Synthetic Biology Opportunities in the Cotton Industry

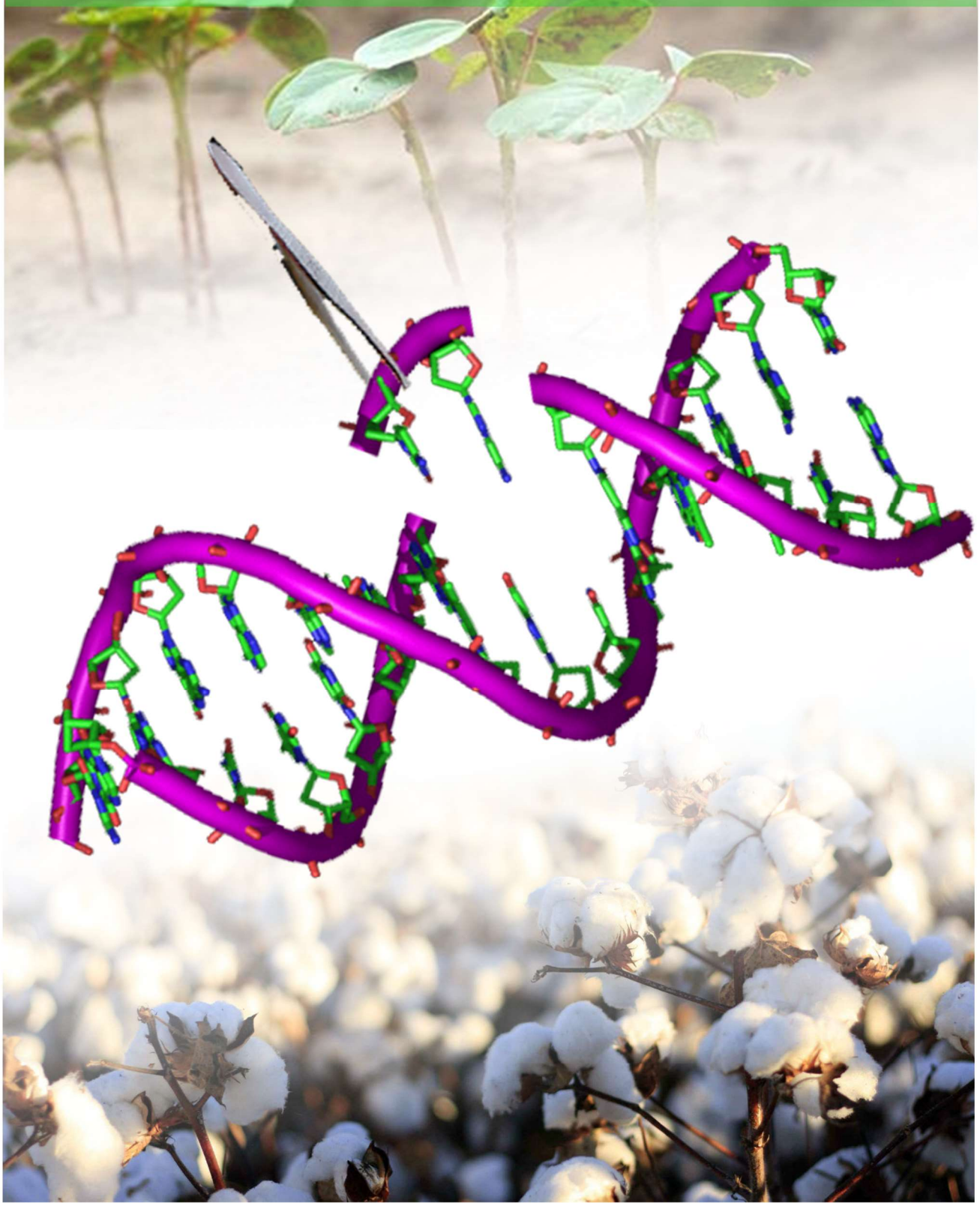

Figure 1. Synthetic Biology in Cotton. Image credits (top to bottom): AgFax.com, Wikimedia, Business Insider.

Synthetic Biology Opportunities in the Cotton Industry

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Executive Summary

Synthetic biology (SynBio) encompasses approaches that design and construct new biological elements (e.g. enzymes, genetic circuits, cells) or redesign existing biological systems to build new and improved functions. SynBio 'upgrades' the potential of genetic engineering, which involves the transfer of single genes from one organism to another. SynBio enables the introduction of multiple genes in a single transgenic event, either from a donor organism or synthetically generated. It can also enable the assembly of novel genomes from the ground up from a set of standardised genetic parts, which can then be transferred into the target cell or organism. SynBio also offers a number of non-breeding applications, such as topical applications in replacement of chemical pesticides, which can be further utilised by stable transformation.

Conventional breeding techniques have successfully introduced several beneficial agronomic traits into cotton, such as fibre quality attributes, crop maturity and disease resistance. The adoption of modern biotechnology approaches has enabled developments beyond the capacity or efficiency of conventional breeding, such as broad-scale insect and herbicide resistance. However, cotton yields continue to be challenged by abiotic and biotic factors. In addition, while progress in traditional breeding is yet to reach a ceiling, genetic diversity in cultivated cotton germplasm is limited (Wendel et al., 1992, Iqbal et al., 2001). Therefore, more advanced cotton cultivar development approaches are required to maintain and improve cotton yields and production efficiency, especially as climate change increases the incidence of biotic and abiotic challenges.

This report describes several applications of synthetic biology to the cotton industry. The most promising synthetic biology tools and approaches are discussed. Five major areas of potential application of SynBio are discussed, with the following conclusions:

Insect pests: *Potential long-term investment in developing RNAi trait against silverleaf whitefly.*

Fungal diseases: *Currently constrained by significant fundamental knowledge gaps.*

Carbon assimilation: *Research currently supported by CRDC in preparation for SynBio application in the next 3 - 6 years. Additional opportunities (i.e. aquaporins) exist, warranting further research.*

Nutrient acquisition: *Long and short-term (scoping) investment opportunities in fundamental research towards microbe attracting/enhancing root exudates via CRISPR-Cas9 or Golden Gate.*

Seed oil quality: *Currently an unviable investment opportunity in Australia.*

Importantly, the challenges facing SynBio application in cotton include the need for more in-depth fundamental genetic information and the need for a transformation system that is available for elite cotton germplasm.

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Synthetic Biology: An Overview

What is Synthetic Biology?

A consensus definition of synthetic biology (SynBio) was drafted by a group of European experts more than a decade ago: "Synthetic biology is the engineering of biology: the synthesis of complex, biologically based (or inspired) systems, which display functions that do not exist in nature" (Synthetic Biology: Applying Engineering to Biology: Report of a NEST High Level Expert Group). This engineering perspective may be applied at all levels of biological organisation, from the molecular level to entire organisms. SynBio enables the rational and systematic design of biological systems (Serrano, 2007). It encompasses approaches that design and construct new biological elements (e.g. enzymes, genetic circuits, cells) or redesign existing biological systems to build new and improved functions. These approaches can occur in two subfields: 1) using existing biological building blocks to create combinations not present in nature; and 2) create non-natural building blocks to replicate natural functions or develop novel functions. Through its evolution, synthetic biology has adopted many of the commonly used engineering terms such as 'switch', 'rewire', and the 'design, test and redesign cycle'.

Defining what is classified as SynBio is heavily debated as many tools and approaches can be considered synthetic. Furthermore, the evolution of technology and terminology has seen different labels applied to similar scientific fields (i.e. biotechnology, genetic engineering, synthetic biology). SynBio 'upgrades' the potential of genetic engineering, which involves the transfer or modification of single genes or components (Serrano, 2007, Roell and Zurbriggen, 2020). This enables the development of complex multigenic traits through the introduction of multiple genes (Roell and Zurbriggen, 2020), either from a donor organism or synthetically generated. Therefore, SynBio can more rapidly develop transgenic material with more complex modifications. For example, the development of C_4 rice required the introduction of six genes. This transformation would have taken years through traditional genetic engineering. SynBio techniques (Golden Gate) enabled this complex transformation to occur in six months (Ermakova et al., 2020a). SynBio can also enable the assembly of novel genomes from the ground up from a set of standardised genetic parts, which can then be transferred into the target cell or organism (Serrano, 2007). Gene editing is a promising technique which allows for an organism's genome to be modified without the introduction of foreign genetic material (Pixley et al., 2019). Topical application of dsRNA to elicit gene silencing through RNA-interference (RNAi) is another tool within the toolbox with great potential for agricultural application, for example, as a biopesticide. Topical RNA viral transfection can similarly be applied to crops to transiently alter

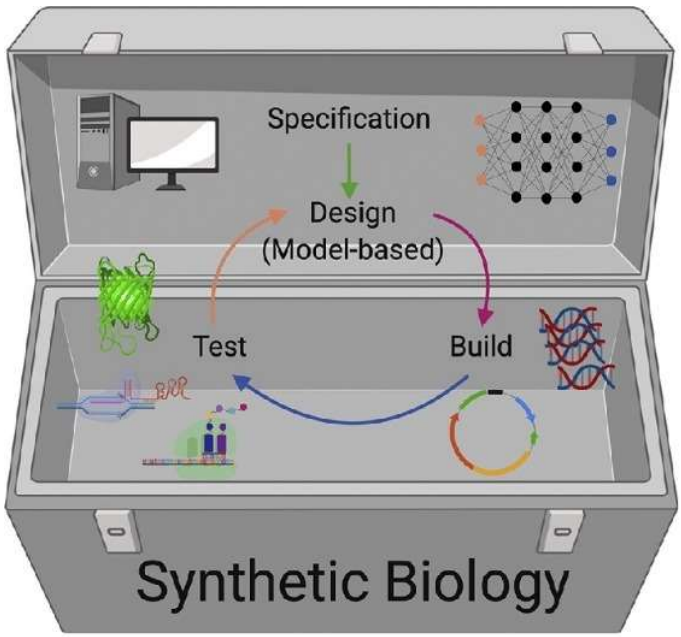

Figure 2. The "design, build, test" cycle of synthetic biology. Image from Roell and Zurbriggen, 2020.

agronomic traits, such as flowering time and stress responses by transiently expressing or silencing regulatory genes (Torti et al., 2021). There are many SynBio tools and techniques suitable for application in agricultural settings (Table 1) with the potential to develop novel agricultural products and significantly improve agricultural management, productivity and sustainability. In extension to this, new artificial promoter development will make it possible to turn genes on and off depending on the presence of a chemical or biotic and abiotic elicitor.

Table 1. SynBio tools with potentially valuable applications in agriculture.

CRISPR-Cas9	Targeted *in vivo* gene editing. An efficient tool for silencing, changing or enhancing specific genes.
Golden Gate	Simultaneous and directional *in vitro* assembly of multiple DNA fragments into a single construct. A valuable tool for stacking multiple genes for complex, multi-gene traits.
RNAi	Targeted gene silencing by RNA-interference (RNAi). Useful for silencing undesirable genes (i.e. toxic compound in edible tissues) or silencing critical processes in undesired organism (i.e. infection mechanisms of fungal diseases).
Gene Drives	Promoting deleterious alleles (i.e. lethal or sterile alleles in insect pests).
Gene Synthesis	Rapid assembly and cloning of identified genes into DNA constructs.
Regulated Promoters	Regulated promoters can temporally control gene expression by activating or deactivating downstream genes under specific conditions, such as environmental stress or phenological development.

Synthetic biology offers a range of research applications that can be classified as either 'fundamental' or 'applied'. Significant advancements in understanding fundamental biology have been and continue to be achieved using synthetic biology. However, there are numerous possible practical applications, from medicine to agriculture. Agricultural industries continue to face severe challenges, particularly those associated with climate change, while demand for agricultural products continues to rise to support the ever-growing population. This challenge could be tackled using synthetic biology techniques that enable even the most complex biological systems to be efficiently and effectively redesigned.

Is Synthetic Biology an Opportunity for the Cotton Industry?

Conventional hybridization and mutation breeding have successfully introduced several beneficial agronomic traits such as maturity and growth habits suited to a range of season lengths and production regions (Kandhro et al., 2002, Xanthopoulos and Kechagia, 2001), improved fibre quality (Muthusamy and Jayabalan, 2011), photo-insensitivity (Raut et al., 1971), fungal pathogen resistance (Ganesan and Jayabalan, 2006), herbicide tolerance (Rajasekaran et al., 1996) and heat tolerance (Rodriguez-Garay and Barrow, 1988, Trolinder and Shang, 1991). In addition to conventional breeding, the adoption of modern biotechnology approaches has genetically engineered cotton varieties with additional traits such as insect and herbicide resistance.

Despite these advancements over the years, cotton yields continue to be challenged by the occurrence of pests, weeds, pathogens and abiotic stresses. Introducing novel properties and additional genetic diversity are required. However, the ability to further overcome these challenges through conventional breeding is limited by the available diversity in the *Gossypium hirsutum* and closely related species (Gingle et al., 2006, Wendel et al., 1992). Overcoming the genetic barriers and reducing genetic drag (introducing unfavourable traits) for novel trait development is possible and efficient through synthetic engineering, which is beginning to be investigated by CSIRO. Novel cotton fibre properties such as stretchy, waterproof and coloured cotton and the introduction of nitrogenases for improved nitrogen fixation are prominent examples of research currently underway at CSIRO. Further adoption of new and innovative approaches using a SynBio toolkit are required to address current and upcoming challenges to maintain Australia's standing as one of the leading producers of the highest quality cotton.

This report will focus on the practical applications of synthetic biology in the cotton industry. It will elaborate on popular and promising synthetic biology tools and approaches, and examples of how they can be used to address some of the most significant challenges in the cotton industry. The aim is not to exhaustively list every possible application of synthetic biology. Table 1 in the review by Roell and Zurbriggen (2020) presents a more comprehensive list of possible applications of synthetic biology in applied agricultural research (Appendix 1). Rather, the aim here is to outline some of the most impactful applications of synthetic biology in the cotton industry. Some of the major limitations and considerations of synthetic biology in agriculture will also be outlined.

Promising SynBio Tools, Techniques and Approaches

CRISPR-Cas9 (gene editing)

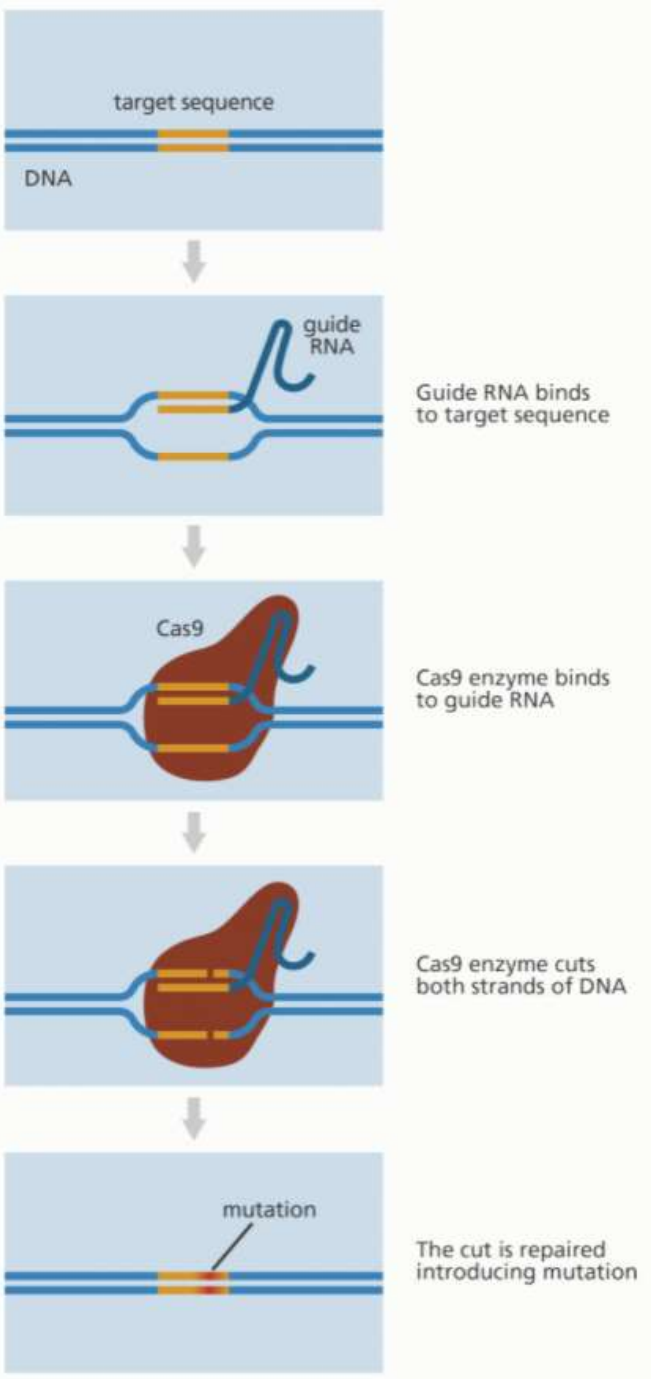

Figure 3. Illustration of how CRISPR-Cas9 editing tool functions. Image credit: Genome Research Limited.

CRISPR-Cas9 is one of the fastest, easiest and cost-effective gene editing tools. It is favoured as an alternative to classical plant breeding and transgenic methods for its simple design and easy construction of reagents (Belhaj et al., 2015). This technique is the application of a 'natural process' that functions as an immune system/defence mechanism in bacteria and archaea. During viral infection of bacteria, CAS proteins stitch pieces of viral DNA into the bacteria's CRISPR region. This allows the bacteria to record and recognise the infecting virus. These regions are translated into guide RNA which bind to the Cas9 protein. Cas9 uses the viral copy RNA to recognise subsequent matching invading viruses, destroying the invading virus' DNA (Fig. 3). This mechanism is being exploited by researchers by using Cas9 and a guide RNA to identify any specific sequences of DNA and edit it to another specific sequence. This process can be used to knock out specific genes (e.g. disease-causing genes) or 'fix' genetic errors. This technique can also be modified to promote gene transcription by deactivating Cas9 so it can't cut DNA, and adding transcriptional activators (Konermann et al., 2015). CRISPR can also be used for gene silencing. Gene editing through such technology is viewed favourably in part because single-gene knockouts or single base-pair mutations may avoid regulation (USDA, 2018). The Australian government declared in 2019 that gene editing techniques in plants and animals that do not introduce new genetic material (i.e. incisions by CRISPR allowed to be repaired naturally without guide RNA) will not be regulated. Editing techniques that do incorporate new genetic material, such as the introduction of new amino acids or genes, will require regulation by the Office of the Gene Technology Regulator (OGTR). However, all Australian state governments except Tasmania are now allowing the use of GMO's.

Golden Gate (gene introgression)

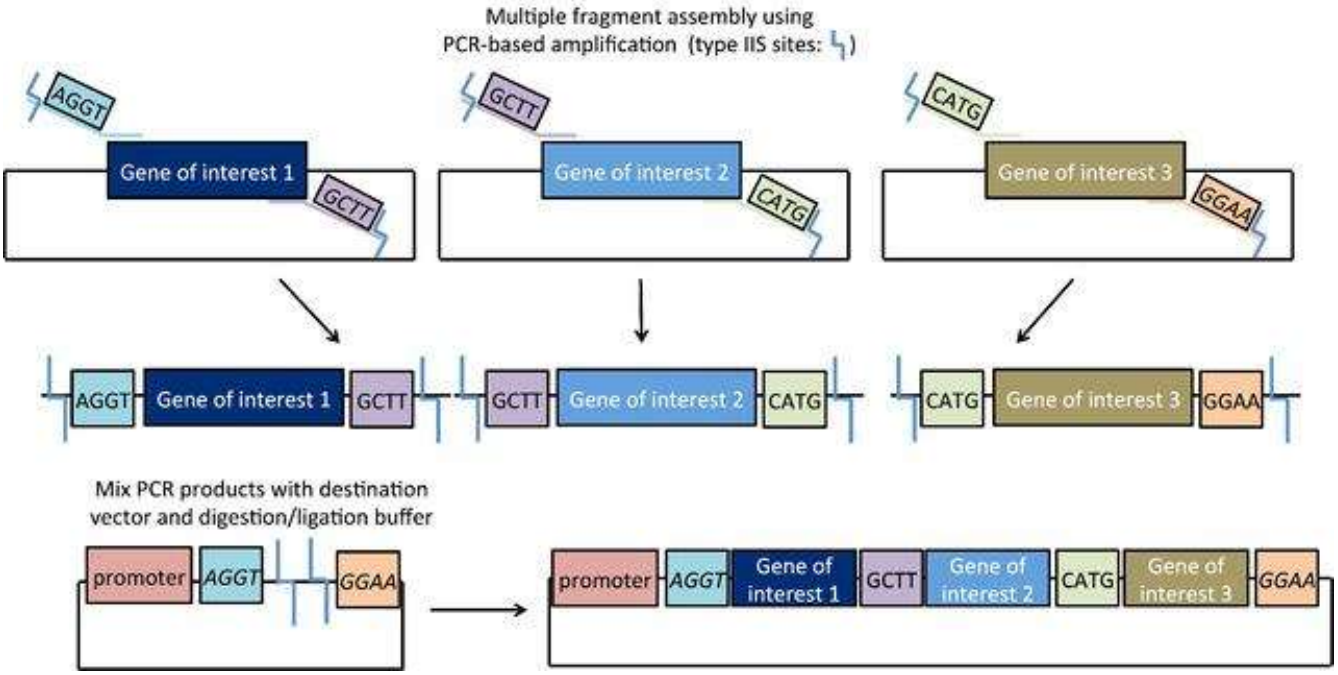

Figure 4. Golden Gate assembly of desired DNA fragments into a destination vector. Polymerase Chain Reaction (PCR) is used to multiply the number of copies of the desired DNA fragments with appropriate restriction (cutting) sites. Image from ADDGENE.

This approach enables the assembly of multiple fragments of DNA into a single vector backbone (a DNA molecule used as a vehicle to transfer genetic material into a cell) in a 'single tube' reaction. The destination vector is designed to contain recognition sequences to allow precise assembly of desired fragments (Fig. 4). Recognition sites are incorporated into the desired DNA fragments that facilitate its precise insertion into the destination vector. A short digestion (enzymatic DNA-cutting) step ensures that only correctly assembled products remain intact, and a final ligation (DNA-joining) step can remove fragments that are no longer required in the final assembled product. This is a desirable approach for stacking multiple genes for efficient multi-trait transfer with appropriate regulatory sequences. This design enables gene parts to be selected that control gene expression and the ability to incorporate multigene constructs that include metabolic pathways into plants.

RNA interference (RNAi; gene silencing)

Post-transcriptional gene silencing (PTGS) mechanisms are a naturally occurring pathways found in eukaryotic organisms to protect against viruses and/or pathogens producing aberrant RNA molecules. This process involves the recognition of the aberrant RNA which is converted into double-stranded RNA (dsRNA). dsRNA is the elicitor of the RNAi response which the DICER enzyme cleaves into 21 nucleotide small interfering RNA (siRNA) molecules. The siRNA molecules are then bound to an argonaut protein and used as a guide strand to recognise specific regions of messenger RNA (mRNA) for degradation (Fire, 1999). Ultimately, this process affects the translation of specific genes. RNAi has been targeted as a process to

silence genes for various agricultural applications such as inducing sterility or lethality in insect pest and suppressing toxin production in edible crop tissues (Chen et al., 2015, Jørgensen et al., 2005, Kola et al., 2015, Liu et al., 2017, Worrall et al., 2019, Zhang et al., 2015).

Gene drives (promoting deleterious alleles)

Increases the frequency of deleterious alleles by inserting enzymes via CRISPR to destroy non-desired genes in chromosomes, thus enabling the desired deleterious gene to be copied and inherited. Some types of gene drives can be reversible and spatially restricted. This technology could be used to target pests, weeds and diseases i.e. introduce sterility in insect pests or inhibit seed setting in weeds.

Gene synthesis

Gene synthesis has revolutionised construction of plasmid DNA used for biotechnology and SynBio. This process has increased the speed at which DNA constructs can be made, drastically reducing the time required for even complex multi-gene constructs. Ultimately, gene synthesis has enabled any gene identified from RNA sequence data to be cloned.

Regulated promoters

Understanding how promoters switch on and off is important for next generation solutions. This has important agricultural applications, enabling genes to be activated or deactivated during specific conditions. For example, water conservation genes can be activated during the detection of drought stress. This is an efficient strategy, particularly for genes that may be energetically expensive, for example, thus limiting their expression to when they're most critically required will limit their trade-offs.

Opportunities in Cotton

Insect Pests

Opportunities:
Complex, multi-gene resistance to current pests
New pest resistance traits for emerging pests & 'Northern cotton'
RNAi Biopesticides & Gene Drive sterile pests

New, robust solutions to control insect pests and their ability to develop resistance is required to sustain and improve cotton production. SynBio technologies present multiple solutions to combat insect pests. Notable success has been achieved historically through the introduction of genes for toxin production in targeted crop tissues. The introduction of the Cry protein genes from *Bacillus thuringiensis* to develop *Bt* cotton is a prime example of the use of 'traditional' genetic engineering techniques to achieve resistance against cotton bollworm (*Helicoverpa armigera*) and other Lepidoptera (Cousins et al., 1991, Fitt and Wilson, 2005, Downes et al., 2016). Since 1992, total farm income gain has increased by approximately AUD$180 per hectare and insecticide application has reduced by around 97% as a result of adoption of *Bt* cotton varieties in Australia (Cotton Australia, 2021). Although resistant individuals are emerging, *H. armigera* and other Lepidoptera continue to be controlled by new *Bt* varieties (Downes et al., 2016, Tabashnik and Carrière, 2017).

The *Bt* trait offers no protection against significant secondary pests such as mirids, whitefly, mites, aphids and thrips. The cessation of insecticides previously used to control *Helicoverpa* spp. has resulted in the increased significance of these secondary insect pests. This is largely because the *Bt* traits do not offer protection from these secondary pests which were previously controlled by pesticides targeting *Helicoverpa* spp. (Wilson et al., 2013, Wilson et al., 2018). Efforts are underway to protect cotton against these secondary insect pests. A new genetically engineered trait has been developed by Bayer - ThryvOn - to provide increased protection against mirids/lygus and thrips (Ellsworth et al., 2021). Further development of cotton cultivars with control over a broader range of major insect pests is required. Incorporating genes for resistance against additional pests such as two-spotted spider mite and silverleaf whitefly into *Bt* cultivars is underway in the CSIRO cotton breeding program and would be highly valuable (Wilson et al., 2018). Silverleaf whitefly would be a particularly valuable target due to its ability to develop resistance against insecticides (da Silva Oliveira et al., 2021).

Technologies that enable more rapid development of novel protection is required as cotton becomes exposed to new insect pests. 'New' pests, such as the fall armyworm, are emerging from other regions, or through the expansion of cotton into new areas such as Northern Australia (Wilson et al., 2018). Interest in growing cotton in Northern Australia is increasing, thus exposing "southern bred" cotton to new insect pests that these varieties have not been

developed to withstand. The main pests of these Northern areas include the pink bollworm and cluster caterpillar, which contributed to the collapse of the cotton industry in the Ord River during the 1970s (Yeates et al., 2014). The pink bollworm is not effectively controlled by *Bt* cotton due to evolved resistance against two of the three *Bt* genes (Mathew et al., 2018). Therefore, the pink bollworm is a significant risk, and thus a valuable target for developing Northern cotton varieties.

SynBio offers several solutions to insect pests in the cotton industry. These technologies offer the unique ability to rapidly introduce multiple genes for more complex protection against existing and new insect pests. This trait stacking technology may also enable development of more complex protection to limit the development of resistance in pests such as *H. armigera*. Stacking traits (e.g. through Golden Gate cloning) is likely to be the most efficient way of

Figure 5. Newly emerged silverleaf whitefly on cotton. Image from IPM Guidelines (cottoninfo.com.au).

developing new varieties with resistance to multiple insect pests that aren't controlled by the *Bt* traits, such as whitefly and mites. Although this may be possible through breeding (Miyazaki et al., 2013, Trapero et al., 2016), progress could be more rapid and efficient through SynBio trait-stacking approaches. This would enable development of cultivars with resistance against multiple insect pests. This technology limits the number of transformations required to introduce multiple or multi-faceted traits such as resistances against a new suite of pests. This highly targeted approach minimises genetic drag (i.e. of unfavourable genes) that can occur with breeding from diverse genetic material.

RNA interference (RNAi) technology offers two different approaches to combat insect pests. Firstly, through the production of biopesticides that are targeted to specific insect pests. These highly specific topical applications reduce the need for chemical pesticides that may be damaging to the environment and non-target organisms. Bioclays are an option for deploying RNAi biopesticides that are being explored by CRDC for use against insect pests including whitefly in the cotton industry. Bioclays are foliar sprays that are developed by loading dsRNA molecules into layered double hydroxide nanoparticles for more stable delivery of the RNA compared to "naked RNA" applications (Mitter et al., 2017, Worrall et al., 2019). However, this field continues to be constrained by the inadequate synthesis of enough

dsRNA or bioclay particles for large-scale applications. More rapid and efficient synthesis procedures is required for topical RNAi to be viable for agricultural applications.

Secondly, insecticidal dsRNA molecules can be expressed by cotton itself, potentially overcoming the manufacturing bottleneck of topical RNAi. Consumption of diet containing dsRNA and siRNA synthesised from genes such as an actin ortholog, ADP/ATP translocase, α-tubulin, ribosomal protein L9 and V-ATPase A subunit have resulted in whitefly mortality (Upadhyay et al., 2011). Expression of such genes can be temporally controlled or spatially controlled. Temporal expression includes constitutive (continuous) or inducible (i.e. upon detection of herbivorous damage) expression. Inducible expression of an insecticidal dsRNA to induce RNAi is possible by introducing a promoter alongside the gene to activate it upon damage by insect pests (Senthil-Kumar and Mysore, 2010). Spatial expression includes ubiquitous (all tissues) or tissue-specific expression. Tissue-specific expression may be particularly valuable for the control of sucking pests that feed on the phloem (sap). Expressing insecticidal dsRNA with a phloem-specific promoter could enable specific expression in the phloem to target sucking insect pests such as whitefly (Upadhyay et al., 2011). Phloem-specific promoters have been identified in plants, including those used to protect against bacterial disease (Dutt et al., 2012) and sucking insects (Javaid et al., 2016). Eakteiman et al. (2018) deployed RNAi in Arabidopsis with a phloem-specific promoter to target a glutathione S-transferase gene, *BtGSTs5*, in whitefly, but with sublethal effects. Full efficacy of RNAi applications will rely on increasing the lethality of these molecules (Shelby et al., 2020), particularly to at least the equivalence of an insecticide for topical dsRNA to outcompete insecticides. Ultimately, further fundamental research (e.g. to identify genes for specific and lethal toxin production and promoters for transient or tissue-specific expression) is required to improve the impact of this approach. A review by (Shelby et al., 2020) summarises the strategies and considerations for controlling whitefly and other insect pests using RNAi.

SynBio offers several other potential solutions to combat insect pests. Gene drives (promotion of deleterious alleles) can enable effective and self-sustaining control of insect pest populations through increasing the frequency of deleterious alleles such as sterility or lethal alleles. Gene drives could also be used to revert resistant insect populations back to susceptible. However, gene drives in insects are less favourable as controlling the travel of the genetically modified insect population is almost impossible, relative to the control that can be implemented for a GMO plant. Targeting the sensory ability of insect pests is also an option, albeit not as prevalent as developing traits and topical applications. For example, the insect pest could be modified to remove its ability to sense cotton. Similarly, the chemical profile of cotton could be modified (or masked by a topical application) or make cotton "invisible" or "repulsive" to the insect, an area under investigation by Michelle Rafter (CSIRO). Given the complex challenge of controlling insect pests, a multi-faceted approach incorporating IPM strategies is required for optimal control, likely through combining cultivar resistance with management practices and topical applications.

Fungal Diseases

Opportunities:
Dual resistance to multi-pathotype diseases
Topical gene silencing sprays

Fusarium and Verticillium wilt are two of the most concerning diseases in the global cotton industry (Li et al., 2017b). These soil-borne fungi can exist in many different forms in the soil, crop debris, other crops and weeds, and the severity of Verticillium wilt tends to worsen with cold, wet conditions (Li et al., 2017b). These diseases can be managed to some degree through integrated agronomic practices, while significant advances towards disease-resistant cotton germplasm have been achieved through the CSIRO's cotton breeding program. Genes encoding antifungal proteins and signalling pathways have been reported to improve cotton's resistance against fungal pathogens (Emani et al., 2003, Murray et al., 1999, Parkhi et al., 2010) such as Verticillium wilt (Tian et al., 2010, Wang et al., 2004). However, it does not appear that any of these studies have resulted in the significant levels of resistance required to be incorporated into cultivars. There is great need for resistance to these diseases, and currently high levels of activity globally in this area of research and development, particularly by CSIRO. However, full resistance to these diseases is yet to be achieved.

Figure 6. Verticillium wilt symptoms in cotton. Image from https://www.dpi.nsw.gov.au/about-us/media-centre/releases/2017/dpi-research-protecting-australian-cotton-from-verticillium-wilt.

Several challenges constrain the development of Verticillium- and Fusarium-resistant cotton. In Verticillium, for example, one of the biggest challenges to the cotton industry is the existence of two pathotypes of Verticillium wilt, defoliating and non-defoliating Verticillium

wilt (Li et al., 2017b), which can co-occur (Le et al., 2020). Breeding efforts thus far have been unable to develop dual resistance to both pathotypes. There are no fungicides identified to date for their control, and host plant resistance in combination with management practices is the most economical and environmentally-friendly approach to managing Verticillium wilt (Göre et al., 2017, Li et al., 2017b). Additionally, varieties that are more resistant to one of the diseases tend to be susceptible to the other (Li et al., 2017b).

Trait stacking through Golden Gate could be beneficial for the development of cultivars with dual resistance to both pathotypes of Verticillium. Dual resistance to both Verticillium and Fusarium would also be highly valuable, albeit an ambitious task, but worth long-term investment. Gene editing of cotton cultivars by CRISPR-Cas9 could also be an effective approach to developing resistance, as this technology has been used to combat bacterial blight (*Xanthomonas*) infection in cotton (Cox et al., 2017). However, these approaches rely on the identification of genes for resistance to introduce into cotton cultivars, or genes to target in the Verticillium genome to inhibit infection or survival. Although numerous genes, quantitative trait loci (genomic regions) and proteins have been identified as potential contributors to some level of resistance to Verticillium in some cotton cultivars and species (Dong et al., 2019, Cheng et al., 2016, Duan et al., 2016, Jun et al., 2015, Li et al., 2014, Li et al., 2018, Mo et al., 2015, Yang et al., 2015, Yang et al., 2018, Zhang et al., 2014, Zhang et al., 2017), the precise combination of genes and the location of their expression for conferring optimal resistance remain elusive. Further research is required to understand the pathogenicity of Verticillium and identify genes that may confer resistance and their function. Additionally, the location of their expression (i.e. in the root hairs or xylem) could be critical for effective resistance. This could be aided or fast-tracked through using CRISPR-Cas9 to identify gene functions, while Golden Gate would enable gene combinations and expression patterns to be tested. Additionally, although some wild species (from the A and D genomes) have been screened for resistance, the full genetic diversity of the cotton (*Gossypium*) genus of over 50 species is yet to be exploited. Screening more distantly related species (i.e. from the B, C, E, F, G and K genomes) may be insightful, however, growing these species in field experiments is likely to be challenging.

Topical RNAi can downregulate genes required for survival or initial infection and spread of fungal diseases in the host (Majumdar et al., 2017). Topically applied double-stranded RNA have been shown to induce antiviral RNAi in cowpea, inhibiting the bean common mosaic virus (Worrall et al., 2019). However, topical applications are unlikely to contact, and thus effectively control, soil-borne fungal pathogens present deep in the soil. Instead, host-induced gene silencing is a more promising strategy to defend cotton against soil-borne diseases such as Verticillium wilt. This approach gives plants the capacity to generate their own molecules to elicit an RNAi response to target invading organisms, which has successfully suppressed Verticillium wilt in transgenic tomato and tobacco (Song and Thomma, 2018). Molecules that elicit RNAi could be designed to target specific functions involved in pathogenicity. For example, velvet regulatory proteins involved in root colonisation and propagation (Höfer et al., 2021) could be targeted and silenced by host-induced RNAi. Expressing these molecules in the correct tissues (i.e. the root hair or xylem) will be critical,

which can be controlled through promoters targeted to these specific tissues. Designing antifungal root exudates that express dsRNA or siRNA, or are comprised of certain antifungal compounds could also be an effective approach to control soil-borne fungal diseases (Zhang et al., 2020).

Case Study

Synthetic biology has been used to protect the Cassava industry from Cassava brown streak disease (CBSD) that devastated production in Uganda, the Demographic Republic of Congo. This disease caused crop damage of up to 70%, resulting in economic losses of $75 – 100 Mil. and higher annually (Manyong et al., 2012, Ndyetabula et al., 2016). Transferring some resistance was possible through conventional breeding; however, this process took a long time to combine CBSD resistance while maintaining good root and harvest qualities (Jennings, 2003). Similar to the issues around Verticillium wilt in cotton, cultivars resistant to one virus species were not resistant to the other virus species responsible for causing CBSD (Patil et al., 2011). Stable and strong resistance to CBSD were achieved through transferring genes of resistance from related species (Jennings, 2003, Patil et al., 2015). This research is being advanced with the use of CRISPR-Cas9 genome editing, altering multiple genes simultaneously to develop cassava cultivars with multi-gene tolerance to CBSD (Gomez et al., 2019). RNAi has also been shown to efficiently control CBSD, conferring complete resistance in 85 % of transgenic lines (Patil et al., 2011).

Carbon Assimilation

Opportunities:
Enhancing productivity & thermotolerance through improving photosynthetic efficiency
Water-use efficient cotton through CAM photosynthesis & aquaporins

Targeting improved photosynthesis is one of the next frontiers for improving crop productivity, resource-use efficiency and abiotic stress tolerance (Ainsworth and Ort, 2010, Betti et al., 2016, Furbank et al., 2020, Long et al., 2006, Posch et al., 2019, Simkin et al., 2019, Sharwood, 2017). Photosynthetic pathways and abiotic stress responses are highly complex, impacting multiple pathways and processes. Therefore, traits that target improved photosynthetic performance and resilience under abiotic stresses will require targeted integration of multiple genes and possibly new reaction pathways. Enhancing photosynthetic pathways would rely on the SynBio toolkit that can efficiently transfer large gene constructs with specified expression patterns. Examples include enhancing photosynthetic enzymes, improving water-use efficiency (WUE) by introducing novel aquaporins and modifying cellular anatomy to improve mesophyll conductance (the diffusion of CO_2 into photosynthetic chloroplasts). Heat shock proteins (Reddy et al., 2016) and altering root traits (Hu and Xiong, 2014) are also targets for improving cotton heat and drought tolerance and WUE that should be considered, and could be particularly powerful when combined with photosynthetic enhancements.

Enhancing productivity and thermotolerance through improving photosynthesis

Improving carbon assimilation and thermotolerance is likely to rely on modifying several key photosynthetic enzymes (in bold below) involved in the Calvin-cycle (carbon fixation, reduction and regeneration) and the electron transport chain ("light photosynthesis"). **Rubisco** catalyses carbon fixation (carboxylation) inside the chloroplast and is a long-standing target of photosynthetic enhancement for yield gain. Carboxylation by Rubisco is aided by its 'helper protein' **Rubisco activase** which prevents Rubisco becoming inactivated and is thermolabile under abiotic stress (Kumar et al., 2009, Kurek et al., 2007, Sharwood, 2020). Carbon assimilation and crop biomass and yield have been improved under heat stress by introducing more thermostable Rubisco activase (Kumar et al., 2009, Kurek et al., 2007, Scafaro et al., 2019) or a catalytically superior Rubisco (Long and Ort, 2010, Zhu et al., 2010) or by modifying both Rubisco and Rubisco activase (Qu et al., 2021). **SBPase** is involved in the regeneration of RuBP during the Calvin-cycle, the substrate for carboxylation by Rubisco. Overexpression and manipulation of SBPase can enhance photosynthesis under heat stress in transgenic rice (Feng et al., 2007), prevent heat-induced yield reduction in soybean (Köhler et al., 2016) and improve vegetative biomass and seed yield in Arabidopsis (Simkin et al., 2017). Overexpression or introduction of novel SBPase can also improve crop WUE (López-Calcagno et al., 2020). **Cytochrome b_6f** is one of the four major light-harvesting protein complexes in the chloroplast membrane, involved in electron transport reactions that provide

energy for carboxylation by Rubisco. Overexpression of light harvesting complexes such as b_6f offer opportunities to improve carbon assimilation (Ermakova et al., 2019, Yamori et al., 2016).

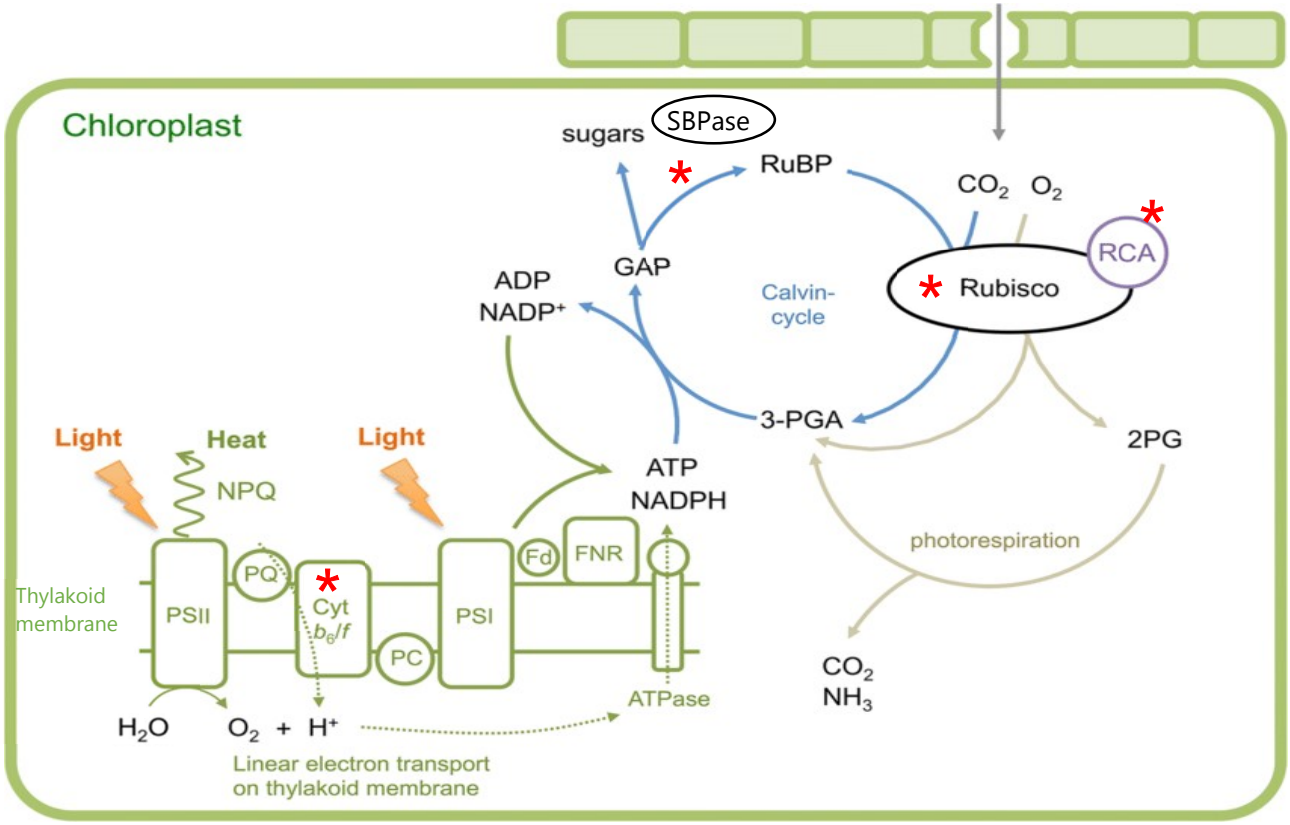

*Figure 7. The electron transport chain (in thylakoid membrane of chloroplast) and the Calvin-cycle (in the chloroplast). Key enzymes targeted for photosynthetic enhancement indicated by *. RCA: Rubisco activase. Image modified from Adachi et al., 2020.*

Improving crop water use efficiency and drought tolerance

Developing more productive, drought stress resilient and water-use efficient cotton is required for productivity to continue in a warmer and drier world. SynBio can facilitate the transfer of novel drought tolerance and improved WUE traits that are not possible through conventional breeding.

Modifying C_3 crops like cotton to have a more water-use efficient photosynthetic metabolism is one such strategy that is only possible through more advanced technologies offered by SynBio (DePaoli et al., 2014). CAM (crassulacean acid metabolism) and C_4 photosynthesis are renowned for being more water-use efficient than C_3 photosynthesis (DePaoli et al., 2014, Borland et al., 2014, Ermakova et al., 2020b). This is due to the presence of carbon-concentrating mechanisms (CCM) that reduce the need for as much stomatal opening, thus transpiring less water while maintaining carbon assimilation rates (Borland et al., 2014). Consequently, CAM plants can use 20 – 80% less water to produce the same amount of biomass compared to C_3 and C_4 plants (Antony and Borland, 2009, von Caemmerer et al., 2012). An added bonus of this mechanism is that its expression can be induced. Facultative CAM species are capable of expressing mRNA encoding for CAM enzymes in response to abiotic stresses, "switching on" this mechanism when it is most needed. This could be

exploited to develop cotton cultivars capable of switching to more water-use efficient metabolisms when water is scarce. The potential productivity impact of introducing water-conserving mechanisms needs to be carefully considered. Water-preserving traits are likely to be most beneficial in rainfed production systems, particularly if inducing a yield penalty is avoided.

Aquaporins are proteins that facilitate the movement of CO_2 and/or water in plants (Uehlein et al., 2003). Expression and overexpression experiments revealed the influence of aquaporins on photosynthesis, mesophyll conductance (Ermakova et al., 2021), stomatal conductance and root hydraulic conductivity, and thus productivity and water use (Sade et al., 2009). Consequently, aquaporins have emerged as another target for developing water-use efficient and drought resistant crops.

Photosynthetic enhancement will rely on SynBio

If traits aren't found in closely related species suitable for crop breeding, photosynthetic enhancement will rely on SynBio approaches. Photosynthetic manipulation is complex, requiring the introgression or modification of multiple genes to improve biochemical pathways. Therefore, rapid and efficient insertion of multiple transgenes into target crops will be paramount (Castilho, 2015, Simkin et al., 2019). SynBio has enabled multiple genetic modifications to occur in a single event, thus enabling effective and efficient improvement of photosynthetic efficiency to improve crop performance, heat and drought resilience and WUE (Kromdijk and Long, 2016, Kubis and Bar-Even, 2019, Ort et al., 2015, Shih et al., 2014, Simkin et al., 2019). Multiple targets, such as Rubisco, Rubisco activase and possibly also their supporting chaperones (Aigner et al., 2017), would be required to successfully enhance photosynthesis and abiotic stress resilience. Additionally, tissue-specific expression would be required. Stacking genes through Golden Gate would provide an efficient approach to this multi-gene modification (Maurino and Weber, 2013, DePaoli et al., 2014). An alternative or addition to gene stacking, gene editing through CRISPR-Cas9 could be used to edit amino acids that confer a catalytic switch, enhancing photosynthetic activity of enzymes such as Rubisco (Whitney et al., 2011, Sharwood, 2017).

The SynBio toolkit also offers the unique ability of specific regulated promoters to “switch on” abiotic stress genes that improve CO_2 assimilation (i.e. increase Rubisco or Rubisco activase activity) under high temperature (Venter, 2007). Further, this system could be designed to "switch off" at certain developmental phases, to prevent the plant remaining in the vegetative phase. This is a concern that must be addressed when aiming to stimulate photosynthesis in non-determinant crops like cotton to avoid continued vegetative production. Careful promoter design could enable high photosynthetic rates for rapid canopy establishment, followed by 'normal' photosynthesis for flowering and boll production. Regulated promoters may also be extremely valuable for preventing yield penalties for conservative mechanisms such as water-preserving mechanisms that may hinder yield under prolonged expression. This approach would need to be experimentally tested through trialling a prototype under abiotic stress conditions in the field.

Nutrient Acquisition

Opportunities:
Nitrogenase introgression for nitrogen-fixing cotton
Establish & enhance plant-microbe interactions

Currently, productivity in western agriculture is sustained by a massive use of fertilisers. The excessive use of fertilisers is environmentally damaging and consumer interest in more sustainable products is increasing. Additionally, this practice is unsustainable due to expected future rising energy costs to produce fertilisers, the low nitrogen-use efficiency of crops and finite availability of macronutrients such as phosphorus (Heuer et al., 2017, Perchlik and Tegeder, 2017, Rogers and Oldroyd, 2014). This challenge can be overcome by improving crop nutrient-use efficiency, uptake or assimilation (Roell and Zurbriggen, 2020). Due to the complexity of these traits and systems, SynBio offers some of the most efficient and effective solutions. Some of the most supported approaches include engineering both crops and their associated microbes to improve the fixation, mobilisation and uptake of macronutrients such as nitrogen and phosphorus (Roell and Zurbriggen, 2020). Symbiotic relationships have evolved between some plant species - most notably, legumes - and nitrogen-fixing bacteria. This interaction delivers around 120 kg per ha of fixed nitrogen directly into the plant's roots (Salvagiotti et al., 2008). Engineering maize to fix the equivalent of 50 kg (N) per ha could substantially improve crop yield (Rogers and Oldroyd, 2014), as demonstrated through modelling (Folberth et al., 2013). In addition to yield improvement, engineering crops to fix their own nitrogen, will significantly reduce fertiliser use.

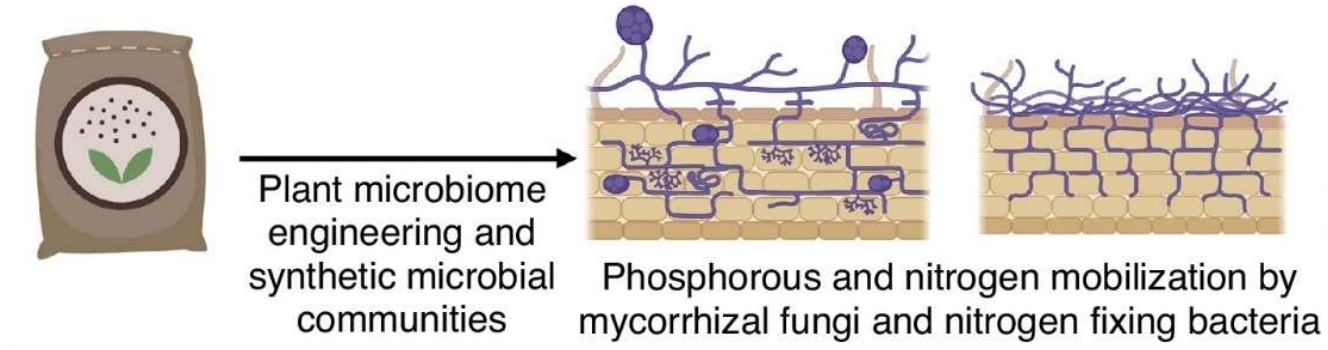

Figure 8. The application of SynBio to improve nutrient acquisition in crops and reduce fertiliser usage. Figure from Roell and Zurbriggen (2020), Current Opinion in Biotechnology.

Engineering crops to fix their own nitrogen is a promising crop modification to improve crop nitrogen-use efficiency. Crop utilisation of nitrogen and phosphorus could be significantly enhanced through several ways by looking towards microbes:

1) **Introducing enzymes** such as nitrogenases or phytases. Nitrogenases are enzymes that naturally occur in some bacteria and Archaea, enabling them to fix nitrogen directly from the atmosphere. SynBio approaches could enable nitrogenases to be introduced directly into plant cell organelles. There is great interest in this area of research, with developments

currently underway at CSIRO and other global research institutions. Cotton has been successfully transformed to improve phosphorus (P) utilization through targeting the phytase enzyme. Phytase activity was enhanced through inserting the *pyk10* promoter from *Arabidopsis thaliana* fused to a phytase gene (*phyA*) from the bacterium *Aspergillus ficuum* in cotton. This modification improved the utilization of phytate as a P source, improving P nutrition in P-deficient conditions. However, a cotton cultivar is yet to be developed and released with this trait.

2) **Enhancing existing microbial activity**. Introducing new microbes to agricultural soils (identified in nature or engineered) has been raised as an opportunity to improve nutrient assimilation in crops. However, the difficulty in establishing a foreign microbe into a niche that is likely already occupied by 'local' microbes adapted to local conditions raises concerns around the feasibility of this approach. Instead, approaches that manipulate already-present microbes presents a more feasible approach. SynBio offers the opportunity to enhance the nitrogen fixation of soil microbes to increase the access of cotton to plant-available nitrogen and improve cotton's nutrient use efficiency (Waltz, 2017). This could be achieved through

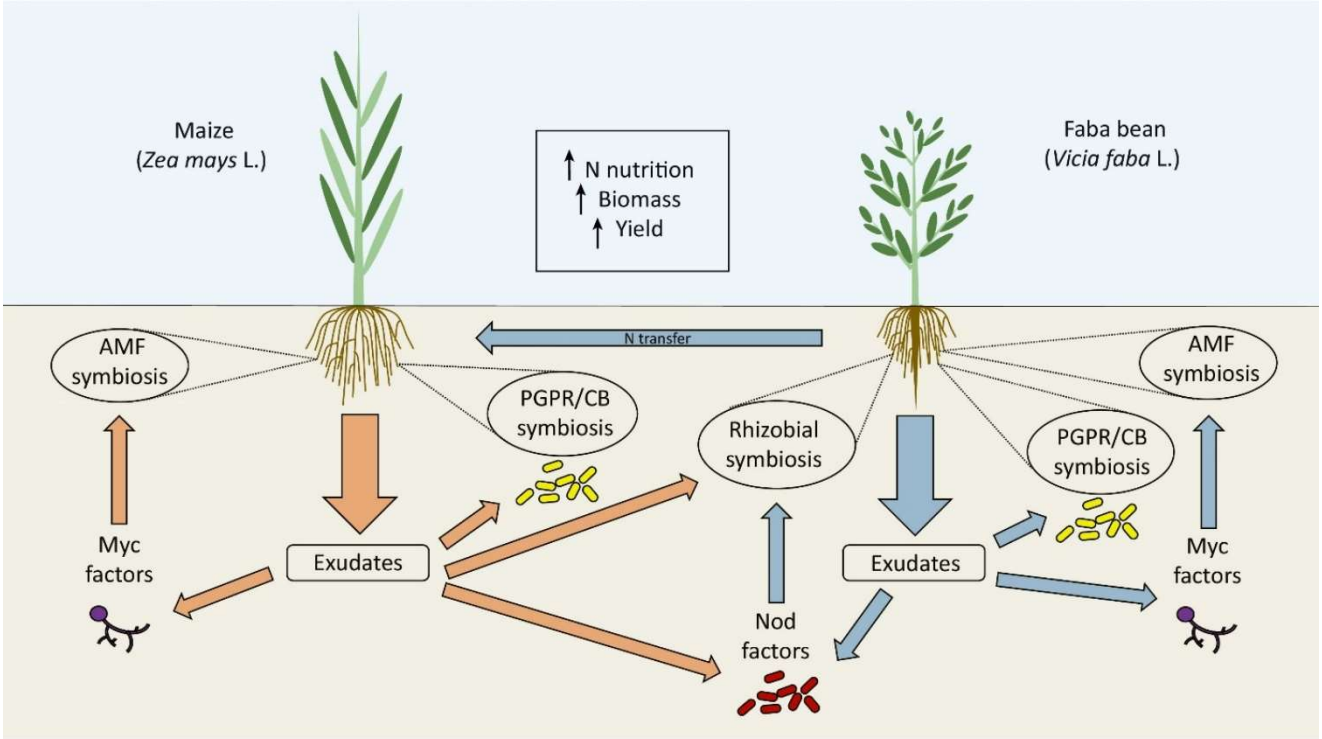

Figure 9. The influence of root exudates on symbiotic microbes in an intercropping system (from Coskun et al., 2017, Trends in Plant Science). AMF: arbuscular mycorrhizal fungi. PGPR: plant growth-promoting rhizobacteria. CB: cyanobacteria.

SynBio in two ways: i) engineer and re-introduce existing microbes, or ii) engineer crops to release favourable root exudates to manipulate or support microbial activity. The former has been identified as a "risky" option, due to the issue of controlling the location of genetically engineered material in microbial communities. Therefore, targeting ability of plants to attract beneficial microbes or manipulate microbial pathways and thus the nitrogen cycle through the production of root exudates (Bardgett et al., 2014, Coskun et al., 2017, Finzi et al., 2015) is a more feasible agronomic application. This approach will rely on an improved

understanding of species-specific interactions between cotton and available microbes, and the composition of root exudates (i.e. carbohydrates, flavonoids and terpenoids identified through metabolomics or transcriptomics) required to attract beneficial microbes or stimulate biological nitrogen fixation and belowground nitrogen transfer (Coskun et al., 2017). After this, the genes associated with such exudate components will need to be identified (i.e. through genomics, transcriptomics or CRISPR-Cas9) in order to be upregulated or modified through SynBio (i.e. through Golden Gate cloning or CRISPR-Cas9). Optimising root exudate release to improve crop nitrogen use efficiency could also reduce nitrogen loss via leaching, runoff and denitrification, thus mitigating nitrogen pollution (Coskun et al., 2017) from cotton production. An important consideration of this research would be to avoid making root exudates that are also attractive or beneficial to pathogens such as Verticillium. However, this interaction also highlights the opportunity to target root exudates for Verticillium wilt resistance (Zhang et al., 2020). An ideal SynBio approach to address both soil-borne fungal diseases and nutrient assimilation would be to express root exudates with the optimal composition to attract beneficial microbes and deter/inhibit pathogenic microbes (personal communication: Jonathan Plett).

3) **Establishing rhizobium-legume-like interactions**. Inducing nodule-formation and microbe-recruitment in non-leguminous crops to mimic rhizobium-legume symbiosis (Rogers and Oldroyd, 2014, Huisman and Geurts, 2020) could be another opportunity to enhance cotton's nutrient assimilation while improving soil fertility. This would require the expression of particular root exudate compounds such as flavonoids that induce the expression of nodulation factors in local microbes that trigger root nodule formation (Beatty and Good, 2011, Oldroyd et al., 2009). Despite the immense interest and research in this field, developing nodulation in non-leguminous crops is yet to be achieved. Hundreds of genes involved in nodulation in legumes have been identified, but selecting the combination required to induce nodulation in a non-nodulating crop has proven challenging (Huisman and Geurts, 2020). CRISPR-Cas9 and RNAi have been suggested as tools to help narrow this search and identify genes that are required for nodulation, while Golden Gate cloning technology is likely the most efficient approach to transferring the large number of genes that are likely to be required to successfully induce and support nodulation and nitrogen fixation (Huisman and

Figure 10. Nodulation on legume roots. Image from https://biology.anu.edu.au/news-events/news/nodulation-legumes.

Geurts, 2020). Huisman and Geurts (2020) presents a comprehensive review outlining the limitations and requirements of engineering nodulation in crops.

Enabling crops to fix their own nitrogen, particularly when the crop needs it rather than when it is convenient for the grower to apply, would be highly valuable and environmentally beneficial. Introducing nitrogenases and modifying plant-microbe interactions are highly complex, thus will likely require the transfer of large gene constructs with regulated promoters for specific expression patterns. For example, introducing nitrogenases requires the coordinated and localised expression of at least 16 *nif* genes (Dixon et al., 1997, Temme et al., 2012) in the mitochondria (Vicente and Dean, 2017, and references therein) to overcome its oxygen sensitivity issues (Allen et al., 2017). Therefore, more advanced techniques such as Golden Gate would be superior over traditional genetic engineering approaches (Rogers and Oldroyd, 2014). One potential criticism of inducing nitrogen fixation in crops is the potential yield penalty associated with increased demands on photosynthetic productivity required to support nitrogen fixation. This supports the need to also target photosynthetic enhancement, as discussed in the previous subsection. SynBio offers the most efficient means of complex multi-trait development required to overcome these challenges, that have the additional benefit of stimulating significant yield gains.

Seed oil

Opportunities:
Healthier/fortified cottonseed oil
Stable (high palmitic/stearic acid) cottonseed oil

Cottonseed oil is a major contributor to total vegetable oil production, ranked the 2nd major source of vegetable oil globally (Ashokkumar and Ravikesavan, 2013). Although it is considered a valuable by-product of cotton lint production, breeding efforts tend to focus on fibre production and overlook oil quality. This oversight has left cottonseed oil behind other major oilseed crops in terms of consumer demand, resulting in recent reductions in market shares (Sharif et al., 2019, Shockey et al., 2017). Cottonseed oil is comprised of ~55 % polyunsaturated fatty acids (PUFA; linoleic acid), ~17 % monounsaturated fatty acids (MUFA; oleic acid) and ~26 % saturated fatty acids (primarily palmitic and stearic acid; Lukonge et al 2007). Cottonseed oil is considered a "heart oil" due to its fatty acid profile and absence of cholesterol. It is also favoured as a cooking oil due to its favourable taste and cooking qualities associated with its saturated and unsaturated fatty acid ratios (Agarwal et al., 2003).

Figure 11. Cottonseed oil. Image from istockphoto.com/TolikoffPhotography.

Although cottonseed oil is considered moderately rich in PUFA and MUFA, further enhancing the nutritional properties (e.g. high oleic/DHA/omega content) would improve the health status of this oil to compete with the most highly regarded "healthy" oils. For example, cottonseed oil contains less than half the amount of oleic acid of peanut or sunflower oil, and not as rich in lineoleic acid as safflower oil (Sekhar and Rao, 2011). The production of these fatty acids could be stimulated through SynBio approaches. For example, the RNAi gene

silencing platform used to increase oleic acid production up to 80 % in linseed (Chen et al., 2015) could be deployed in cotton to increase the production of desirable fatty acids.

Gene silencing through RNAi has been shown to improve cottonseed oil quality. The toxic terpenoid compound gossypol has been removed from seeds through the generation of glandless cotton (Risco and Chase, 1997, Sunilkumar et al., 2006). This was achieved through RNAi to disrupt the gossypol biosynthesis pathway (Sunilkumar et al., 2006). Early attempts resulted in increased susceptibility of cotton plants to insect pests and pathogens due to the important protective role of gossypol (Sunilkumar et al., 2006). However, tissue-specific reduction of gossypol in seeds can be achieved through the adoption of gene silencing by RNAi with tissue-specific promoters. Despite the potential value of gossypol-free cottonseed oil, this is yet to be incorporated into cultivars.

In contrast, improving the stability of cottonseed oil through increasing the proportion of stable fatty acids such as stearic and palmitic acid is also desirable. Although this would reduce the "healthy" status of cottonseed oil, this would still be a healthier substitute for partially hydrogenated vegetable oils (*trans* fats) and serve as a more sustainable replacement of palm oil in baking and food processing (Hayes and Pronczuk, 2010, L'Abbé et al., 2009). This would also further improve the value and application of cottonseed oil. Using RNAi, Liu et al. (2017) successfully increased the palmitic acid and oleic acid content in cottonseed oil; however, this was at the expense of stearic acid. Lukonge et al. (2007) noted similar difficulties in developing cotton cultivars with higher percentages of stearic and linoleic acid through breeding. Additional research, likely in the field of SynBio rather than conventional breeding, is required to optimise the content of these stable fatty acids in cottonseed.

However, there are multiple challenges that have led to cottonseed oil becoming an undesired product, which is a likely explanation for why these potentially valuable seed oil traits haven't been incorporated into cotton. Cottonseed is no longer being crushed for oil in Australia in part because the value of cottonseed as stockfeed is higher than cottonseed oil, and cottonseed must be dehulled to process into oil. Cottonseed oil production, and thus investment in its improvement, would only be viable if its value exceeds the value of stockfeed cottonseed, but the volatility of the value of each of these products renders this area risky for investment.

Benefits, Limitations & Considerations of SynBio in Agriculture

Benefits:

SynBio is viewed as a field of biology that could be the key to achieving the "next Green Revolution" that is required to sustainably feed a growing population in increasingly challenging circumstances. It is currently believed that traits and properties that can be selected for should be targeted by breeding approaches rather than SynBio approaches due to the expense and risk of these relatively 'new' approaches (verbal communication, Steve Swain). However, traits that have reached their genetic potential through conventional breeding would benefit from SynBio approaches by targeting traits from distantly related species, other organisms or synthetically generated. SynBio can also generate non-breeding solutions, ranging from topical applications against pests and diseases, generating sterile pests and weeds, and novel properties such as enhanced oil profiles.

SynBio provides a range of tools to develop more complex traits and properties in crops more rapidly than other approaches. A good example of this is C_4 rice, a project that seeks to improve photosynthetic, nitrogen- and water-use efficiency of rice. This goal requires the conversion of its photosynthetic system from C_3 to C_4, a complex strategy that involves complex anatomical and biochemical changes (Ermakova et al., 2020b, Hibberd et al., 2008, Hibberd and Covshoff, 2010, Langdale, 2011, Sedelnikova et al., 2018). Introducing up to 20 genes is required to "completely rewire" rice metabolism and anatomy (Ermakova et al., 2020b). The construction of such large and complex multi-gene vectors has largely been enabled by falling gene synthesis costs, synthetic promoter systems and the establishment of complex DNA assembly techniques such as Golden Gate (Ermakova et al., 2020b, Rogers and Oldroyd, 2014). Gene synthesis through techniques such as Golden Gate have enabled rapid and simple assembly of gene modules often in a 'on pot cloning' approach. The growth and development of synthetic biology in the last 5 years has enabled the C_4 rice project to adopt a more rapid cycle of design, test and prototype coupled to the adoption of a rapid Agrobacterium-based rice transformation system in a rice variety that is fast flowering, day neutral, small and an established model for functional genomics (Ermakova et al., 2020b, Li et al., 2017a). Therefore, synthetic biology offers multiple approaches to achieve complex and ambitious crop modifications that are more rapid than other approaches such as conventional breeding.

Molecular switches (i.e. regulated promoters) are another tool that can have agronomic applications. Molecular switches activate a specific gene under specific conditions, thus activating a trait or response only when needed. This is an efficient system that can reduce resource waste (i.e. chemical defence production) when not needed. This technology can develop "Smart Plants" that can adjust to the environment in new ways (Wright and Nemhauser, 2019). This could be a valuable application in targeting abiotic stress resilience (Degen et al., 2020) through the development of cotton cultivars that 'activate' resilience genes under the initial detection of abiotic stress conditions.

Considerations:

Solutions need to be simple, accurate and affordable, addressing the challenges faced by resource-poor farmers and underserved consumers (Pixley et al., 2019). As agriculture becomes more globalised, issues and solutions would benefit from extending beyond an Australian context in order to be economically viable and impactful. Additionally, equitable access to the benefits of resulting GM crops requires affirmative policies, targeted investments and excellent science (Pixley et al., 2019). Lack of success in some SynBio projects has been due to focusing on humanitarian or environmental sustainability goals that are difficult to monetize (Pixley et al., 2019). Financial benefit to multiple aspects of the value chain need to be carefully considered to ensure SynBio projects are high value and impactful.

Limitations:

One of the biggest limitations constraining the progress of SynBio application is the cost of deregulation and social licencing. Social acceptance is also a significant challenge to the successful adoption of SynBio. Additionally, successful adoption, application and acceleration of SynBio will rely on investment by various sectors.

Another significant limitation that has slowed the progress of applying SynBio is the requirement to identify the precise gene(s) required for a targeted function. This is particularly challenging and prolonged when addressing multigenic functions. Verticillium wilt is a good example of such a challenge. Despite the monumental effort that has gone into the attempted development of Verticillium-resistant cotton varieties, much more research to uncover the genetic information behind this disease is required before SynBio can be applied. Therefore, the application of SynBio will rely on the success of fundamental molecular and functional genomics research.

Development of transgenic cotton plants depends on the optimisation of a suitable transgene transfer and integration procedure. Currently, cotton is predominantly transformed through *Agrobacterium* and particle bombardment-mediated gene transfer. Regeneration of plants from transformed tissue has seen limited success arising from problems such as somaclonal variation which prolongs culture periods, high frequency of abnormal embryo development, low conversion rate of somatic embryos into plantlets and high genotype-dependency (Mishra et al., 2003, Stelly et al., 1989, Sun et al., 2006). Additionally, only one cotton cultivar, Coker, is amenable to transformation. Further research is required to streamline the transformation and regeneration process and develop a transformation pathway in elite germplasm.

Finally, any SynBio opportunity requires significant investment, both in time and finances, to yield a technology, product or trait. Many years, likely a decade or longer, of fundamental and proof-of-concept research, is required prior to the years (likely decades) required to develop a SynBio technology, product or trait. Therefore, committing to a SynBio development opportunity would depend on a partnership or co-investment to invest adequate time, expertise and money into the project to be successful.

Conclusions

The opportunities offered by SynBio to address various agronomic challenges are "endless". However, assessing these opportunities with a *realistic* lens is critical. Despite the substantial efficiency and effectiveness that can be achieved through SynBio techniques, there are numerous challenges that have and continue to limit the successful application of SynBio. Numerous risks, costs, regulations and public perceptions can limit the uptake of these technologies. Additionally, developing SynBio technologies, products and traits requires substantial investment - decades of research and development, and millions of dollars - to be successful. Therefore, significant commitment in just one or two SynBio projects would be advised. Ultimately, these outcomes need to have a clear value proposition, be highly impactful (i.e. significantly improve cotton lint yield (>10 %) or quality) and have a high benefit to cost ratio in order to be feasible.

Overall assessments of the potential SynBio opportunities for the Australian cotton industry are as follows:

Silverleaf Whitefly RNAi
Using RNAi technology to protect cotton against silverleaf whitefly is promising and likely to be highly valuable. Given the challenges of manufacturing substantial quantities of RNAi/Bioclays, the incorporation of RNAi as a spatially expressed trait in cotton is likely to be more successful, and 'self-managed'. Fundamental research is emerging in this field in various crops and various insect pests, which has provided substantial insight into the potential of this approach. Further fundamental research is required to identify gene targets with higher lethality and suitable phloem-specific promoters in cotton. *A long-term investment aided by co-investment would be required.*

Dual Verticillium resistance
The development of dual pathotype Verticillium resistance may be one of the most valuable opportunities for the cotton industry. However, despite substantial activity this area of research, gains are yet to be made. Therefore, substantial fundamental research is required to identify the suite of genes required for resistance against both pathotypes without yield penalty, followed by various trials to determine the optimal location of expression. Future long-term investment could be considered to stack such genes using Golden Gate. However, further understanding around the molecular biology of Verticillium wilt is required (i.e. by CSIRO). *Significant knowledge gaps remain in this field. Investment in the substantial fundamental research required to bring this field to the level of applied SynBio research is likely to require significant investment.*

Enhanced carbon assimilation

Research is currently underway and supported by CRDC (UWS2201) to determine whether modifications to Rubisco, Rubisco activase and other photosynthetic proteins (e.g. SBPase, ATPase, HSPs) can improve the photosynthetic thermotolerance of cotton. This work aims to use Golden Gate to stack genes for superior Rubisco, Rubisco activase and other identified proteins in the next 3 - 6 years. Alternative approaches, such as introducing C_4 or CAM photosynthetic machinery are also possibilities being tested in other crops, but these are much more complex transformations that should only be considered if SynBio applications don't arise from UWS2201. Improving CO_2 diffusion through aquaporins could further enhance carbon assimilation and WUE. Therefore, aquaporins present a new opportunity for investment.

Research currently supported by CRDC in preparation for SynBio application in the next 3 - 6 years. Additional research into the potential for aquaporins to improve cotton WUE could be a promising and comparatively simple future investment in addition to UWS2201.

Improved nutrient acquisition through microbial activity

The substantial research into nodulation is yet to successfully progress the generation of nodulation in various crops, likely due to the complex suite of genes required. Thus, investment in this area in cotton is not advised, unless future fundamental research reveals significant findings.

However, nitrogen-use efficient cotton would be highly valuable economically and environmentally. Improving NUE through nitrogenases is already being investigated by CSIRO. However, enhancing microbial activity or attraction through the modification of root exudates is an underexploited area in cotton that may hold potential and may be a simpler transformation using Golden Gate or CRISPR-Cas9. Additionally, altering root exudate composition to combat Verticillium wilt infection may warrant further exploration of root exudates for multiple valuable agronomic functions. This opportunity would require substantial fundamental research, thus requiring long-term investment. However, an initial screening study could assess the potential of cotton root exudates to improve microbial activity and Verticillium deterrence.

Long and short-term (scoping) investment opportunities of potential value exist. Substantial fundamental research required prior to applied SynBio research.

Improved cottonseed oil quality

Cottonseed oil is an undesired product in Australia - lower in value than stockfeed cottonseed.

Currently an unviable investment opportunity in Australia.

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Nutrient Acquisition	Jonathan Plett (WSU) Oliver Knox (UNE) Blake Palmer (NSW DPI) Shiming Liu (CSIRO) Craig Wood (CSIRO) Eleonora Egidi (WSU) Brajesh Singh (WSU)
Seed Oil	Warwick Stiller (CSIRO) Craig Wood (CSIRO)
General	Warwick Stiller (CSIRO) Steve Swain (CSIRO)

Appendix

Table 2. Synthetic biology applications for future agriculture and food. Table from Roell and Zurbriggen (2020).

Approach	Description	Species	Reference
Improving plant growth and agricultural yield			
Improving carboxylation reactions			
C4 photosynthesis in C3 plants	Implementation of C4 photosynthesis in C3 species includes biochemical and developmental (Kranz-Anatomy) engineering with the most prominent example of the C4 Rice Project (https://c4rice.com).	C3 crop plants (e.g. *Oryza sativa*)	(Schuler et al., 2016)
Implementation of carbon-concentrating-microcompartments	Implementation of algae (pyrenoid) or cyanobacterial (carboxysomes) carbon concentrating mechanism in plant chloroplasts to suppress RubisCO oxygenase activity	C3 crop plants (e.g. *Oryza sativa*)	(Mackinder et al., 2016, Long et al., 2018)
Synthetic pathways for CO_2 assimilation	*In vitro* CO_2 fixation using a synthetic pathway composed of 17 enzymes (CETCH cycle)		(Schwander et al., 2016)
Minimizing (photo)–respiratory CO_2losses			
Chloroplastic photorespiratory bypass	Oxidation of glycolate in the chloroplast to release two CO_2 molecules and native photorespiratory flux knockdown resulted in a 40% biomass increase under field conditions	*Nicotiana tabacum*	(South et al., 2019)
Synthetic CO_2 neutral photorespiration	*In vitro* conversion of glycolate into glycoly-CoA and re-assimilation into the CBBC without CO_2 and nitrogen release. Two enzymes were engineered for substrate and co-factor specificity		(Trudeau et al., 2018)
Minimizing respiratory CO_2 loss	Potential targets: i) optimize protein turnover ii) redesign respiratory metabolism, iii) avoid futile cycle, iv) efficient ion transport		(Amthor et al., 2019)
Improving water use efficiency and photosynthetic light reactions			
Optogenetic manipulation of stomatal kinetics	Guard-cell specific of a synthetic blue light-gated K^+-channel to allow rapid response of stomatal opening under fluctuating light conditions	*Arabidopsis thaliana*	(Papanatsiou et al., 2019)
Accelerating recovery from photoprotection	Overexpression of *PsbS* and xanthophyll cycle enzymes resulted in a faster restoration of maximum CO_2 assimilation from nonphotochemical quenching of chlorophyll fluorescence	*Nicotiana tabacum*	(Kromdijk et al., 2016)

Approach	Description	Species	Reference
Design Breeding			
De novo domestication	Genetic manipulation of several domestication genes in wild type plants enables a timesaving domestication process	*Solanum lycopersicum*	(Zsögön et al., 2018)
Reducing synthetic fertilizer usage in agriculture			
Establish functional nitrogenase or symbiotic nitrogen fixation in crop plants			
Functional nitrogenase in plants	Expression of 16 nitrogenase genes in plant mitochondria	*Nicotiana benthamiana*	(Allen et al., 2017)
Symbiotic nitrogen fixation in crop plants	Requires the expression of four regulatory programs. The SynSym international consortia addresses questions regarding synthetic nitrogen fixation (https://synthsym.org)	Several crop plants	(Rogers and Oldroyd, 2014)
Synthetic microbiota for improved nutrient utilization			
Cultivation with growth promoting plant microbiome bacteria	Different Rhizobiales isolated supported growth of *Arabidopsis*. In particular, taxonomic groups containing nitrogen-fixing nodule symbionts	*Arabidopsis thaliana*	(Garrido-Oter et al., 2018)
Plant microbiome composition	Identification of root-associated fungus in non-mycorrhizal plants to improve phosphorous utilization	*Arabis alpina*	(Almario et al., 2017)
Construction of synthetic microbiota for crops	Within the private sector engineering the microbiome of crops is already addressed	Several crop plants	(Waltz, 2017)
Increasing the nutritional value of crop plants			
Increase provitamin A content	GoldenRice project (http://www.goldenrice.org)	Oryza sativa	(Beyer, 2010)
Increase VLC-PUFA content	Seed-specifically expression of VLC-PUFAs biosynthetic genes	*Brassica napus Canola sativa*	(Ruiz-Lopez et al., 2014, Napier et al., 2019)
Remove cyanogenic glycosides	RNA interference targeting two cytochrome P450 genes	*Manihot esculenta* Crantz	(Jørgensen et al., 2005)
Increased anthocyanin content	Fruit-specific expression of two transcription factors (*Del* and *Ros1*) inducing anthocyananin biosynthesis	*Solanum lycopersicum*	(Butelli et al., 2008)
Reduced gluten content in wheat	CRISPR/Cas9 mediated knockout of up to 45 genes in wheat to lower gluten content	*Triticum aestivum*	(Sánchez-León et al., 2018)
Vitamin B_{12} biosynthesis in plants	Engineering *E. coli* for *de novo* vitamin B_{12} biosynthesis		(Fang et al., 2018)
Photoautotrophic organisms as production platform			

Approach	Description	Species	Reference
Vaccine and cosmetic production	Use mosses as green cell factory for the production of vaccines and cosmetics	*Physcomitrella patens*	(Reski et al., 2018)
Scalable production of artemisinin in biomass crops	Chloroplastic expression of the core artemisinic acid biosynthesis pathway and additional enzymes to improve flux through the pathway	*Nicotiana tabacum*	(Fuentes et al., 2016)
Improving saccharification efficiency	TALEN-mediated mutagenesis of more than 100 caffeic acid O-methyltransferase alleles in polyploid sugarcane to improve the saccharification efficiency for biofuel production	*Saccharum officinarum*	(Kannan et al., 2018)
Synthetic or biohybrid systems	Construction of artificial leaves and synthetic photosynthetic cell as solar energy driven production platforms		(Nocera, 2012, Berhanu et al., 2019)

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