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REVIEW PAPER

Synthetic biology approaches to engineering the nitrogen symbiosis in cereals

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Abstract

Nitrogen is abundant in the earth's atmosphere but, unlike carbon, cannot be directly assimilated by plants. The limitation this places on plant productivity has been circumvented in contemporary agriculture through the production and application of chemical fertilizers. The chemical reduction of nitrogen for this purpose consumes large amounts of energy and the reactive nitrogen released into the environment as a result of fertilizer application leads to greenhouse gas emissions, as well as widespread eutrophication of aquatic ecosystems. The environmental impacts are intensified by injudicious use of fertilizers in many parts of the world. Simultaneously, limitations in the production and supply of chemical fertilizers in other regions are leading to low agricultural productivity and malnutrition. Nitrogen can be directly fixed from the atmosphere by some bacteria and Archaea, which possess the enzyme nitrogenase. Some plant species, most notably legumes, have evolved close symbiotic associations with nitrogen-fixing bacteria. Engineering cereal crops with the capability to fix their own nitrogen could one day address the problems created by the over- and under-use of nitrogen fertilizers in agriculture. This could be achieved either by expression of a functional nitrogenase enzyme in the cells of the cereal crop or through transferring the capability to form a symbiotic association with nitrogen-fixing bacteria. While potentially transformative, these biotechnological approaches are challenging; however, with recent advances in synthetic biology they are viable long-term goals. This review discusses the possibility of these biotechnological solutions to the nitrogen problem, focusing on engineering the nitrogen symbiosis in cereals.

Key words: Maize, nitrogen, nitrogen fixation, nodulation, sub-Saharan Africa, symbiosis, synthetic biology.

Introduction

The global population has rapidly expanded from 2.5 billion in 1950 to over 7 billion people today and is predicted to rise to over 9 billion by 2050 (UN DESA, 2013). The associated increased demand on food production was met in part through the Green Revolution of the 1960s when yields were substantially raised in many parts of the world through the increased use of chemical fertilizers and high-yielding varieties. Today around 120 teragrams (Tg) of nitrogen are chemically fixed every year, around 80% of which is used as agricultural fertilizer (Galloway *et al*., 2003, 2004). Typical nitrogen-use efficiencies for wheat, rice, and maize indicate that around 66% of this nitrogen is lost to the environment (Raun and Johnson, 1999), either in the form of nitrous oxides, which are potent greenhouse gases, or as soluble nitrates that find their way into aquatic systems (Glendining *et al.*, 2009). Around 100 million tonnes is deposited globally in terrestrial, freshwater, and marine environments every year, a three-fold increase over preindustrial levels (Galloway *et al.*, 2008; Rockström *et al.*, 2009). Nutrient excesses are especially large in China, northern India, the USA, and Western Europe, leading to widespread nutrient pollution (Foley *et al.*, 2011). In addition, the energy-intensive

Abbreviation: SYM pathway, symbiosis signalling pathway.

production of nitrogen fertilizers is the greatest use of fossil fuels in agriculture, with predictions that this process will consume around 2% of global energy by 2050 (Glendining *et al.*, 2009). For all these reasons it is desirable to reduce agricultural reliance on nitrogen fertilizers.

However, reducing reliance on agricultural fertilizers must be balanced with the predicted requirement of doubling agricultural productivity to meet the demands of a growing global population with changing consumption patterns (Beddington, 2010). With nearly 40% of the earth's ice-free land already under agricultural production (Ramankutty *et al.*, 2008) and crop yields in the developed world reaching capacity (Mueller *et al.*, 2012) there is little scope to meet this demand through agricultural expansion or yield improvements in the West. Even today, with all the agricultural intensification nearly a billion people remain chronically malnourished across the globe, a quarter of whom reside in sub-Saharan Africa (Foley *et al.*, 2011). The mean baseline maize yield in sub-Saharan Africa is 1.58 Mg·ha^{-1} (Folberth *et al.*, 2013) compared to 10 Mg·ha^{-1} reported for the USA (USDA NASS, 2013; Fig. 1). The primary reason for low yields in sub-Saharan Africa is the limitations in the production and supply of nitrogenous and phosphorus fertilizers (Mueller *et al.*, 2012). The world average fertilizer application rate for maize is 134 kg·ha^{-1}, but rises to 220 kg·ha^{-1} in parts of the developed world. This contrasts with sub-Saharan Africa where application rates average only 3–5 kg·ha^{-1} (Foley *et al.*, 2011; Folberth *et al.*, 2013).

Increasing cereal yields through the increased availability and use of fertilizer in sub-Saharan Africa could address both the chronic malnutrition and support economic development in this region, while helping to meet the global demand for increased food production (Tilman *et al.*, 2011). But, as has been seen in other parts of the world, this agricultural transformation would come with significant environmental costs on both a regional and global scale.

A biotechnological approach where cereal crops are engineered to fix nitrogen has the potential to reduce fertilizer use in the developed world and greatly reduce the environmental impact of increasing yields in the developing world. However, replacing nitrogen fertilizer in the developed world would require levels of nitrogen fixation in cereals equivalent to those that occur in legumes, and would therefore be extremely challenging. A much more feasible goal from a biotechnological approach would be to initially aim to provide levels of fixed nitrogen equivalent to 25–50 kg·ha^{-1}. Figure 1 (adapted from Folberth *et al.*, 2013) models the yield responses of maize to this level of nitrogen under two scenarios: high-yielding cultivars with sufficient irrigation and cultivars commonly used in sub-Saharan Africa, under typical irrigation conditions. Engineering lines that are capable of providing fixed nitrogen equivalent to 50 kg·ha^{-1} would more than double yields in

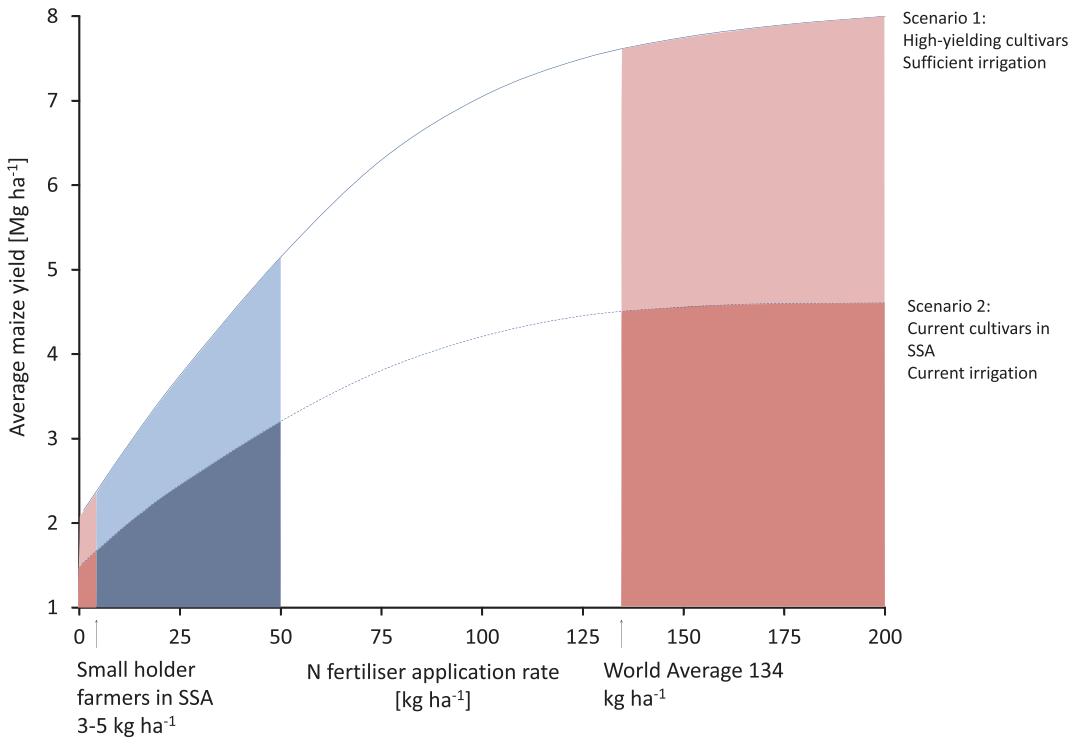

Fig. 1. Simulated maize yields at different rates of N fertilizer application in sub-Saharan Africa (SSA). Yields of maize were estimated for fertilizer increases to different levels under sufficient irrigation for a high-yielding cultivar (solid line) and under current levels of irrigation for the low-yielding cultivars commonly used in SSA (dashed line). Yields increase linearly up to 50 kg·ha^{-1} for current irrigation and cultivar inputs and up to 100 kg·ha^{-1} for conditions of sufficient irrigation and a high-yielding cultivar. The blue regions represent opportunities to double or triple yields in SSA by supplying nitrogen equivalent to these low application rates. Reprinted from Folberth C, Yang H, Gaiser T, Abbaspour KC, Schulin R. 2013. Modeling maize yield responses to improvement in nutrient, water and cultivar inputs in sub-Saharan Africa. *Agriculture, Ecosystems and Environment* **119,** 22–34. With permission from Elsevier.

either scenario. As engineered plants would receive nitrogen directly to the roots of plants, the absolute amount of fixed nitrogen required to mimic the response to applied nitrogen would be much lower. Folberth *et al.* (2013) observed that under the scenario for current irrigation and existing cultivars the increased production would provide about 10 times more maize than the net import of maize and food aid (cereals) to this region, going a long way towards delivering food security for sub-Saharan Africa. While shorter-term opportunities exist for increasing yields to small-holder farmers in the developing world (Vanlauwe and Giller, 2006), there is significant potential for yield improvements through biotechnological solutions that can reduce environmental impacts as agriculture is intensified in these regions.

One potential criticism for moving the legume symbiosis into cereals is the potential for a yield penalty associated with the increased demand on photosynthates required to support nitrogen fixation. This is likely to be an issue in situations where one is attempting to replace inorganic fertilizers, for instance in intensive agricultural systems in the developed world. However, in low-input agricultural systems, such as smallholder farming in sub-Saharan Africa, the limits on nutrient availability are almost certainly limiting photosynthetic capacity. In such low-yielding environments the introduction of nitrogen fixation should facilitate improved photosynthetic capability and this is likely to far outweigh any increased demand on photosynthates associated with nitrogen fixation. Thus, we would anticipate yield increases for smallholder farmers at least at the lower end of engineered nitrogen fixation (Fig. 1), with yield penalties only likely to have an impact with increasing levels of nitrogen fixation.

Legumes have evolved the capability to associate with nitrogen-fixing bacteria, which are housed inside nodules on the roots of the plant (Oldroyd and Downie, 2006). This interaction delivers fixed nitrogen to the plant in the region of 120 $kg \cdot ha^{-1}$ (Salvagiotti, 2008) and reveals what is feasible for engineering into cereals. There are multiple biotechnological approaches currently being explored that could deliver fixed nitrogen to cereal crops (Beatty and Good, 2011; Oldroyd and Dixon, 2014). One scenario focuses on engineering a nitrogen-fixing symbiosis in cereal roots, either through transferring the legume-rhizobial interaction to cereals or through improving pre-existing associations in cereal roots. Alternatively, the nitrogenase enzyme itself could be introduced into organelles of plant cells to create a new nitrogen-fixing capability. This is an attractive solution and is currently underway in at least two major projects but has two notable challenges. Firstly, nitrogenase is a highly complex enzyme and would require the coordinated expression of at least 16 *nif* genes (Dixon *et al.*, 1997; Temme *et al.*, 2012). Secondly, nitrogenase activity has high energetic demands but while aerobic respiration is therefore essential, nitrogenase is irreversibly denatured by oxygen. Unlike symbiotic nitrogen fixation, no eukaryotes have evolved a nitrogen-fixation capability, despite plastids being derived from endophytic cyanobacteria. This might indicate there are fundamental barriers to nitrogenase activity in plant plastids. Engineering a nitrogen-fixing symbiosis can provide solutions to these problems found in nature, by adapting existing signalling and developmental mechanisms to provide a suitable environment for nitrogenase activity in the plant nodule.

Both of these approaches are highly challenging and it is unlikely that in the short term any will deliver the levels of fixed nitrogen equivalent to fertilizer application rates in the developed world. However, even low levels of nitrogen fixation could be transformative for crop yields in the developing world (Fig. 1). It is hoped that these biotechnological approaches may gradually reduce the requirement in agriculture for inorganic fertilizer. Work is currently underway in major projects addressing the challenges of both these biotechnological approaches. For an assessment of both approaches see the recent review by Oldroyd and Dixon (2014). In this review we focus on one approach: recapitulating the nitrogen-fixing capability of legumes in cereals.

Transferring the rhizobium–legume symbiosis

The rhizobium–legume symbiosis is initiated by flavonoids released from the plant roots that stimulate rhizobia to produce the signalling molecule Nod factor (Long, 1996). The perception of Nod factor drives a host of developmental responses in the compatible plant host that prepares the plant to accommodate the endosymbiotic partner. Perception of Nod factor initiates two coordinated genetic programmes: the initiation of cell divisions in the root cortex and the development of an infection thread (or IT) in the root epidermis (Madsen *et al.*, 2010). The infection thread is an invagination of the plasma membrane and cell wall that extends from the tip of the root hair carrying rhizobia into the root cortex towards the site of the developing nodule (Murray, 2011). The nodule is a specialized root organ that provides a suitable environment for the enzymatic function of bacterial nitrogenase. Within the cells of the nodule bacteria differentiate into nitrogen-fixing organelle-like structures known as bacteroids, contained within a plant-derived symbiosome membrane (Udvardi and Poole, 2013).

To completely reproduce the nitrogen symbiosis in cereals four co-ordinated genetic programmes would have to be introduced into cereals: recognition of Nod factors; organogenesis of the root nodule; bacterial infection and establishment of a suitable environment for nitrogenase activity inside the nodule. These four processes are not necessarily mutually exclusive; however, they provide a useful framework for structuring the different aspects of work necessary in this biotechnological approach. Our knowledge is currently most advanced to approach the first step: engineering Nod factor signalling in cereals. Hence, this review focuses mostly on this aspect.

Within the larger legume family there is a wide diversity of nodule structures and mechanisms of rhizobial infection (Sprent, 2001). Nodules can range from simple swellings on the surface of the root/stem to highly differentiated structures. Bacterial infection mechanisms are equally diverse, ranging from entry through cracks (Capoen *et al.*, 2010) and

colonization of intercellular spaces through to the most complex forms of infection that involve root hairs and infection threads (Sprent, 2001). In all of these interactions, from the most primitive to the most highly evolved, the plant benefits from fixed nitrogen. However, it is very likely that greater complexity in the symbiotic interaction improves the efficiency of nitrogen fixation and thus provides greater benefit to the plant. This evolutionary trajectory reveals benefits to the plant even in the most primitive symbiotic structures and this is useful when considering engineering this process in cereals (Charpentier and Oldroyd, 2010). We might anticipate that, once a close association between the cereal root and the rhizobial bacteria has been achieved, some level of fixed nitrogen may be supplied to the plant. An engineering process could therefore follow this evolutionary path, whereby improvements in the complexity of the association provide efficiency gains and thus increased levels of fixed nitrogen. However, along this route of engineering a nitrogen-fixing nodule on a cereal root there may be effective delivery of fixed nitrogen at earlier stages.

The rhizobium–legume symbiosis is a relatively recent evolutionary innovation which has recruited mechanisms established to support a much more ancient endosymbiotic relationship with arbuscular mycorrhizal fungi (Markmann and Parniske, 2009). The commonalities between the two symbioses are extensive. Recent work has revealed that the Nod factors, the lipochitooligosaccharide (or LCO) signals of rhizobia, are structurally very similar to those mediating signalling during the mycorrhizal symbiosis, known as Myc factors (Maillet, 2011). Just like Nod factors, Myc factor perception leads to the activation of a common symbiosis signalling (SYM) pathway (Figs 2 and 3). Engineering nodulation signalling would therefore be engineering the perception of Nod factors to activate the SYM pathway and engineering the outputs of this pathway for nodulation-specific gene expression. The SYM pathway itself is well conserved between legumes and monocotyledons (Banba *et al.*, 2008; Gutjahr *et al.*, 2008) but we will first consider possible challenges that may exist in recruiting this existing signalling machinery in cereals to successfully transduce the newly engineered signal.

Engineering the common SYM pathway

In legumes a significant part of the Nod factor signalling pathway is required for both nodulation and mycorrhizal signalling, with at least 10 genetic components having dual functions. (reviewed in Oldroyd, 2013). Central to the SYM pathway is signalling via oscillations in calcium known as calcium spiking. Upstream of calcium spiking is a leucine-rich-repeat receptor kinase (SymRK; Endre *et al.*, 2002; Stracke *et al.*, 2002), two cation channels (CASTOR and POLLUX; Ané *et al.*, 2004; Imaizumi-Anraku *et al.*, 2005; Charpentier *et al.*, 2008), and three components of the nuclear pore (NENA, NUP85, and NUP133; Kanamori *et al.*, 2006; Saito *et al.*, 2007; Groth *et al.*, 2010). Meanwhile, downstream of calcium spiking is the calcium-activated kinase CCaMK (Catoira *et al.*, 2000; Lévy *et al.*, 2004; Mitra *et al.*, 2004) and its interacting partner IPD3/CYCLOPS (Yano *et al.*, 2008).

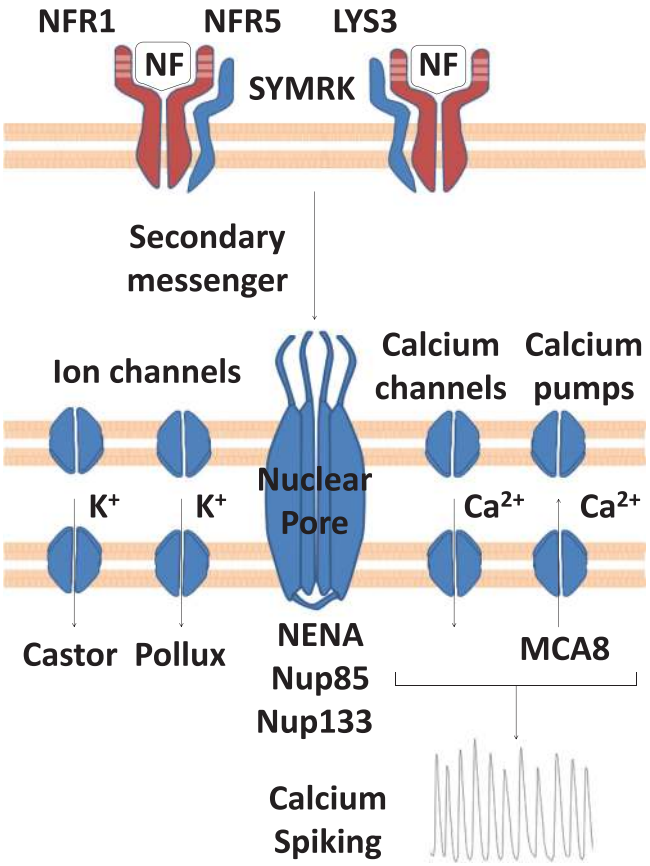

Fig. 2. Nod factor perception. Nod factor is recognized by the Nod factor receptors NFR1, NFR5, and LYS3 embedded in the plasma membrane. Perception also leads to the activation of SYMRK. Activation of this receptor complex leads to the formation of a secondary messenger which initiates calcium spiking driven by proteins in the nuclear envelope. Calcium spiking is dependent on three nuclear pore proteins, NENA, Nup85, and Nup133, the cation channels Castor and Pollux, as well as the calcium pump MCA8 and calcium channels which have yet to be identified. Components functioning in mycorrhizal signalling (blue) are already present in cereals, driving calcium spiking in response to Myc factors. The nodulation-specific components (red) are the targets for cereal engineering.

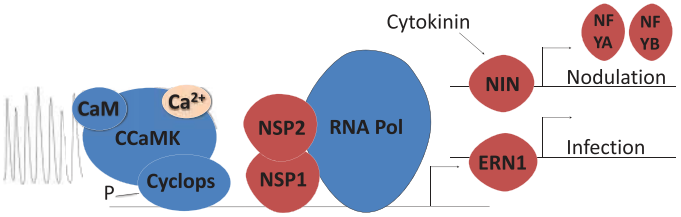

Fig. 3. Nodulation signalling downstream of calcium spiking. Calcium spiking is perceived by the binding of calcium and calmodulin (CaM) to CCaMK. The activation of CCaMK results in the phosphorylation of Cyclops. Signal transduction downstream of CCaMK involves the transcription factors NSP1 and NSP2. ERN1 is activated by NSP1 and NSP2 and is required for development of infection threads. NIN activation drives the transcription factors NF-YA and NF-YB that are associated with nodule organogenesis. Cytokinin signalling is also essential for nodule organogenesis and can direct induction of NIN. Nodulation signalling components are marked in red and mycorrhizal signalling components in blue, although NSP1 and NSP2 have been shown to be required for some aspects of mycorrhizal signalling.

The current understanding is that Nod factor perceived at the plasma membrane through NFR receptors, along with activation of the SymRK receptor, leads to the production of a secondary messenger which initiates perinuclear calcium spiking requiring the ion channels and nuclear pore components.

The SYM pathway has been shown to be present in cereals and essential for supporting the mycorrhizal symbiosis (Gutjahr *et al.*, 2008). Importantly, a significant number of the rice SYM signalling components have been shown to be capable of complementing reciprocal legume mutants, not only for mycorrhization, but also for nodulation (Banba *et al.*, 2008). CASTOR, CcaMK, and CYCLOPS all fall within this group and are unlikely to need to be engineered into cereals. Nucleopore-complex components NUP85, NUP133, and NENA are conserved in cereals and even in non-symbiotic species such as *Arabidopsis* (Roth *et al.*, 2012; Wiermer *et al.*, 2012). Although it has not been directly demonstrated, they are likely to have non-symbiotic roles and would probably function without modification in engineered cereals. Two other putative symbiosis specific SYM components, POLLUX and NSP2, are also present in non-symbiotic Brassicaceae (Heckmann *et al.*, 2006; Venkateshwaran *et al.*, 2013). These proteins may have a non-symbiotic function or may function in bacterial associations recently described in metagenomic studies in *Arabidopsis* (Bulgarelli *et al.*, 2012; Lundberg *et al.*, 2012). OsPOLLUX, while being structurally conserved, is unable to complement *dmi1* for mycorrhization or rhizobial infection (Markmann and Parniske, 2009); however, this has recently been explained by subtle differences in the pore complex between POLLUX and DMI1 and at least in *Lotus japonicus* this deficiency is compensated by the presence of CASTOR. OsSymRK is able to complement the legume mutant for mycorrhization, but not for nodulation phenotypes. This is unsurprising as OsSYMRK differs significantly in its domain structure and length between the legumes and monocots and only longer versions, present in all nodulating species, are able to support nodulation signalling in legume mutants. It has been suggested this is a key evolutionary innovation for the development of nodulation-specific signalling and a key target of engineering nodulation signalling in cereals (Markmann and Parniske, 2009).

The extensive conservation of SYM pathway components in rice (and other cereals) indicates they have an innate potential for engineering the SYM pathway to allow recognition of nitrogen-fixing bacteria. The impact of engineering this first step of rhizobial recognition and nodulation-specific signalling into cereals is unknown, but may allow a degree of bacterial colonization and would certainly provide a good foundation for the longer-term goal of engineering a nodule-based symbiosis in cereal crops.

Engineering Nod factor perception and the activation of the SYM pathway

Upstream of the common SYM pathway the first nodulation-specific step is the perception of rhizobia and is a function of the binding of Nod factor by two LysM receptor-like kinases, NFR1 and NRF5 in *L. japonicus* and NFP and LYK3 in *Medicago truncatula* (Fig. 2; Radutoiu *et al.*, 2003; Arrighi *et al.*, 2006; Smit *et al.*, 2007; Broghammer *et al.*, 2012). Given the structural similarities, it seems likely that Myc factors produced by arbuscular mycorrhizal fungi must be recognized by similar LysM receptor-like kinases, although the family members have yet to be elucidated in legumes (Young *et al.*, 2011). Recent evidence suggests that a third LysM receptor-like kinase related to NFR1, LYS3, enhances the efficiency of nodule formation in *L. japonicus*, especially under less favourable conditions (Lohmann *et al.*, 2010).

Host specificity of Nod factor recognition is a function of the LysM receptor-like kinases (Madsen *et al.*, 2003; Radutoiu *et al.*, 2003, 2007) and, by transferring the receptors, this specificity can be transferred between legume species (Radutoiu *et al.*, 2007), which bodes well for the engineering of cereals. A more phylogenetically distant transfer of a new bacterial recognition capability through the transfer of specific receptors has been demonstrated for defence signalling (Lacombe *et al.* 2010). This transfer of receptors between species implies that the underlying signal transduction machinery can be recruited by the receptors and we hope that this will also be true for the Nod factor receptors in cereals.

Engineering nodulation-specific outputs of the SYM pathway

The second nodulation-specific component of the legume SYM pathway which would need to be engineered into cereals relates to the suite of transcription factors activated downstream of the SYM pathway. A calcium- and calmodulin-dependent serine/threonine protein kinase (CCaMK) decodes the calcium spiking response (Catoira *et al.*, 2000; Lévy *et al.*, 2004; Mitra *et al.*, 2004). Association of CCaMK with calcium and calmodulin leads to the phosphorylation of CYCLOPS (Yano *et al.*, 2008) that is associated with gene expression. In a downstream or parallel event the GRAS-protein transcription factors NSP1, NSP2 (Kaló *et al.* 2005; Smit *et al.*, 2005), and RAM1 (Gobbato *et al.* 2012) form complexes to drive symbiont-specific gene expression in legumes. NSP1 and NSP2 are sufficient to activate the nodulation-specific ERF-transcription factor *ERN1* (Cerri *et al.*, 2012) and downstream transcription factors *NIN*, *NF-YA*, and *NF-YB* are involved in the initiation of nodule organogenesis (Fig. 3; Marsh *et al.*, 2007; Soyano *et al.*, 2013), along with components of cytokinin signalling (Murray *et al.*, 2007; Tirichine *et al.*, 2007).

Interestingly the deregulation of CCaMK is sufficient to lead to the generation of spontaneous nodules on the roots of legumes (Gleason *et al.*, 2006; Tirichine *et al.*, 2006).

For engineering cereals the gain-of-function CCaMK alleles allow the activation of this part of the SYM pathway without the necessity for Nod factor induction of calcium spiking, allowing analysis of the SYM pathway from calcium to gene expression. The impact of stacking the autoactive CCaMK and the nodulation-specific transcription factors in cereals can be assessed in terms of gene expression from promoter–reporter fusions as well as developmental markers associated with this signalling event.

Challenges in cereal engineering

Among the challenges in this engineering strategy is defining a suite of promoters to achieve stable expression in root tissues, particularly the epidermis and the cortex, for multiple different genes. The same promoter used to express multiple genes in the same line may activate gene silencing. Multiple promoters will have to be characterized and tested in combination. In the past the construction of such large multigene vectors would have been a limiting factor in engineering work. However, with the cost of gene synthesis falling and the establishment of DNA assembly strategies such as Golden Gate (Weber *et al.*, 2011) and Gibson Assembly (Gibson, 2011) it is now possible to rapidly create large numbers of multigene constructs extremely quickly. Modular assembly strategies such as Golden Gate also mean that as new signalling components are identified they can be rapidly synthesized and incorporated into engineering constructs along with the existing engineering modules. The DNA assembly tasks required to test large numbers of promoters individually and in combination are now trivial in the light of these advances in synthetic biology. Furthermore, next-generation sequencing methods allow the gene expression changes of any engineering process to be assessed quickly and comprehensively. The major limiting factor for engineering cereal species is now the testing of constructs *in planta*. Even where transformation is efficient a single generation in a species such as maize takes approximately a year and this sets the pace for testing constructs in these species. This highlights the importance of developing further good model grass systems such as the wheat relative *Brachypodium distachyon* (Brkljacic *et al.*, 2011) and the panacoid grass *Setaria viridis*, a recently developed model for maize (Doust *et al.*, 2009; Brutnell *et al.*, 2010). Establishing highly efficient transformation procedures in these species or finding ways to transiently express gene constructs in cereals is vital if these engineering strategies are to be successful.

Concluding remarks

Engineering nitrogen-fixing cereals has been a dream since the 1970s and in the past the feasibility of this has been overstated. However, major advances in our understanding of symbiosis signalling and the production of nodules in legumes means that we are now in a good position to initiate this engineering approach. Engineering the nitrogen-fixing capability into cereal crops is still an enormously challenging task but advances in the field of synthetic biology means that some of the technologies are now in place. The ability to discriminate and independently engineer the different parts of the SYM pathway means that we can engineer the component parts in isolation and this is currently the most tractable approach to this engineering problem. The production of cereal crops providing even a small amount of nitrogen could significantly raise yields in the low input agricultural systems of sub-Saharan Africa. If these first steps of engineering Nod factor signalling in maize were to be successful it would be a step forward for genome engineering and a solid foundation on which to build strategies for inducing nodule organogenesis, bacterial infection, and supporting nitrogen fixation in cereal roots.

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