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GE Wheat Ignites Panic, While Myriad Scores a Victory in Oz and Defeat at Home

Phill Jones

Earlier this year, an Oregon farmer used glyphosate herbicide to clear vegetation from a wheat field. When he discovered that a small number of wheat plants tolerated the chemical, he sent herbicide-tolerant plants to an Oregon State University scientist for testing. Soon, the scientist notified US Department of Agriculture officials that the plants contained a protein that conferred tolerance to glyphosate herbicide. The possibility that genetically engineered (GE) wheat was present in fields used for growing wheat commercially posed a serious problem: GE wheat is not approved for commercial sale anywhere in the world.

On May 29, the USDA announced that a small number of volunteer wheat plants growing in an Oregon field had tested positive as GE glyphosate-resistant wheat. Further USDA tests pointed to GE wheat as MON71800, a variety developed by Monsanto.

The news surprised Monsanto. From 1997-2004, Monsanto and academic researchers had studied Roundup Ready® GE wheat in test fields. The fields were grown in 16 states, including Oregon. The company wrapped up the Oregon tests in 2001; GE wheat seeds were either destroyed or sent to a USDA facility in Colorado. Monsanto abandoned the entire GE wheat project in 2004, and discontinued breeding, field research, and product development programs for the wheat. Apparently, the shutdown was driven by the concern that GE wheat grown on US farms would seriously harm the demand for US wheat in foreign markets. The concern was justified.

Quickly after the USDA announced the Oregon farmer's discovery, buyers in Japan, South Korea, Taiwan, and Europe suspended imports of US western-white wheat. The European Union, Japan, and South Korea also announced that any imported US wheat shipments would be tested for GE wheat. US wheat prices fell.

Lawsuits cropped up during the first week of June. A Kansas farmer filed the first lawsuit against Monsanto, alleging that the company's gross negligence hurt US wheat farmers by depressing wheat prices and closing some foreign markets. A Washington State farmer and the Center for Biological Diversity filed their own lawsuits against Monsanto with similar allegations.

Monsanto was not pleased.

"Tractor-chasing lawyers have prematurely filed suit without any evidence of fault and in advance of the crop's harvest," Monsanto executive vice president and general counsel David Snively told the Associated Press.

By mid-June, Monsanto's extensive testing of more than 30,000 seed samples in

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Oregon and Washington State failed to identify evidence of further contamination. The USDA reported that the agency "has neither found nor been informed of anything that would indicate that this incident amounts to more than a single isolated incident in a single field on a single farm. All information collected so far shows no indication of the presence of GE wheat in commerce."

How did the GE wheat end up on that single field? GE wheat seeds could not have hidden dormant in the soil for so many years and then suddenly germinated. Besides, Monsanto did not have a test site at the location where the Oregon farmer found the GE wheat. Did cross-pollination during the time of the trials transfer the glyphosate-tolerant trait to conventional wheat? This seems unlikely. The discovered GE wheat was winter wheat, whereas Monsanto's field trials studied GE white spring wheat; the two varieties of wheat pollinate at different times. Monsanto Chief Technology Officer Robb Farley told reporters that, while old GE wheat seed might have been planted accidentally, sabotage was also a possibility. The origin of the Oregon GE wheat remains a mystery.

A G'day for Myriad Down Under

During February 2013, Justice Nicholas of the Federal Court of Australia decided the case of *Cancer Voices Australia v. Myriad Genetics* in which the plaintiffs challenged the patentability of Myriad's nucleic acid claims. As Justice Nicholas explained, the issue to be decided was whether, under Australian patent law, a valid patent may be granted for a claim that covers naturally-occurring nucleic acid that has been isolated from human cells. The three claims at issue covered isolated nucleic acid molecules encoding certain mutant or polymorphic BRCA1 polypeptides.

"According to the applicants [the plaintiffs]," the judge wrote, "naturally occurring DNA and RNA, even in isolated form, are products of nature that cannot form the basis of a valid patent." The plaintiffs argued that the claims fail to comply with patent law, because they cover isolated nucleic acid that is not materially different from nucleic acid that occurs in nature. The defendants produced evidence that nucleic acid molecules in a human cell differ chemically, structurally, and functionally from isolated nucleic acid molecules of the disputed claims.

"There is no doubt that naturally occurring DNA and RNA as they exist inside the cells of the human body cannot be the subject of a valid patent," Justice Nicholas said. "However, the disputed claims do not cover naturally occurring DNA and RNA as they exist inside such cells. The disputed claims extend only to naturally occurring DNA and RNA which have been extracted from cells obtained from the human body and purged of other biological materials with which they were associated." The judge concluded that the claimed nucleic acid molecules are patentable under Australian law.

The plaintiffs appealed the decision. Soon, the case will be aired before a full bench of the Federal Court.

Oops! They Did it Again: Supreme Court Injects Confusion into Patent Law

The case, *Association for Molecular Pathology v. USPTO*, asked if claims to DNA molecules are patentable under US law. The dispute began in May 2009 when, on behalf of the Association for Molecular Pathology and other groups, the American Civil Liberties Union and the Public Patent Foundation sued the US Patent and Trademark Office, Myriad Genetics, and Directors of the University of Utah Research Foundation. The plaintiffs alleged the invalidity of Myriad's patent claims to molecules with nucleotide sequences of the BRCA1 and BRCA2 genes, two genes linked to susceptibility for breast cancer.

In the first leg of a four-year journey, a district court judge decided that the DNA molecules are not patentable. The Court of Appeals for the Federal Circuit reversed the decision about DNA claims. "[W] e conclude," the court wrote, "that the challenged claims are drawn to patentable subject matter because the claims cover molecules that are markedly different – have a distinctive chemical identity and nature – from molecules that exist in nature." The plaintiffs appealed to the Supreme Court, which vacated the Federal Circuit's decision and sent the case back to the Federal Circuit for reconsideration in view of the Court's enigmatic rule in *Mayo Collaborative Services et al. v. Prometheus Laboratories, Inc.* (2012). Once again, the Federal Circuit judges decided in favor of Myriad's DNA claims. The plaintiffs appealed the decision to the Supreme Court for a second time.

On June 13, 2013, the Supreme Court published its decision in *AMP v. USPTO*. "[W]e hold," Justice Thomas wrote, "that a naturally occurring DNA segment is a product of nature and not patent eligible merely because it has been isolated, but that cDNA is patent eligible because it is not naturally occurring." Retroviruses might have a different view on whether cDNA molecules do not occur in nature.

An example of a claim that no longer qualifies for patent protection is a Myriad claim to an isolated DNA molecule encoding a BRCA1 polypeptide that has a particular amino acid sequence. The Supremes stated that they did not "consider the patentability of DNA in which the order of the naturally occurring nucleotides has been altered. Scientific alteration of the genetic code presents a different inquiry, and we express no opinion about the application of [US patent law] to such endeavors." Rather, they decided that "genes and the information they encode are not patent eligible ... simply because they have been isolated from the surrounding genetic material."

The scope of this decision will have to be determined by further lawsuits. For instance, how much alteration of the genetic code is required to shift a protein-encoding DNA molecule into the patent-eligible category? As another example, does AMP v. USPTO mean that proteins isolated and purified from cells are not entitled to patent protection?

John Sterling, editor-in-chief of *Genetic Engineering News*, offered one interpretation of the Supremes' decision.

> One lawyer I spoke to compared the ruling to King Solomon's decision to cut the baby in half. This is to say the lawyer believes the Court's ruling was driven by the U.S. Justice Department, which had come to the conclusion that all biotech patents were destined to be invalidated due to an ongoing and vigorous anti-patenting campaign by the ACLU and other groups opposed to patents. According to this legal expert, by at least allowing synthetically produced genetic material to be patented, the Supreme Court basically avoided throwing the baby out with the bathwater.

However, some fail to see the value in cutting the baby in half. "[O]ne industry analyst I spoke to," Sterling wrote, "literally fears for the future of the life science industry, saying who is now going to sink big bucks into promising new biotech discoveries if there will no longer be strong patent protection for those discoveries and a significant financial return to those investors for their support?"

For the record, King Solomon did not actually bisect a baby. He only threatened to cut a baby in half to provoke a response from two women who claimed to be the baby's mother. But then, the king was known for his wisdom.

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Current Developments of Intragenic and Cisgenic Crops

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Introduction

The full potential of genetically engineered crops can probably not be realized without an increased acceptance by the general public. Likewise, the costly and lengthy procedures for obtaining approval of these crops are major barriers for implementation. New strategies and approaches are therefore required in the development of the genetically engineered crops of the future.

Several public surveys have shown that one of the major concerns among the general public is the combination of genetic elements derived from different organisms that cannot be crossed by natural means. Intragenesis and cisgenesis are two transformation concepts developed to meet this concern. In contrast to transgenesis where genes and DNA sequences are moved between any species, cis-/intragenesis is based on genetically engineering using only the plants own genetic material or genetic material from closely related species that can be intercrossed. Additionally, foreign sequences such as selection marker genes and vector-backbone sequences should be absent in the final intragenic and cisgenic plants.

The definition of the intragenic transformation concept was introduced by Rommens in 2004[1]. Intragenesis allows for the design of cassettes combining specific genetic elements from plants belonging to the same sexually compatibility gene pool (**Fig. 1**). Accordingly, coding regions of one gene (with or without introns) can be combined with promoters and terminators from different genes from the same sexually compatibility gene pool. Additionally, silencing constructs can be designed by combining several different genetic elements from the same sexually compatibility group (**Fig. 1**).

The more restrictive cisgensis concept was introduced by the Dutch researchers Schouten, Krens, and Jacobsen in 2006[2]. For cisgenesis, *in vitro* rearrangements are not permitted, and the cisgene has to be an identical copy of the endogenous gene, including the promoter, introns and the terminator in the normal-sense orientation (**Fig. 1**). However, in contrast to intragenesis, which requires that the T-DNA border sequences used for Agrobacterium-mediated transformation are isolated from the sexually compatible DNA pool (P-borders), the T-DNA borders originating from Agrobacterium can be used in cisgenesis (**Fig 1**). Although there are several variants and mixes of these two definitions, all definitions are based on the exploitation of the same gene pool as the one used in conventional breeding of a crop (a more detailed description on the variant definitions is included in the article in *Plant Biotechnology Journal*[3]).

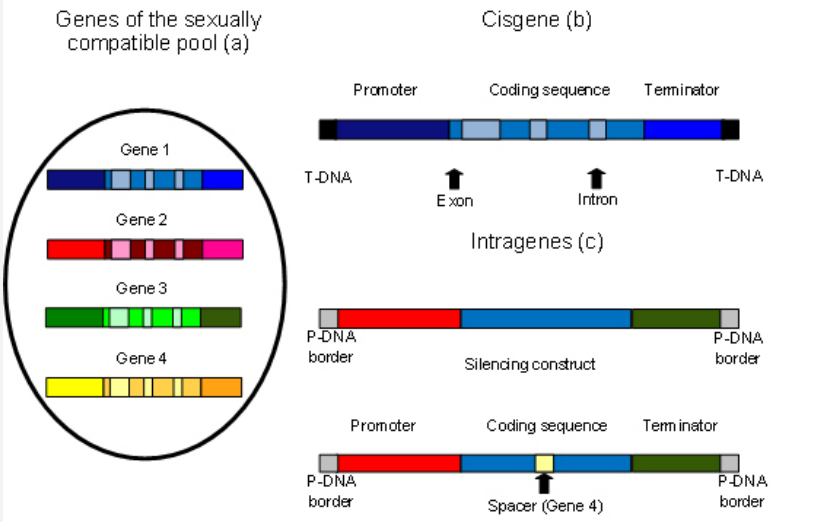

Figure 1. Illustration of cisgene and intragene constructs as defined by Schouten and coworkers in 2006[2] and Rommens in 2004[1], respectively. The cisgene is an identical copy of a gene from the sexually compatible pool including promoter, introns and terminator (a, b). When using *Agrobacterium* mediated transformation, the cisgene is inserted within *Agrobacterium* derived T-DNA borders. Intragenesis allows *in vitro* recombination of elements isolated from different genes within the sexually compatible gene pool (a, c). Furthermore, there is no requirement for introns and cDNA or fragments of genes can be used. Thus both expression and silencing intragenic constructs can be designed. According to the definition of Rommens, the intragene should be inserted within borders isolated from the sexually compatible DNA pool (P-DNA borders), when using *Agrobacterium* mediated transformation. The figure is copied from the *Plant Biotechnology Journal*, reference number 3.

Limitations and potentials of the two concepts

Compared to transgenesis, one of the limitations shared by both concepts is that traits outside the sexually compatible gene pool cannot be introduced. It is also evident that the generation of intra-/cisgenic crops requires additional expertise and time compared to transgenic crops. Hence, the desired genes or fragments of genes may not be readily available but have to be isolated from the sexually compatible gene pool. Also, the production of selection marker-free plants often requires the implementation or development of new methods, since such methods may not be readily available for the crop to be engineered. Finally, since 20 – 80% (depending on the species) of the transformants contain vector-backbone sequences, many transgenic lines have to be discarded. Thus, considerable efforts have to be spent, especially on crops with low transformation efficiencies to produce high numbers of transformants.

However, the disadvantages described above for intragenesis and cisgenesis are, in our opinion, more than compensated by their potential to overcome some of the limitations of classical breeding. Both concepts confer a faster and more precise tool for transfer of genes between related species than classical backcross breeding. Equally important, the linkage drag often seen in conventional backcross programs is avoided. The intra-/cisgenic concepts can also overcome limitations of classical breeding when it comes to improving traits with limited natural allelic variation. A higher expression level of a trait can be obtained through cisgenesis by inserting an additional gene copy of the trait or through intragenesis by introducing a hybrid gene containing an advantageous promoter and terminator isolated from the sexually compatible gene pool. Lower expression levels can be obtained through intragenesis by the introduction of different silencing constructs.

The present and future potential use of these concepts to produce intragenic and cisgenic crops is encouraged by the several surveys and focus group interviews in the US and Europe that clearly show that both intragenic and cisgenic crops are acceptable to a greater number of people than transgenic crops[3]. Further encouragement for commercial application would be derived from less rigid regulatory measures compared to the current regulation for transgenic crops.

Crops and traits currently engineered by intragenesis and cisgenesis

Even though the intragenic and cisgenic concepts were introduced only recently (nine and seven years ago, respectively), several different traits in a variety of crops have been engineered according to these concepts (**Table 1**). As indicated in **Table 1**, some of the crops are still under development and therefore contain DNA elements from non-crossable species at this stage. Individual crops and traits engineered through cisgenesis or intragenesis are briefly described below, with a more detailed description available in the *Plant Biotechnology Journal* article[3].

Crops that contain commercially widespread clones, i.e., potato, apple, strawberry, and grapevine, were

among the first crops in which cis/intragenic approaches for improvements were attempted. Transformation as a breeding tool has substantial advantages for these heterozygous crops since classical breeding by out-crossing with varieties carrying the desired new traits creates new genotypes. Direct insertion of the desired gene through genetic transformation ensures that the genotypes remain intact.

The development of a high-amylopectin potato was the first attempt to generate a crop that is very close to the definition of an intragenic crop. A construct in which almost all DNA fragments were only obtained from the sexually compatible DNA pool was designed to silence the granule bound starch synthase gene (*GBSS*) gene responsible for the synthesis of amylose, which together with amylopectin constitutes the starch components in potatoes. Moreover, the potato did not contain selection marker genes and vector-backbone sequences (**Table 1A**). However, the construct was terminated by the nopaline synthase gene terminator (*nos*) originating from *Agrobacterium*. Therefore the high-amylospectin potato cannot be considered fully intragenic.

Later, several true intragenic potatoes with improved processing qualities were developed by Rommens and coworkers at J.R. Simplot (US). Improved processing qualities have been obtained through silencing of a polyphenol oxidase gene (*Ppo*) to reduce black spot bruise (**Table 1B**) and through silencing of two genes involved in starch degradation (water dikinase (*R1*) and amyloplast-targeted phosphorylase-L (*PhL*)) to reduce cold-induced sweetening and also limit acrylamide formation (**Table 1C**). An even greater reduction of acrylamide levels in processed potatoes was obtained by silencing the tuber-specific genes in asparagine biosynthesis (*StAs1* and *StAs2*) (**Table 1D, E**).

Enhancement of disease resistance through both intragenic and cisgenic approaches has also been attempted in these crops. Cisgenic potatoes and cisgenic apples have been developed through transfer of resistance genes from wild related species. This has resulted in potatoes with increased resistance to late blight and apples with increased resistance to scab (**Table 1F, G**). Disease resistance has also been obtained through overexpression of already existing resistance genes present within the crop itself. The development of intragenic strawberry with increased resistance to gray mould was attempted through overexpression of a polygalacturonase inhibiting protein to reduce the effect of the fungal polygalacturonase. Polygalacturonase is used by the fungus during infection to break down strawberry cell walls (**Table 1H**). Likewise, a cisgenic approach is currently under development in grapevine to enhance fungal disease resistance though the insertion of a grapevine pathogenesis-inhibiting protein (**Table 1I**).

Woody plants, which are highly heterozygous and have very long generation times, can also greatly benefit from genetically engineering. To date, one cisgenic approach has been performed in poplar in which overexpression of growth-related poplar genes resulted in plants with a variety of different growth types (**Table 1J**).

The intra-/cisgenic approach to trait improvement has also been or is currently attempted in the forage crops alfalfa and perennial ryegrass. Both forage crops are perennial outcrossing species that can readily cross with wild or uncultivated relatives and in which the spreading of transgenes to the environment via pollen flow is a major concern. Pastoral Genomics (New Zealand) is currently developing perennial ryegrass with improved drought tolerance through the insertion of a H^+-pyrophosphatase gene isolated from ryegrass, arguing that this ryegrass, when fully developed, could be grown in New Zealand with few ecological concerns beyond those faced by their classically bred counterparts (**Table 1K**). Similarly, an intragenic approach was used to produce alfalfa with increased forage quality through silencing of the caffeic acid o-methyltransferase gene (*Comt*), resulting in reduced levels of lignin in the plants (**Table 1L**).

The seed-propagated self-pollinated cereals barley and wheat have also been subjected to genetic engineering based on intra-/cisgenic approaches. Although classical backcross-programs can be used in these crops to introduce genes from related species into elite cultivars of barley and wheat without making large changes to the original genotype, genetic engineering is still a more precise tool of gene transfer. Different ploidy levels of the related species further complicate classical backcrossing. Thus a cisgenic approach was used for a fast and precise transfer of the gene for the *1Dy10* HMW glutenin subunit from hexaploid bread wheat (not present in durum wheat) to tetraploid durum wheat (**Table 1M**). Intragenesis and cisgenesis can also

be used in these crops to overcome limited natural allelic variation for a trait. One example is the rather constant mature grain phytase activity observed between different barley varieties, which restricts classical breeding for improvement of this trait. Hence, a cisgenic approach was taken. A barley phytase gene (*HvPAPhy_a*) preferentially expressed during grain filling was isolated and a copy of this gene was introduced into barley. The outcome of the insertion of an extra genomic copy of the barley gene was a 2.8-fold increase in the phytase activity in the mature grain (**Table 1N**).

Field trials with intragenic and cisgenic crops

The numerous examples of useful crop traits developed through intra-/cisgenic approaches clearly demonstrate the potential of these concepts. In order to assess how many of these intra-/cisgenic crops are currently nearing commercial cultivation, we search the online databases for field trial applications and approvals in different countries. Searching the websites from the EU (http://gmoinfo.jrc.ec.europa.eu) and US (http://www.isb.vt.edu/) showed that high amylopectin potatoes, potatoes with resistance to late blight, apple with increased resistance to scab, and barley with improved phytase activity are currently in field trials in the EU, while potatoes with improved processing qualities are undergoing field trials in the US. Furthermore, JR Simplot recently petitioned the U.S. Department of Agriculture (USDA) to deregulate a potato with all the previously described intragenic modifications, i.e., reduced black spot bruise, slow degradation of starch to sugar during storage, and low asparagine to reduce the acrylamide level in processed potatoes. Thus, although intra-/cisgenic crops are still regulated under the same regulatory guidelines as transgenic crops worldwide, one of the intragenic developments may presently be close to commercial application.

Implication for agbiotechnology

The future developments of intra-/cisgenic crops will, however, depend on a lessening of rigid regulatory measures compared to the current regulation for transgenic crops. This would reduce the cost of approval and thus enable smaller breeding companies to use intragenesis and cisgenesis as additional tools for crop improvement. Presently, there actually seems to be willingness in the EU to at least make assessments on whether deregulating these technologies could be feasible in the future. Recently, the European Commission (EC) requested EFSA (European Food Safety Authority) to evaluate possible hazards posed by crops generated through cisgenesis and intragenesis as compared to crops generated by conventional breeding and transgenesis. In the resulting report, EFSA concluded that modifications made by all four techniques can produce unintended hazardous effects, attributable to the source of the gene, the trait, and general changes in structure of the plant genome. Importantly, in comparison to conventional breeding they concluded that cisgenesis will lead to similar hazards while both intragenesis and transgenesis may lead to novel hazards not associated with conventional breeding[18]. Similarly in the US there seems to be a willingness to discuss these techniques and a subgroup of cisgenic crops engineered to express plant protectants was recently proposed to be exempted from regulation by the US Environmental Protection Agency[19].

The future developments of intra-/cisgenic crops will ... depend on a lessening of rigid regulatory measures compared to the current regulation for transgenic crops.

It still remains to be seen if these evaluations and suggestions will lead to less stringent regulation of cisgenic and/or intragenic plants. It also remains to be seen if the two concepts are going to remain independent, or if a more unified and internationally harmonized concept comprising both will have to be defined in order to achieve less stringent regulations and to permit global trade of these products without difficulties.

However, although a less ridgid regulation may not be achieved in the near future, the development of intra-/cisgenic crops may yet address the concerns of the public, and may therefore facilitate a new dialogue among scientists, breeding companies, and the public regarding genetically engineered plants. Increased confidence in these novel crops may facilitate a qualified discussion of the potential environmental, economic, and health benefits of genetically engineered plants, and of their possible contribution to address the global need for more efficient and sustainable crop production.

Table 1. Intragenic/cisgenic crops developed or currently under development.

	Intra-/Cis-genesis	Type	Gene	Trait	References
	Crops with commercially widespread clones				
A	Potato (Intra)	Silencing	*GBSS**	High amylopectin	4
B	Potato (Intra)	Silencing	*Ppo*	Preventing black spot bruise	5
C	Potato (Intra)	Silencing	*Ppo, R1, PhL*	Limiting degradation of starch. Limiting acrylamide formation	6
D	Potato (Intra)	Silencing	*StAs1,StAS2*	Limiting acrylamide formation	7
E	Potato (Intra)	Silencing	*StAs1*	Limiting acrylamide formation	8
F	Potato (Cis)	Genes from related species	*R-genes*	Late blight resistance	9
G	Apple (Cis)	Gene from related species	*HcrVf2*	Scab resistance	10
H	Strawberry (Intra)	Overexpression	*PGIP*	Gray mould resistance	11
I	Grapevine (Cis)	Gene from related species	*VVTL-1***	Fungal disease resistance	12
	Woody species				
J	Poplar (Cis)	Overexpression	Genes involved in growth**	Different growth types	13
	Outcrossing species				
	Perennial ryegrass (Intra)	Overexpression	*Lpvp1****	Drought tolerance	14
L	Alfalfa (Intra)	Silencing	*Comt*	Reduced levels of lignin	15
	Seed propagated self-pollinating crops				
M	Durum wheat (Cis)	Gene from related species	*1Dy10*	Improved baking quality	16
N	Barley (Cis)	Overexpression	*HvPAPhy_a*	Improved grain phytase activity	17

Crops with asterisks are only partially intragenic or cisgenic: * - Contains the *nos* terminator originating from *Agrobacterium*. ** - Still contains a selection marker gene. *** - Still contains selection marker genes, promoters and terminators from unrelated species.

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Using Synthetic Biology to Retarget Biosynthetic Pathways to the Chloroplast for Direct Access to the Products of Photosynthesis

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Introduction

Oxygenic photosynthesis in cyanobacteria, algae, and plants converts solar radiation into chemical energy in the form of reducing equivalents (NADPH) and energy rich ATP. The reducing power and energy is subsequently used to fix CO_2 into carbohydrates, reduce NO_3^- to NH_4^+ and perform wide range of other essential chloroplast functions. Together this energy conversion process is essential for the growth of the organism and the production of biomass, which we depend upon as food, feed, fibers, and fuel.

In recent years the awareness of the finite reserves of fossil fuel reserves has sparked renewed interest in photosynthesis and how it can be improved and exploited in order to increase crop yield or directly channeled into a fuel carrier such as hydrogen (H_2) or isoprene[1].

In addition to biomass, the chloroplast also provides the energy and carbon building blocks required for synthesis of a wealth of bioactive natural products of which many have potential uses as pharmaceuticals or other mid-to-high-value products.

However, in the course of plant evolution, the energy generation and biosynthetic capacities of high-value natural products have been compartmentalized. Photosynthesis is located in the chloroplast, whereas cytochrome P450 monooxygenases (P450s), involved in the synthesis of a wide spectrum of bioactive natural products, are located in the endoplasmic reticulum (ER), where they are powered by single electron transfers from NADPH. In addition, P450s are present in low amounts, and the reactions proceed relatively slowly due to limiting concentrations of NADPH.

We have recently demonstrated that it is possible to break the evolutionary compartmentalization of energy generation and P450-catalyzed biosynthesis by relocating an entire P450-dependent pathway to the chloroplast and driving the pathway by direct use of reducing power generated by photosynthesis in a light-dependent manner[2]. This demonstrates the potential of transferring the pathways for structurally complex high-value natural products to the chloroplast and directly tapping into the reducing power generated by photosynthesis to drive the P450s using water as the primary electron donor.

Bioactive natural products.

Plants are able to synthesize more than 200,000 different bioactive natural products, which, in the plant, fulfill many different roles, e.g., the adaptation of plants to environmental challenges and to ecological niches, contributing to the plasticity of plants. Some of these compounds act as chemical defense compounds, others as attractants of pollinators or seed dispersers, and some as protection against environmental stresses. Their properties also make them industrially useful as pharmaceuticals, flavors, fragrances, colors, and even biopesticides. Some of the compounds have found uses as drugs (e.g., artemisinin, taxol, and thapsigargin) and are considered high-value compounds, mainly because they may only be present in low or highly variable amounts in the plant or are challenging to isolate. In addition, biosynthesis of the compounds is typically tightly regulated and their formation is restricted to specific tissues or developmental stages of the plant.

In the formation of bioactive natural products, cytochrome P450 monooxygenases play a key role in catalyzing the regiospecific and stereospecific hydroxylations that are often difficult to achieve using chemical synthesis approaches. As can be guessed from the variety of natural products produced, plants are an especially rich source of P450s[3]. The P450-catalyzed monooxygenations are typically dependent on electron donations from NADPH, mediated by the diflavin protein NADPH-cytochrome P450 reductase. In the plant cells, the P450s are membrane bound and typically localized in the endoplasmic reticulum.

Using sunlight: Photosynthesis and the photosynthetic complexes.

In the chloroplast of plants or algae or within the cytoplasm of cyanobacteria, the light-driven reactions of photosynthesis are mediated by the two photosystems, photosystem I and II (PSI and PSII), localized in specialized membranes, the thylakoids[4]. In the thylakoids, PSII and PSI operate in series to extract electrons from water and reduce $NADP^+$ (**Fig. 1**). Photons absorbed by PSII are used to oxidize water in the oxygen-evolving complex connected to PSII. The extracted electrons are passed on to PSI via the plastoquinone (PQ) pool, the cytochrome b6f protein complex, and lumenal plastocyanin (PC). Upon light excitation, PSI transfers electrons from the lumenal side to the stromal side of the membrane, where is uses them to reduce the soluble electron transfer protein, ferredoxin (Fd). Fd transfers electrons to various proteins in the chloroplast, for instance ferredoxin-$NADP^+$ oxidoreductase (FNR), which reduces $NADP^+$ to form NADPH. The pH gradient formed over the thylakoid membrane by light-driven electron transport through PSII and PSI is utilized for ATP formation. When the Calvin-Benzon cycle is fully active, the vast majority of ATP and reducing equivalents in the form of NADPH are used to drive the reductive steps of the CO_2 fixation.

Both PSI and PSII are membrane-bound multi-subunit protein-pigment complexes. Their core architecture is conserved from cyanobacteria to plants, and current knowledge of their structure and function is extensive.

The PSII core consists of at least 20 protein

subunits and contains the Mn_4CaO_5 cluster that catalyzes the splitting of water, the extraction of electrons, and the production of O_2. The core in plants is surrounded by a large number of light-harvesting complexes (LHCII, trimers of Lhcb1-3 proteins). As mentioned above, PSI catalyzes light-driven electron transport from plastocyanin in the thylakoid lumen to $NADP^+$ in the stroma, and it does so with a quantum yield of nearly 1. This efficiency is unmatched by any other biological or chemical system, which means that each photon captured by one of the approximately 170 chlorophyll molecules in the PSI complex of higher plants results in excitation of an electron in the reaction center of PSI[4]. PSI is also composed of a core complex that is responsible for charge separation and the first steps of electron transport and a peripheral antenna system, which are involved in light-harvesting and transfer of excitation energy to the reaction center[5]. In plants, PSI is a monomer consisting of at least 16 different proteins to which the four peripheral light-harvesting proteins (Lhca1 to Lhca4) are attached in a crescent on one side of the core complex[4].

Synthetic biology: Redirecting photosynthetic electron transport

Synthetic biology is a relative new concept in which the biological building blocks (i.e., genes, enzymes, pathways or regulatory circuits) are thought of as modular, well-characterized biological parts that predictably can be combined to construct novel genetic devices and complex cell-based systems following engineering principles[6]. In this context, photosynthetic complexes like PSI can be considered one building block and a biosynthetic pathway as another building block. Thus, it is possible to incorporate the biosynthetic pathway in the thylakoids in close proximity to the PSI reaction center. This allows the biosynthetic enzymes to tap directly into the photosynthetic electron transport immediately after PSI.

Using transient expression in tobacco as an experimental system, we obtained proof-of-concept of the possibility of transferring the P450-catalyzed dhurrin pathway from the cytosol to the chloroplast. This was achieved using gene constructs encoding fusion proteins located between the transit peptide of the stroma-localized Fd and the coding regions of the two P450 enzymes and the UDP glucosyl transferase[2]. The chloroplast was also capable of providing both the heme cofactor for proper functional assembly of the P450s and UDP-Glucose as the substrate for the final glucosylation step. In this way, the energy-demanding P450-catalyzed synthesis was driven by directly tapping into the ultimate, renewable source of energy—the Sun. In summary, P450s can be targeted to the chloroplast and be driven by photosynthesis, and are able to generate dhurrin in significant amounts. This constitutes a proof-of-concept for establishing new biosynthetic pathways in plant chloroplasts and thereby bringing the biosynthetic machinery close to the source of reducing power and carbon skeletons. The next obvious step is to establish stably transformed plants, algae, or cyanobacteria.

High-value products: Diterpenoids

The dhurrin pathway was expressed in chloroplasts to provide proof of concept and to show that PSI can deliver its electrons via the soluble electron transporter, ferredoxin, to the two P450s. Future research is directed towards expressing potentially more valuable compounds, such as terpenoids. The terpenoids constitute a large and diverse class of naturally-occurring metabolites in plants. They are derived from five-carbon isoprene units that can be assembled and modified in numerous ways, and most terpenoids are multi-cyclic structures with specific functional groups and carbon skeletons. Plants contain several classes of terpenoids, including monoterpenoids (C10), diterpenoids (C20), and carotenoids (C40)[7].

The global market for terpenoids is estimated to be in the billion dollar arena[8], comprising fragrances, food ingredients, fine chemicals, and terpenoids for therapeutic products. Therapeutic products include drugs for the treatment of diabetes, Alzheimer's disease, coronary disease, eye diseases, and (increasingly) various forms of cancer. For instance, the diterpenoid Taxol is used clinically in the treatment of numerous cancers (annual market $260M and rising). Thus, there is great potential in producing large amounts of well-defined diterpenoids in a sustainable manner.

With over 44,000 known compounds, diterpenoids are the most ancient and structurally diverse class of plant natural products. Plants harboring these compounds have a long history in traditional medicine due to their pharmacological properties. However, as already pointed out, availability of the diterpenoid compounds is generally very limited.

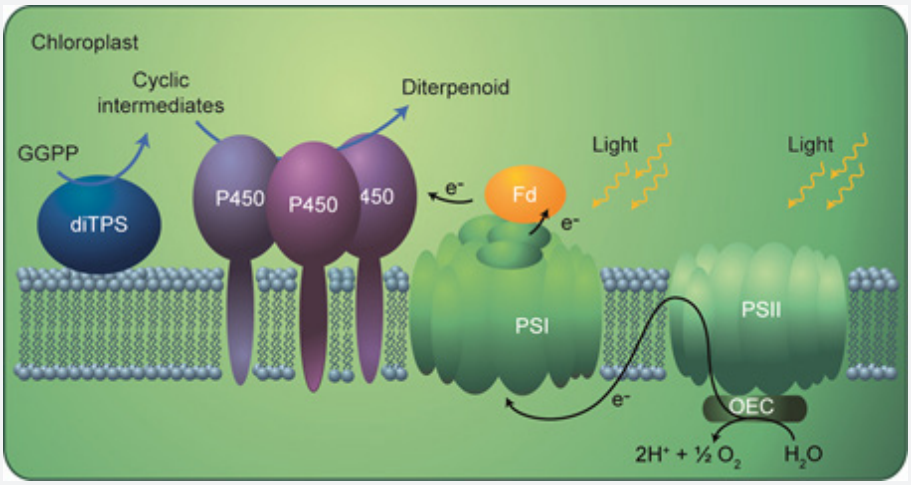

Figure 1. Proposed schematic model of a light-driven diterpenoid metabolon. The right-hand side of the figure depicts the photosynthetic electron flow from water-splitting at the oxygen evolving complex (OEC) of photosystem II (PSII) to photosystem I (PSI). PSI delivers the electrons in a light-driven way to ferredoxin (Fd), which carries them to the P450 enzymes involved in the diterpenoid production. GGDP is generated from photosynthetic fixed CO_2 and made available to the diterpene synthase (DiTPS). DiTPS converts GGPP into a cyclic intermediate, which in turn is decorated by one or more P450s, each catalyzing specific regio- and stereospecific hydroxylations. The combination of DiTPS and P450s produces the finale high-value bioactive products.

While diterpenoid phytochemicals are structurally complex, the biosynthetic routes follow a relatively simple enzymatic scheme catalyzed by two key enzyme families, diterpene synthases (diTPS) and cytochromes P450. Diterpenoids have a C-20 hydrocarbon backbone, cyclized by the diTPS from the universal substrate C-20 geranylgeranyl pyrophosphate (GGPP) (**Fig. 1**). GGPP is naturally synthesized and present in the chloroplast. The reactions catalyzed by the diTPS and P450s are invariably difficult or impossible to mimic by organic chemical synthesis. As a direct result, most diterpenoids used today are extracted from native plant species using laborious procedures, often yielding minute quantities. Production of target diterpenoids from native plant resources also suffers from several limitations beyond the quantities produced. Challenges include cultivation, harvest, extraction, and finally the purity of the target compound, which often represents a fraction in a mixture of related but undesired side products. Likewise, diterpenoid production in the plant may be dependent on or induced by unknown environmental factors.

Production: Choice of organism and organelle

Plant-based systems offer alternatives to classical biotechnological hosts like bacteria and yeast. Production in a photosynthetic organism offers a true sustainable alternative, because the carbon skeletons, energy, and reducing power are derived from photosynthesis via CO_2 fixation and light-driven electron transport.

The moss *Physcomitrella patens* is an ideal biotechnological host for diterpenoid production because it does not require the phytohormone gibberellic acid, which is diterpenoid, and it has thus been possible to develop a diterpene-free moss line that is highly suitable for expression of novel foreign diTPS and production of terpenoids[9]. The chloroplast is a well-suited compartment for expression of foreign genes. Manipulation of the chloroplast or the plastid genome can be routinely performed in the unicellular green alga *Chlamydomonas reinhardii*, the moss *Physcomitrella patens*, the higher plant *Nicotiana tabacum*, and a few other plant species[10,11].

Chloroplast genome transformation has been used successfully to manipulate endogenous genes and open reading frames and to introduce new genes and multigene constructs into the plastid genome. Using the plastid genome for the expression of foreign genes is attractive for several reasons: the plastids have a potential for high-level foreign protein expression; it is possible to express multiple genes from operons; and there are no reported position effects or epigenetic gene silencing mechanisms in the chloroplast[10]. Integration of foreign DNA into the plastid genome occurs exclusively by homologous recombination, thus facilitating precise engineering approaches such as the exchange of large genomic regions by synthetic pieces of DNA constructs, and targeted insertion of new genes and operons into preselected genomic locations. Using the vitamin E biosynthetic pathway as an example, a synthetic operon has been designed and successfully introduced into the tobacco chloroplast[12], thus enabling use of chloroplasts as production factories in synthetic biology.

Cyanobacteria resemble the evolutionary precursor of chloroplasts and thus have very similar biochemistry

to plants. Many species of cyanobacteria are easy to manipulate genetically and it grows robustly in liquid cultures. Thus, cyanobacteria can also be engineered for production of the target terpenoids.

Both *Physcomitrella* and cyanobacteria can be grown in photobioreactors, and engineering these organisms to produce large quantities of specific terpenoids is therefore highly relevant, since this allows growth of genetically engineered organisms in contained photobioreactors.

In **Figure 1**, a proposed light-driven diterpenoid metabolon is presented. The diterpene synthases are naturally located in the chloroplast where they convert the common C20 precursor geranyl-geranyl-di-phosphate (GGPP) into a cyclic intermediate that will be specific for each diterpenoid synthase or set of diterpene synthases. Next, a set of P450s that have been redirected to the chloroplast are inserted into the thylakoids near the photosynthetic reactions centers. The proposed synthetic metabolon will by-pass inherent production limitations in the natural plant sources and will significantly boost product levels.

The proof-of-concept study with the dhurrin pathway has opened up new opportunities for design and development of environmentally benign "plug-and-play" platforms for the production of high-value compounds such as the structurally complex diterpenoids. The key P450 enzymes can be expressed in much larger amounts in the highly abundant thylakoids compared to the ER, and when driven by solar energy, they have an essentially limitless supply of reducing power. The new platforms will consume lower amounts of carbon-based fuels, offer simpler, more efficient extraction and purification procedures, and leave more land available for agriculture and food production.

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