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The role of membrane transport in metabolic engineering of plant primary metabolism

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Plant cells are highly compartmentalized and so is their metabolism. Most metabolic pathways are distributed across several cellular compartments, which requires the activities of membrane transporters to catalyze the flux of precursors, intermediates, and end products between compartments. Metabolites such as sucrose and amino acids have to be transported between cells and tissues to supply, for example, metabolism in developing seeds or fruits with precursors and energy. Thus, rational engineering of plant primary metabolism requires a detailed and molecular understanding of the membrane transporters. This knowledge however still lags behind that of soluble enzymes. Recent advances include the molecular identification of pyruvate transporters at the chloroplast and mitochondrial membranes and of a new class of transporters called SWEET that are involved in the release of sugars to the apoplast.

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Introduction

Metabolic engineering targets the alteration of networks of biochemical reactions to modify the quality or the yield of desired products. This includes adjustment of the amount and kinetic properties of metabolic enzymes as well as the regulatory networks that govern the expression of pathway components, which in most cases is achieved through genetic engineering [1,2]. Thanks to the development of mathematical models of metabolism, the concepts of metabolic engineering have been successfully applied to the engineering of microbial metabolism, for example to increase the amount of desired amino acids or fermentation products [3], as well as the production of plant specialized metabolites in yeast, such as artemisinic acid [4]. Progress in plant metabolic engineering still lags behind achievements in the microbial field, which is partly owing to the complexity of plant metabolism, a fact that impedes the development of genome scale metabolic models for important crop and model plants [5,6]. Two major goals can be defined for plant metabolic engineering: (i) Increasing the yield or quality of plant products, such as oils and fats, cell walls, sugars and starch, proteins or specialized metabolites that have health promoting properties or serve in defense against or attraction of biotic interactors; (ii) Increasing photosynthetic and resource-use efficiency to optimize the amount of plant products that can be achieved with a given amount of water, fertilizer, and land. The latter point is of special concern given the need to increase plant productivity to meet the demands of a growing human population with respect to food, feed, fiber, and energy [7]. This review highlights the importance of a detailed understanding of the molecular mechanisms of metabolite transport within and between cells and organelles for rational engineering of plant metabolism. Given the importance of the chloroplast as the site of photosynthetic carbon assimilation and of multiple other anaplerotic routes, special emphasis will be put on recent findings on the transport into and out of the chloroplasts and other plastid subtypes.

Improving photosynthetic efficiency – a metabolite transport perspective

Several principal avenues can be envisaged for increasing plant productivity, including the alteration of plant architecture or life history traits. We focus on improving photosynthetic efficiency through (i) increasing the efficacy of photosynthetic carbon assimilation and through (ii) optimizing sink strength, thereby relieving feedback inhibition of photosynthesis.

Improving photosynthetic carbon assimilation

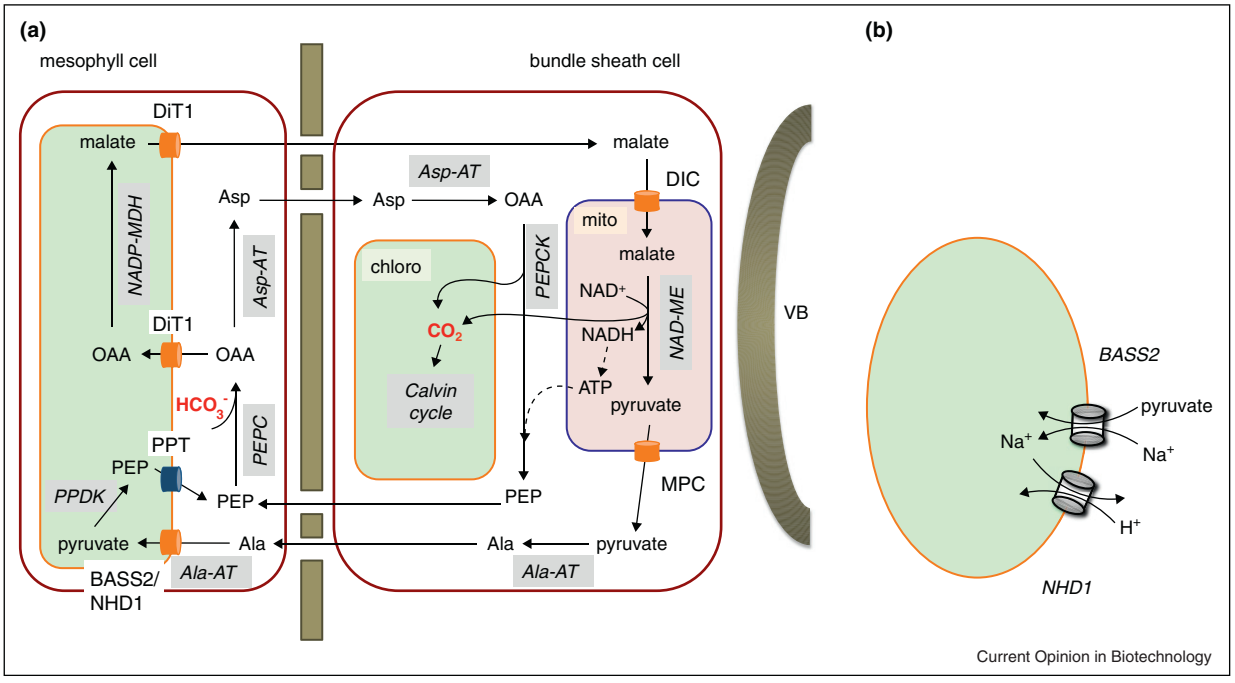
Photosynthetic carbon assimilation proceeds through carboxylation of the carbon dioxide acceptor ribulose 1,5-bisphosphate (RubP) in the chloroplast stroma, a reaction that is catalyzed by the enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) as part of the reductive pentose phosphate pathway (RPPP). Rubisco is a bifunctional enzyme that not only catalyzes the productive incorporation of carbon dioxide into organic matter, but also oxygenation of RubP and as a consequence the loss of carbon from plant biomass in the form of carbon dioxide through photorespiration [8]. To minimize the rate of carbon loss through photorespiration and to thereby increase photosynthetic efficiency, the concentration of carbon dioxide at the active site of the Rubisco enzyme

must be increased. Several means to this end have evolved in plants and algae, such as the bicarbonate pumping systems found in many algae and cyanobacteria [9], and the C_4 photosynthetic CO_2 pump in land plants [10]. Computational approaches have identified additional, potentially superior carbon fixation routes that do not currently exist in nature but that might be implemented through synthetic biology [11••,12]. Major efforts are currently underway to introduce carbon concentrating systems into C_3 crops plants, such as wheat and rice, with special emphasis on converting C_3 plants to C_4 plants [13]. Alternative approaches include shortcutting photorespiration by detoxifying the product of the Rubisco oxygenation reaction, 2-phosphoglycolate (2PG), within the chloroplast stroma [14,15]. Interestingly, many cyanobacteria possess a complete pathway for converting 2PG to 3-PGA via tatronate-semialdehyde [16,17], similar to the one that has recently been implemented in plants through genetic engineering [18]. Apparently, this pathway was lost during the evolution of chloroplasts from cyanobacteria by endosymbiosis. Possibly, the formation of glycine from glycolate during photorespiration was of selective advantage, which favored the loss of the tatronate semialdehyde route that does not produce glycine as a pathway intermediate. While shortcutting photorespiration has already been demonstrated to lead to increased biomass production at least under short day conditions in engineered Arabidopsis plants in two independent studies [18,19•], a proof of concept for transforming a C_3 to a C_4 plant is still lacking. As outlined below, the implementation of both, shortcutting photorespiration and transforming C_3 to C_4, will benefit from a better understanding of intracellular solute transport.

C_3 to C_4 – transporters required for a functional pathway

Three distinct biochemical subtypes of C_4 photosynthesis have been described and named after the enzyme that carries out decarboxylation of the C_4 acid: the NAD-malic enzyme (NAD-ME) type, the NADP-malic enzyme (NADP-ME) type, and the phospho*enol*pyruvate carboxykinase (PEP-CK) type. Accumulating evidence indicates that significant flexibility exists between these decarboxylation pathways within a particular C_4 species, depending on environmental and developmental cues [20•,21]. The NADP-ME type is frequently considered to be the most efficient subtype of C_4. However, it also requires the largest amount of transmembrane transport steps [22]. From the perspective of membrane transport,

Figure 1



(a) Schematic outline of a phospho*enol*pyruvate carboxykinase (PEP-CK)/NAD-malic enzyme (NAD-ME) type C_4 photosynthetic pathways, including the six known transporters required to run the pathway: PPT, phospho*enol*pyruvate/phosphate translocator; DiT1, plastidic oxaloacetate/malate transporter; DIC, mitochondrial dicarboxylate transporter; MPC, mitochondrial pyruvate carrier; BASS2, plastidic pyruvate:sodium symporter; NHD1, plastidic sodium:proton antiporter.
Additional abbreviations: Ala, alanine; Ala-AT, alanine aminotransferase; Asp, aspartate; Asp-AT, aspartate aminotransferase; NAD-ME; NAD-dependent malic enzyme; NADP-MDH, NADP-dependent malate dehydrogenase; OAA, oxaloacetate; PEP, phospho*enol*pyruvate; PEPC; phospho*enol*pyruvate carboxylase; PPDK, pyruvate,phosphate dikinase; VB, vascular bundle. **(b)** Function of the plastidic pyruvate double translocator system. Pyruvate is imported into plastids together with sodium ions by BASS2; the sodium ions are excreted from the plastid in counter-exchange with protons by the sodium:proton antiporter NHD1.

this is the subtype that is most difficult to engineer, a process hindered by the fact that the transporters catalyzing the import of malate into and pyruvate out of bundle sheath cell chloroplasts have not yet been identified at the molecular level. The situation is different for the PEP-CK and also for the NAD-ME subtypes. With the recent discovery of the plastidial pyruvate:sodium symporter [23••], all metabolite transporters required for implementing a rudimentary PEP-CK C_4 are now known. The PEP-CK mini-cycle itself requires no intracellular transport steps (Figure 1). Carboxylation of PEP by PEP-carboxylase yields oxaloacetate (OAA) that is then aminated to aspartate (Asp) by aspartate aminotransferase (AspAT). Driven by a concentration gradient from mesophyll to bundle sheath cells, Asp is transported to bundle sheath cells where it is converted back to OAA by AspAT and decarboxylated to PEP by PEPCK. CO_2 resulting from this decarboxylation is entering the bundle sheath cell chloroplasts where it is fixed by Rubisco. PEP returns to the mesophyll cells for another round of carboxylation. This mini-cycle would however generate an ammonia imbalance between MC and BSC because the amino acid Asp is imported into BSC and PEP is returned to MCs, leaving one amino group in the BSCs per cycle. This is compensated by a parallel-running NAD-ME cycle, which brings malate to the BSCs and returns the amino acid alanine (Ala), thereby returning the amino group to the MCs (Figure 1). The compensatory cycle requires the increased expression of four solute transporters in the chloroplast envelope membranes of mesophyll cell chloroplasts, three of which are required for PEP recycling: (i) the pyruvate:sodium symporter (BASS2), and (ii) a sodium:proton antiporter (NHD1) that maintains plastidial sodium homeostasis, and (iii) the PEP/phosphate antiporter (PPT). BASS2 imports pyruvate into the chloroplast (jointly with sodium, which is subsequently transported to the cytosol by NHD1, in counter-exchange with protons, Figure 1b) where it is converted to PEP by pyruvate:phosphate dikinase. PEP, the primary CO_2 acceptor in C_4 photosynthesis, is exported from the chloroplast in counter-exchange with inorganic *ortho*-phosphate (Pi) by PPT. The NAD-ME cycle also requires an OAA/malate antiporter at the MC chloroplast envelope, which imports OAA into MC chloroplasts where it is reduced to malate by NADP-malate dehydrogenase and exports the resulting malate to the cytosol. This step is catalyzed by the plastidial dicarboxylate transporter 1 (DiT1), which can exchange malate for OAA and 2-oxoglutarate [24•]. Malate diffuses along a concentration gradient to the BSCs where it enters the mitochondria, probably by a mitochondrial dicarboxylate translocator [25], and is decarboxylated by NAD-ME, yielding pyruvate, NADH, and CO_2. CO_2 is entering the BSC chloroplasts to be fixed by Rubisco. Pyruvate leaves the mitochondria and is converted to Ala by AlaAT. Ala is returned to MCs where it is converted to pyruvate by AlaAT and pyruvate enters the MC chloroplasts to be converted to PEP, thereby closing the branched PEP-CK/NAD-ME cycle. Until recently, it was unknown how pyruvate is transported across the mitochondrial membrane. However, mitochondrial pyruvate carriers (MPCs) were recently identified in humans, yeast, and Drosophila [26••,27••] and orthologous proteins also exist in plants (with Arabidopsis At5g20090 and At4g14695 being the most closely related proteins to mammalian MPC1 and MPC2, respectively). Thus, in contrast to the NADP-ME subtype, candidate genes for all required transporters to implement a PEP-CK/NAD-ME cycle have now been identified (see Table 1 for a listing of AGIs of the candidate genes in Arabidopsis). Maintaining ion and metabolite balance across the plastid envelope as demonstrated by the BASS2/NHD1 system with regard to sodium may prove critical for other approaches to increase photosynthetic efficiency, such as adding cyanobacterial bicarbonate transporters to the chloroplast envelope of plants [28]. Candidate transporters, such as BicA and SbtA catalyze the co-transport of bicarbonate with sodium ions [29], which require the activity of a plastidial sodium-transporter to drive the process. NHD1 fulfills this requirement, although its expression level might need to be adjusted to meet the required flux.

Priming the NADP-ME C_4 pump by photorespiration

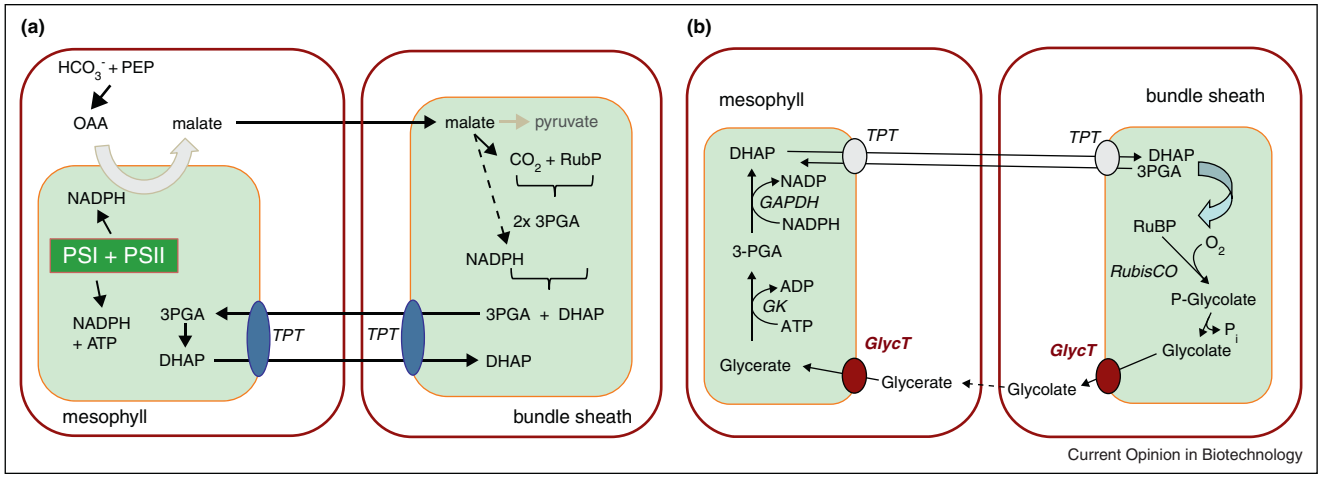
The NADP-ME subtype depends, at least in maize and sorghum, on shuttling of 3-PGA and triose phosphates

Table 1

Arabidopsis Gene Identifiers (AGIs) of transporters required for the implementation of a PEP-CK/NAD-ME C_4-type photosynthesis

AGI	Membrane location	Name of transporter	Acronym	Substrate(s)
At5g33320, At3g01550	Chloroplast	Phosphoenolpyruvate phosphate translocator	PPT1, PPT2	Phosphoenolpyruvate, phosphate
At2g26900	Chloroplast	Pyruvate sodium symporter	BASS2	Pyruvate, sodium
At3g19490	Chloroplast	Sodium proton symporter	NHD1	Sodium, proton
At5g12860	Chloroplast	Dicarboxylate translocator 1	DiT1	Oxaloacetate, malate, 2-oxoglutarate, fumarate, succinate
At2g22500, At4g24570, At5g09470	Mitochondrion	Dicarboxylate carrier	DIC1, DIC2, DIC3	C4 acids, phosphate
At5g20090, At4g14695	Mitochondrion	–	–	Pyruvate

Figure 2



(a) The 3-PGA/triosephosphate shuttle between bundle sheath and mesophyll cells in NADP-ME-type C_4 photosynthesis. Bundle sheath cells in some NADP-ME C_4 plants such as maize and sorghum have little photosystem II activity in bundle sheath cells and thus are dependent on redox import from the mesophyll cells. Redox power in bundle sheath cells is not sufficient to reduce all 3-PGA in the reductive pentose phosphate cycle to triose phosphates (DHAP), hence part of the 3-PGA must be exported to the mesophyll cells for reduction to DHAP by the triose phosphate translocator TPT. Because the TPT catalyzes the strict counter-exchange of 3-PGA with DHAP (or *ortho*-phosphate), this shuttle-system is dependent on the availability of suitable counter-exchange substrates on *cis*-site and *trans*-site of the chloroplast envelope membrane. **(b)** Priming the NADP-ME C_4 redox shuttle by photorespiratory metabolites. Glycerate kinase (GK) localized in mesophyll cell chloroplasts provides a means to build up a pool of counter-exchange substrates for TPT. Photorespiration in the bundle sheath generates glycerate that is transported to the mesophyll cells and taken up into the mesophyll cell chloroplasts in counter-exchange with protons by the plastidial glycolate/glycerate transporter (GlycT). Glycerate is converted to 3-PGA by GK and further reduced to DHAP by GAPDH. This builds up a pool of triose phosphates that can be exchanged for 3-PGA coming from bundle sheath cells, thereby priming the 3-PGA/DHAP shuttle.

(TPs) between BSC and MC chloroplasts (see [22] for review). While the required transporters for the steady state operation of the 3-PGA/TP shuttle are known, the shuttle might require one additional transport protein to get the cycle started at the beginning of the light phase. The 3-PGA/TP shuttle operates via the 3-PGA/TP/Pi antiporter TPT, which catalyzes the strict counter-exchange of TPs with either 3-PGA or Pi [30]. 3-PGA that cannot be reduced to TPs in the BSC chloroplasts owing to lack of redox equivalents is exported by the TPT in counter-exchange with TPs and transported to the MC, where it is taken up into chloroplasts, again by the TPT, in counter-exchange with TPs. In essence, 3-PGA is transported from BSCs to the MCs, reduced to TPs and then shuttled back to BSCs (Figure 2A). However, in the morning, MC chloroplasts probably do not contain the required counter-exchange metabolites (either TP, 3-PGA, or Pi) and thus are not able to take up 3-PGA via the TPT. They need to be 'preloaded' by a metabolite uniporter, similar to priming a pump. As initially suggested by Bauwe and co-workers, a photorespiratory glycerate shuttle between BSCs and MCs might achieve the priming [31•]. With the exception of the chloroplast-localized enzyme glycerate kinase (GK) that converts glycerate to 3-PGA, all enzymes of photorespiration are localized in the BSCs of the C_4 plant maize. In contrast to all other known GK enzymes in plants, the maize mesophyll GK is regulated by thioredoxin, being activated in the light [31•]. The priming is hypothesized to work as follows: Oxygenation of RubP in the BSC generates phosphoglycolate, which is converted to glycerate by the photorespiratory enzymes in the BSCs and then transported to the MC where it is taken up into the chloroplasts by the glycerate transporter (Figure 2B). While this transporter has not yet been identified at the molecular level, it has been demonstrated in biochemical studies to exchange glycerate for protons [32], hence it would be able to load glycerate into chloroplasts in counter-exchange with protons. Glycerate is then converted by GK to 3-PGA and further to TP by GAPDH, which in maize MC chloroplasts cannot enter the RPPP as in C_3 plants but needs to be exported to the BSCs. Hence, through import of glycerate into MC chloroplasts and its conversion to 3-PGA and/or TPs, a metabolite pool inside MC chloroplasts is established that enables the operation of the 3-PGA/TP shuttle via the TPT. It is important to note that this priming of the NADP-ME 3-PGA/TP shuttle via intercellular transport of photorespiratory intermediates at this point is hypothetical and awaits experimental testing.

Optimizing the photorespiratory bypass through modification of plastidial glycolate transport capacity

As noted above, biomass production of Arabidopsis plants could be increased by shortcutting photorespiration

[19•,33]. In both currently known synthetic photorespiratory bypasses, 2-PG, the toxic product of the Rubisco oxygenation reaction, is detoxified within the chloroplast, instead of converting it to 3-PGA in the complex photorespiratory cycle that involves peroxisomes, mitochondria, and cytosol [15]. Both synthetic shortcuts use glycolate as the initial substrate, converting it either to 3-PGA or to CO_2 within the chloroplast. Chloroplasts however possess a very active glycolate transporter [34]; that is, the transgenically introduced pathways for glycolate metabolism will compete with the glycolate transporter for substrate. To fully assess the potential of the photorespiratory bypasses for increasing photosynthetic efficiency, requires reducing or blocking the flux of glycolate from the chloroplast, for example by reducing the activity of the plastidial glycolate transporter. The protein catalyzing this flux is however currently unknown.

Optimizing sink strength and source-to-sink allocation of carbohydrates

Increasing photosynthetic efficiency through reducing photorespiration, as outlined above increases the source capacity, which is essential to increase the productivity of crops such as rice in which sink strength has apparently outgrown source capacity [13]. In other crops, such as potato, sink strength might still represent a limiting factor and increasing transport capacity can potentially contribute to overcoming this limitation. Supportive evidence for this comes from the observation that increasing the transport capacity of potato tuber amyloplasts for glucose 6-phosphate and ATP was associated with a large increase in starch contents of the tubers and total tuber yield [35]. This was achieved by simultaneous overexpression of the glucose 6-phosphate/phosphate translocator GPT and the ATP/ADP antiporter NTT [35]. Overexpression of the single transporters did not influence yield, indicating a co-limitation in tuber starch biosynthesis by energy in the form of ATP and by carbon in the form of glucose 6-phosphate [35,36]. Also the long-distance transport of carbohydrates, such as sucrose, from source to sink tissues might limit sink strength. One of the players in this pathway, the transporter catalyzing the efflux of sucrose from leaf mesophyll cells into the apoplastic space of apoplastic loaders was unknown until recently, which prevented an assessment of the role of sucrose unloading in the source-to-sink translocation. Using an elegant approach, the sucrose unloaders were recently identified [37••]. Human cell culture cells were engineered to co-express fluorescence–resonance energy transfer (FRET) sucrose-specific nano-sensors jointly with candidate proteins (SWEETs) that were identified in a previous similar screen that employed glucose nano-sensors [38••]. Several candidate genes could be identified from Arabidopsis (AtSWEET 10–15) and rice (OsSWEET 11 and 14) and it was shown that an Arabidopsis double mutant carrying T-DNA insertions in AtSWEET 11 and 12 showed reduced export of carbon from the leaf, increased accumulation of starch, and reduced photosynthetic capacity. This clearly demonstrates that not only phloem loading, but also unloading of sucrose from the leaf mesophyll is an important factor in source-to-sink translocation of sucrose. Of similar importance is the long-distance transport of amino acids between sources and sinks, although the mechanisms of amino acid export from source cells are not yet understood [39].

Conclusions

Engineering the transport capacity of cellular membranes is an important and frequently underappreciated aspect in efforts to engineer plant primary (and probably also specialized) metabolism. For example, converting C_3 to C_4 photosynthesis requires a massively increased metabolic flux for some metabolites across the chloroplast envelope by at least one order of magnitude [40]. Proteomic and transcriptomic approaches contributed to identifying candidate proteins carrying this flux [23••,41•,42], which now enables the implementation of a complete C_4 carbon concentration mechanism. Still, the current knowledge about intracellular and intercellular metabolite transport is rather limited, for example with respect to the intracellular transport of amino acids [39]. Technological breakthroughs, such as the use of metabolite nano-sensors [43] and large-scale electrophysiological screens [44••] are needed to increase the repertoire of membrane transporters that can be employed in engineering approaches.

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