependent manner (Fig. 4C and 4D). The model indicated that pathway productivity was largely independent of the control strategy when optimized under ideal conditions. However, in an uncertain and variable environment, the biofuel-dependent controllers would allow more consistent productivity. A second modeling example examined the control law rather than the controller and described designs for integral feedback controllers (Ang et al., 2010). A simple proportional controller was proposed where an increase in pathway output led to increased expression of a repressor, lowering the pathway expression. This system can be switched from proportional to integral control if the repressor is made to degrade at a

constant rate. A truly constant decay rate is not possible, but pseudo-zeroth order decay can be achieved through saturated enzymatic proteolysis with much greater magnitude than that of first order dilution (Wong et al., 2007). Proportional negative feedback can diminish the effects of disturbances to a pathway, but integral control, particularly in conjunction with proportional control, can completely reject disturbances. However, implementation might be difficult, especially combining controllers with different set points hard coded into their biochemical parameters. These computational models suggest new systems to test, but their predictions await experimental confirmation.

Although the application of sensors linked to actuation within metabolically engineered strains has been largely limited to computational models to date, natural biological systems attest to the value of integrating control into biosynthetic pathways. Control systems allow a pathway to respond to changes in conditions (Zaslaver et al., 2004) while filtering out spurious disturbances (Bennett et al., 2008). This increased reliability will simplify the optimization of a pathway, both at the bench and through the scale up process.

6. Conclusions

Genetically-encoded sensors that detect intracellular metabolite and cofactor levels can play key roles in the analysis and design of engineered biosynthetic pathways. By taking advantage of the modular architectures associated with many sensors and the ability to couple sensors to diverse reporter and actuator elements, these genetic devices can be used for both rapid optimization of pathway components and implementation of dynamic control strategies that allow for more efficient use of cellular resources. Current challenges include the limited availability of ligand-binding elements (input domains) that detect relevant classes of small molecule metabolites. Research efforts in synthetic biology are providing important advances to the modular design of sensors and control systems. Future advances directed to increasing the number of available ligand-binding parts to relevant metabolites and the capabilities for tuning biochemical parameters and resulting control laws will be key to the broader implementation of sophisticated sensing-regulatory strategies in metabolic pathway design.

Abbreviations

Acyl-HSL	Acyl-homoserine lactone
BLA	β-lactamase
bPBP	Bacterial periplasmic binding protein
CFP	Cyan fluorescent protein
Dex	Dexamethosome
DHFR	Dihydrofolate reductase
ER	Estrogen receptor
FRET	Fluorescence resonance energy transfer
GFP	Green fluorescent protein
GR	Glucocorticoid receptor
MBP	Maltose binding protein
Mtx	Methotrexate
RXR	Retinoid X receptor

TCB	1,2,4-trichlorobenzene
YFP	Yellow fluorescent protein

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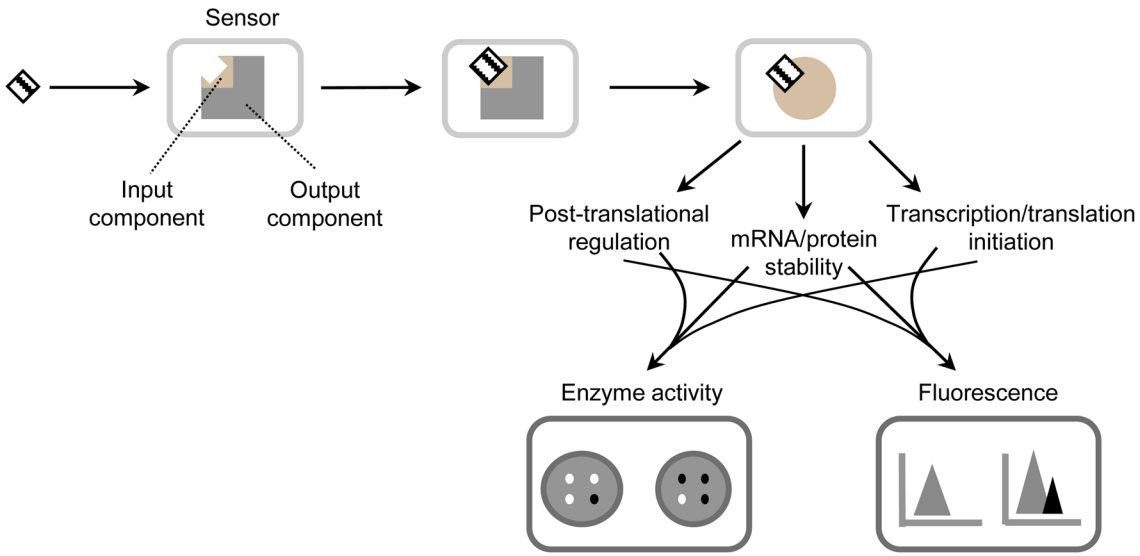

Figure 1. Cellular sensors transmit binding information into differential phenotypes

Cellular sensors are composed of two functional components. The input component detects the small molecule and undergoes a conformational change to modulate the activity of the output component, which in turn mediates regulatory processes through diverse mechanisms. The activity of the output component is translated into measurable genetic outputs, such as fluorescence or enzyme activity.

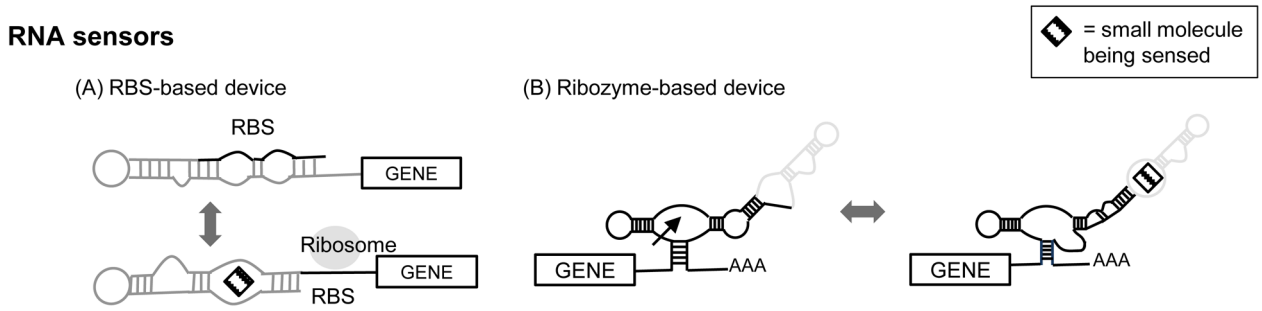

Figure 2. Types of RNA sensors

(A) Example of a RBS-based sensing-regulatory device. An aptamer is linked to a RBS via a linker sequence, which mediates access of the ribosomal subunit to the RBS through a ligand-dependent conformational change (Desai and Gallivan, 2004). (B) Example of a ribozyme-based sensing-regulatory device. An aptamer is modularly linked to a hammerhead ribozyme through a linker sequence capable of strand displacement. The linker allows the device to adopt two functional conformations that are associated with ligand-unbound (ribozyme-active) or ligand-bound (ribozyme-inactive) states (Win and Smolke, 2007).

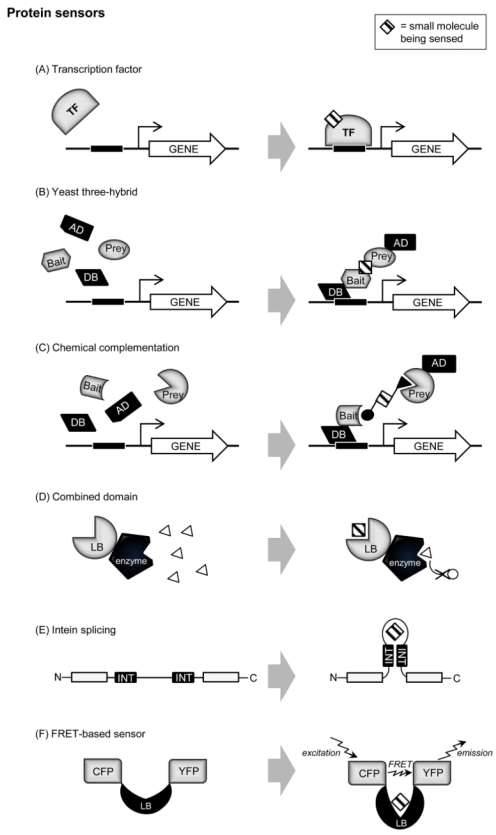

Figure 3. Types of protein sensors

(A) Allosteric regulation of a transcription factor (TF) is one example of a sensing mechanism based on transcriptional regulation, where the presence of a small molecule is linked to expression of a reporter or enzyme by placing the relevant promoter element upstream of the selected gene. (B) Additional sensing functionality can be encoded in a yeast three-hybrid system, where the small molecule-dependent interaction of the bait and prey proteins reconstitutes a transcriptional activator from a DNA-binding domain (DB) and activation domain (AD). (C) By a similar design, chemical complementation senses the small molecule of interest fused within the ligands of the bait and prey (circle and square) and produces a transcriptional readout. (D) A combined domain sensor is an example of sensing mechanism based on post-translational regulation of protein activity, where the binding of the small molecule to the ligand-binding (LB) input domain causes a conformational change that is reported as a change in enzymatic activity of the output domain. (E) By a distinctly different method of sensing, intein sensors use ligand-dependent intein splicing to link the concentration of a target small molecule to the level of active processed protein. (F Finally, FRET-based sensors report the binding of a small molecule to the ligand-binding domain as a change in the level of fluorescence resonance energy transfer (FRET) between a fluorophore pair such as CFP and YFP.

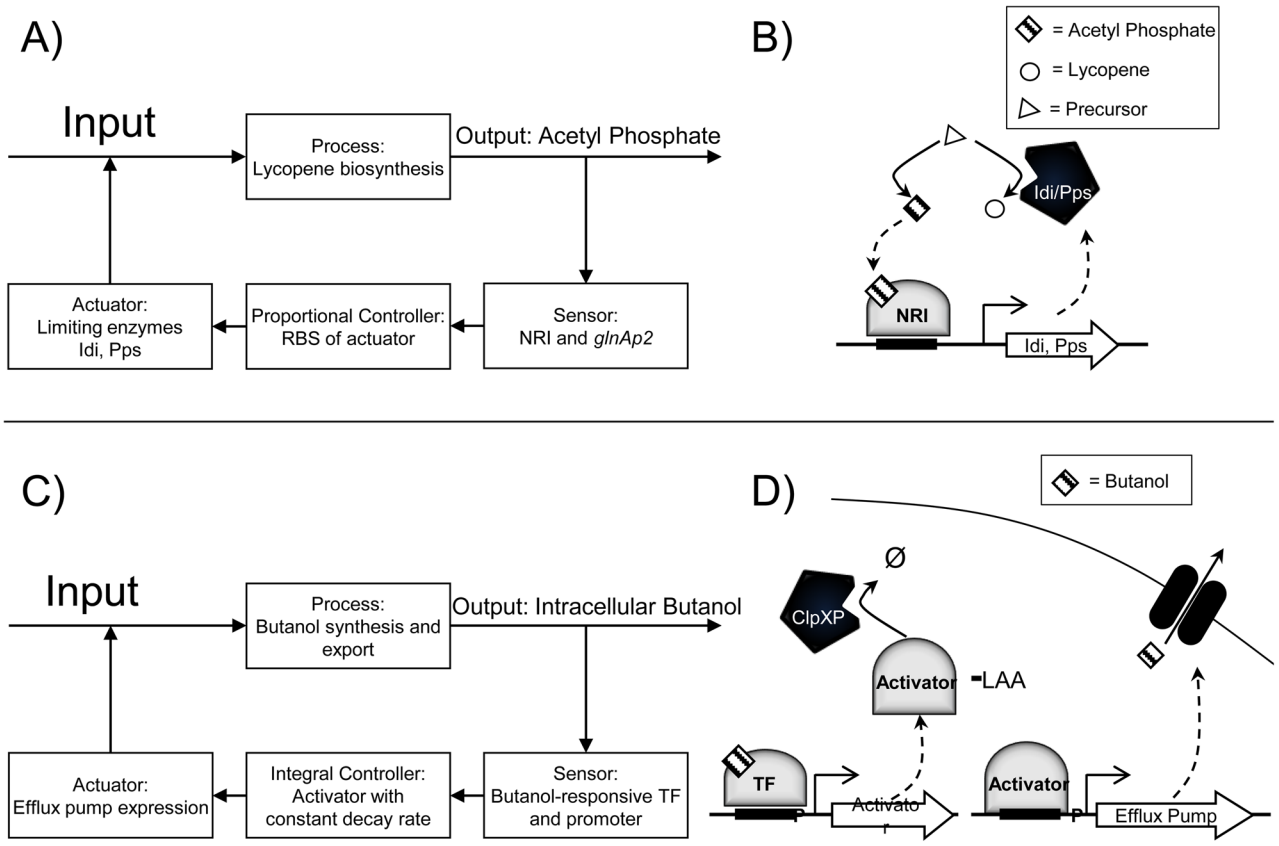

Figure 4. Block diagrams and molecular mechanisms of feedback controller designs

(A) A block diagram for an existing lycopene controller (Farmer and Liao, 2000). The sensor measures the output (acetyl phosphate) and converts it into a modular biological signal (transcription from the *glnAp2* promoter). The controller connects that signal to the actuator, expression of the rate limiting pathway enzymes. (B) Molecular implementation of the lycopene controller. The precursor (triangle) is converted into acetyl phosphate by endogenous enzymes. Overproduction of acetyl phosphate leads to expression of Idi and Pps, diverting flux to lycopene. (C) A block diagram of a hypothetical controller combining a biofuels-responsive controller (Dunlop et al., 2010) and an integral controller (Anesiadis et al., 2008). (D) Molecular implementation of the hypothetical biofuels controller. A butanol-responsive transcription factor is used to regulate activator expression. The activator has a degradation tag, leading to constant degradation by ClpXP. The activator also induces expression of the efflux pump, lowering the intracellular butanol concentration.