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REVIEW

Nitrogen fixation in maize: breeding opportunities

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Abstract

Maize (*Zea mays* L.) is a highly versatile crop with huge demand of nitrogen (N) for its growth and development. N is the most essential macronutrient for crop production. Despite being the highest abundant element in the atmosphere (~78%), it is scarcely available for plant growth. To fulfil the N demand, commercial agriculture is largely dependent on synthetic fertilizers. Excessive dependence on inorganic fertilizers has created extensive ecological as well as economic problems worldwide. Hence, for a sustainable solution to nitrogenous fertilizer use, development of biological nitrogen fixation (BNF) in cereals will be the best alternative. BNF is a well-known mechanism in legumes where diazotrophs convert atmospheric nitrogen (N≡N) to plant-available form, ammonium (NH_4^+). From many decades, researchers have dreamt to develop a similar symbiotic partnership as in legumes to the cereal crops. A large number of endophytic diazotrophs have been found associated with maize. Elucidation of the genetic and molecular aspects of their interaction will open up new avenues to introgress BNF in maize breeding. With the advanced understanding of N-fixation process, researchers are at a juncture of breeding and engineering this symbiotic relationships in cereals. Different breeding, genetic engineering, omics, gene editing, and synthetic biology approaches will be discussed in this review to make BNF a reality in cereals. It will help to provide a road map to develop/improve the BNF in maize to an advance step for the sustainable production system to achieve the food and nutritional security.

Introduction

The world population is expected to grow to ~ 10 billion by 2050. Hence, to fulfil the food requirements agricultural production needs to be increased by 56% (Bloch et al. 2020). Cereals represent biggest share among crops in food basket. However, for sustainable agricultural production, the supply of sufficient nitrogen (N) is the biggest challenge to the cereal crops with 25 to 30% of nitrogen use efficiency (NUE). Worldwide, it has been estimated that plants require approximately 150 to 200 million tonnes of mineral N, out of which only about 100 million tonnes of N is produced through the industrial Haber–Bosch process (Unkovich et al. 2008). Industrial N production creates a number of environmental issues by emission of 3.6 kg CO_2-eq. per kg of N manufactured (Fossum 2014). On the contrary biological nitrogen fixation (BNF) provides a win–win situation under changing climate scenario. In BNF, without any cost the atmospheric N is converted into reduced forms by a group of prokaryotes called diazotrophs, such as cyanobacteria, free-living soil bacteria, associative bacteria (Azospirillum) and symbiotic bacteria (Rhizobium and Bradyrhizobium) (Postgate 1982). It has been evaluated that BNF generates approximately 80–90% of N available to plants diversity (Rascio and Rocca 2013). Through BNF, a total of 139 to 175 million tonnes of N is added to the earth's ecosystem and out of which 35–44 million tonnes N is contributed by symbiotic associations (Chafi and Bensoltane 2009; Bano and Iqbal 2016). Hence, it is one of the major contributors to the total biospheric N accounting for 30–50% of the overall N in crop fields (Ormeno-Orrillo et al. 2013). BNF is an integral process in leguminous crops, while cereal crops lack this ability. Considering the economic and environmental concerns related to N fertilizers, it will be a viable and sustainable approach to harness this mechanism in maize,

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which is produced in maximum quantity in the world, to boost its production sustainably.

Maize is the highest yielding cereal crop being cultivating in about 170 countries globally and covering ~ 194 million hectares area with 1147.6 million thousand tonnes production (FAOSTAT 2020). Maize is principally utilized for feed and industrial purposes, besides directly consumed as food or processed food in various parts of the world (Fig. 1). Its proportion of consumption varies considerably from country to country like in the USA 35.0% of corn is used for feed, 34.7% for ethanol production,13.5% for exports, 8% for dried distiller grains (DDGs), 1.5% for starch, 7.5% for human consumption and 1% for seed (NCGA 2020), whereas in India, ~ 60% of the total maize grain is consumed as feed, 14% for industrial purposes, 17% as food, 7% as processed foods, and 4% for other purposes including seed (Rakshit and Chikkappa 2018). Although maize has wider adaptability, several factors (biotic and abiotic) constrain its production and productivity. Among the abiotic stresses, low nutrient and water availability directly hamper the physiological processes of maize and affect yield in turn (Gonzalez-Dugo et al. 2010).

N is the principal macronutrient playing a major role in plant physiology. Being the prime component of amino acids, chlorophyll, adenosine triphosphate (ATP) and nucleic acids, it is the most vital nutrient required in the largest quantity for maize production (Wang et al. 2017). For the production of one ton of crop biomass, almost 9–11 kg of N is required (Anuar et al. 1995). In maize, the increased application of N is directly proportional to biomass, grain yield, leaf number and shelling percentage (Nunes et al. 1996; Bakht et al. 2006). Its deficiency constrains proper maize development and growth. Although N is the most abundant element in the atmosphere (78.1%) and soil reserves (2–20 t ha^{-1}) (Bockman et al.1990), but its availability for crops is limited as plants utilize it in reduced forms only, viz. nitrate (NO_3^-) or ammonium (NH_4^+) (Huang et al. 2000). The concentration of NO_3^- and NH_4^+ is very dynamic and largely depends on the soil edaphic environment.

Crop plants access N directly either from chemical fertilizers, manures or atmosphere through processes like BNF and lightning (Vance 2001). From 1960s, the application of synthetic nitrogenous fertilizers for crop production has increased nine times, and over the next 40 years, it is expected to increase further by 40 to 60% (Sutton et al. 2013). In 2017, for total agricultural production, approximately 109 million tonnes of the nitrogenous fertilizers were consumed worldwide (FAO 2017). It is projected that by 2050, there will be a need for additional 443 million tonnes of maize production that will require approximately 27–63 million tonnes of N (Alexandratos and Jelle 2012). Maize is a heavy feeder crop and highly responsive to N application; thus, the demand for nitrogenous fertilizers is also expected to increase many folds. Organic fertilizers are insufficient to meet the total N requirement. Hence, excessive use of chemical fertilizers costs us large sums of money in addition to human and environmental health (Glendining 2009). Globally, out of total N applied to crops through fertilizers, only 47% is converted into grains and the remaining is either turned in organic matter or lost through erosion, leaching, runoff and emitted in gaseous forms, viz. ammonia (NH_3), nitrous oxide (N_2O), dinitrogen (N_2) or nitrogen dioxide(NO_2) (Lassaletta et al. 2014). Hence, all these issues necessitate the search for a sustainable alternative approach to supply N for cereal crops production, including maize. In this review suitability of different breeding and biotechnological approaches to turn BNF a reality in maize with more N availability to increase yield and reduced environmental concerns have been discussed. It will give a direction for breeding-based new approaches to reduce the nitrogenous fertilizers dependency and promote the eco-friendly productive system for heavy N executive maize cropping system.

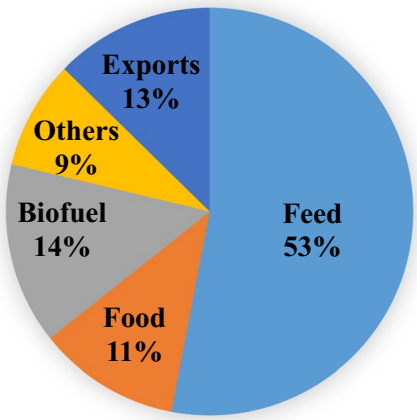

Fig. 1 Global consumption of maize (*Source*: OECD/FAO 2020)

Need for biological nitrogen fixation in maize

For a sustainable solution to nitrogenous fertilizers use, BNF in cereals has been a hope since 1970s. Manufacturing nitrogenous fertilizers is a highly energy-consuming process requiring six times more energy than phosphorus (P) and potassium (K) fertilizers production (Da Silva et al. 1978). Reducing dependence on these fertilizers by developing BNF an integral mechanism in crop development will be a profitable venture. Studies depict that globally, BNF produces nearly 200 million tonnes of N annually (Graham 1992; Peoples et al. 2009) and out of which 50% of crop field N is contributed by diazotrophic bacteria using nitrogenase

enzyme (Ramírez-Puebla et al. 2019). Thus, BNF lures the attention of agricultural researchers to incorporate this trait in staple and economically important food crops (Table 1). However, due to the complex nature of the BNF process, there are various constraints at the technological level to successfully address this trait. Hence, N-fixation in cereals remains an unresolved issue for researchers.

Several research groups diligently worked to achieve this target likewise a project on "Assessing Opportunities for Nitrogen Fixation in Rice (*Oryza sativa*)" was conducted under the aegis of the International Rice Research Institute (IRRI) with many other countries during 1994–2001 (Ladha and Reddy 2000). Under the project, the rice nodulin genes (*ENOD40*) were characterized in addition studied the role of chitin in nodulation, explored genetic programs for root endosymbiosis, and proposed modification of lateral root as a more achievable goal in the short period with BNF ability. Similarly, many other projects have also been initiated to introgress BNF in cereals funded by the Bill and Melinda Gates Foundation (BMGF, USA), the National Science Foundation (NSF, USA), the Biotechnology and Biological Sciences Research Council (BBSRC, UK) and the Indian Council of Agricultural Research (ICAR, India) (Sharma et al. 2016). In 2016, the India-UK N-fixation centre has been launched at the ICAR-Indian Institute of Soil Science, Bhopal (India), with the objectives of genetic engineering of rhizobia and rice endophytes, and improving BNF. They have engineered the rhizobia and characterized endophytes in rice with studying their mechanism of infection (ICAR 2016). Engineering Nitrogen Symbiosis for Africa (ENSA) project funded by BMGF has purposed synthetic biology as a viable approach to engineer N-fixation in cereals (Rogers and Oldroyd 2014). They have discovered a similar pathway for lateral roots and nodule development indicating that a substantial part of nodule formation machinery is already present in cereals which can be further engineered for N-fixation (Katharina et al. 2019). A group of scientist at Lethbridge Research and Development Centre, Canada, have successfully developed *nif* genes cluster with 16 essential genes and directly introgress it in wheat mitochondria. Now

Table 1 Events related to BNF identification and current progress in nitrogenase transfer

Year	Events	References
1836	Detection of nitrogen as a nutrient for plants	Smil (2001)
1886	Hellriegel and Wilfarth demonstrated the legumes ability to fix nitrogen	Santi et al. (2013)
1888	Rhizobia isolation from nodules	Winogradsky et al. (1895)
1893	Isolation of *Clostridium pasteurianum* (free-living N fixers)	Soumare et al (2020)
1901	Isolation of Azotobacter	Smil (2001)
1953	Identification of two novel nitrogen-fixing bacteria: *Beijerinckia fluminensis* and *Azotobacter paspali*	Döbereiner et al. (1966); Baldani (2005)
1972	Isolation of *Enterobacter cloacae* from corn roots	O'Hara (1998)
1975	Isolation of *Spirillum* sp. in maize and demonstration of their nitrogenase activity	Von Bulow and Dobereiner (1975)
1984	Detection of nitrogen fixation in methanogens (archaea)	Belay et al. (1984)
1994–2001	A project on "Assessing Opportunities for Nitrogen Fixation in Rice (*Oryza sativa*)" by International Rice Research Institute (IRRI)	(Ladha and Reddy 2000)
2012	Develop database of all *nifH* sequences	Gaby and Buckley (2011)
2012	Refactoring of nitrogen fixation genes to a simpler and smaller cluster	Temme et al. (2012)
2013	*Nif* gene cluster transfer from nitrogen-fixing bacteria *Paenibacillus* sp. into *Escherichia coli*	Wang et al. (2013)
2014	Creation of an artificial FeFe nitrogenase system in *Escherichia coli*	Yang et al. (2014)
2014	Synthetic biology approach to engineer nitrogen symbiosis in cereals	Rogers and Oldroyd (2014)
2015	Transfer and expression of *Pseudomonas stutzeri* A1501 nitrogen fixation island in *Escherichia coli*	Han et al. (2015)
2016	Transfer *nifH* in mitochondria of *Saccharomyces cerevisiae* (a model of eukaryotic cell)	Buren et al. (2017)
2016	Transgenic tobacco production with nif genes transfer and expression in plant plastids	Ivleva et al. (2016)
2016	N-fixation centre launched at the ICAR-Indian Institute of Soil Science, Bhopal (India)	Sharma et al. (2016)
2018	Detection of minimum three genes (*nifH*, *nifD*, and *nifK)* required for introgression of nitrogenase biosynthesis	Yang et al. (2018)
2020	Applied gene editing on *Kosakonia sacchari* strain isolated from maize to decouple the nitrogenase enzyme biosynthesis from the regulatory networks	Bloch et al. (2020)
2020	Sierra Mixe maize landrace with mucilage production proposed as a model for nitrogen fixation in cereals	Bennett et al. (2020)

the team is working to regenerate the *nif* genes-enriched wheat plantlets (King 2019). Currently, the maize research is mainly focused on multiple traits like high yield, disease-pest resistance, and fertilizer use efficiency, but study regarding the interaction between maize and diazotrophs is a still ignorant area to tap it on.

Various N-fixing bacteria such as *Azospirillum*, *Azoarcus* and *Herbaspirillum* inhabit in endophytic associations with maize in intercellular plant tissues without causing any disease (Rosenblueth and Martínez-Romero 2006). It provides a scope to explore this trait in maize breeding to fix N naturally. Plant-associated diazotrophs also benefit the crop growth and development by promoting the mechanisms like the synthesis of vitamins and phytohormones, stimulation of nutrient uptake (solubilize and mineralize inorganic and organic phosphate), reduction in ethylene synthesis and improving resistance against pathogenic microbes, etc. (Berge et al. 1990; Triplett 1996; Hallmann et al. 1997). Therefore, under suitable conditions plant growth or health can also be benefitted from a diazotrophic association (Boddey et al. 2000; Chelius and Triplett 2000). Considering all these, the integration of the BNF mechanism with maize breeding strategies will be helpful to achieve sustainable agricultural production (Riggs et al. 2001; Kennedy et al. 2004). Maize is not a plant with N-fixation ability. Hence, no experimental data is available to estimate its effect on maize yields. However, an analysis of energy cost on legumes like soybean (*Glycine max*) may give a rough idea of energy cost levied in maize by N-fixation. The assessment has shown that soybean with nodules consume 19% of photosynthate, but this will compensate with N-fixation, nitrate assimilation and other indirect benefits like C sink maintenance that will increase photosynthesis, H_2 released in the process will enhance plant growth and other positive environmental consequences (Ladha and Reddy 2000).

Different approaches available for nitrogen fixation

N-fixation is a dynamic and highly energy demanding process (Fig. 2a). All N-fixing prokaryotes *i.e.* diazotrophic bacteria fix N by owning nitrogenase enzyme system, which hydrolyses 16 ATPs for one molecule of N-fixation, making it a most expensive metabolic process (Simpson and Burris 1984) (Fig. 2b). Diazotrophic bacteria are highly diverse

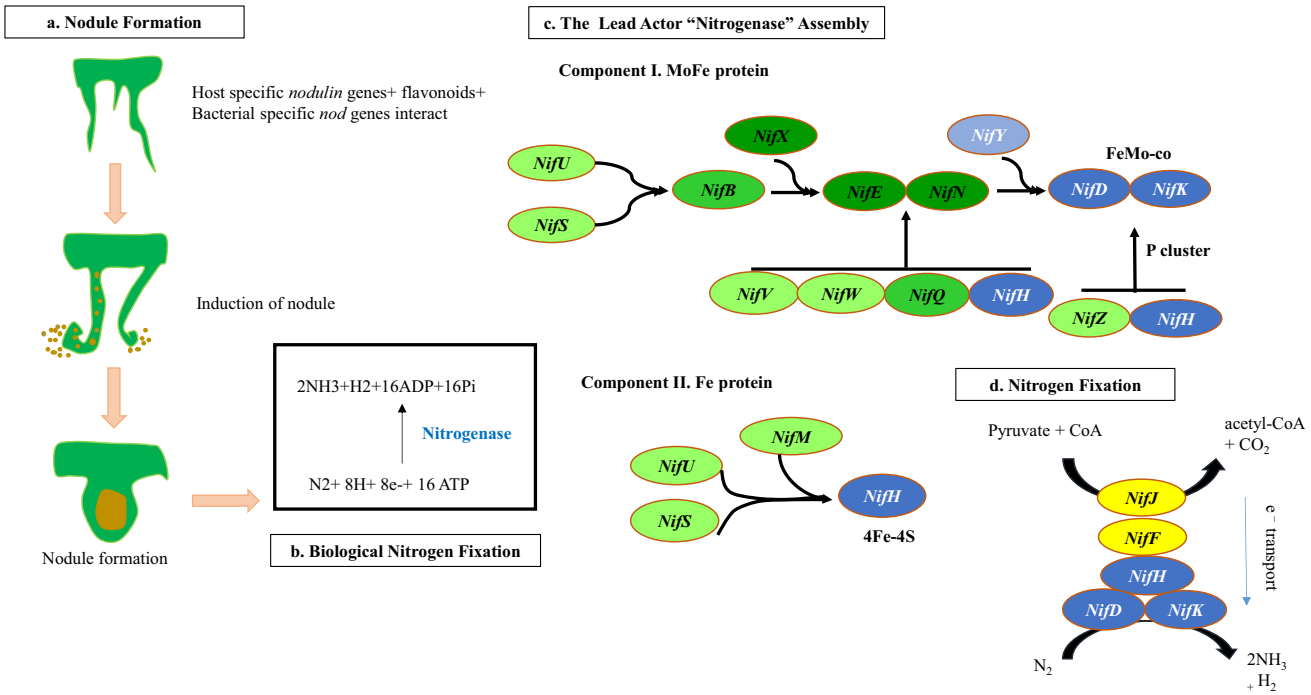

Fig. 2 Schematic representation of biological nitrogen fixation (BNF). **a** For effective symbiotic association development mainly two classes of genes are required: nodulation and nitrogen fixation genes. Nodulation genes, *e.g. nodABC*, interact with host-specific nodulin genes and plant flavonoids. Nitrogen fixation genes possess the structural genes (*nif* and *fix*) to encode nitrogenase (*nifHDK*) enzyme that is the lead actor for nitrogen fixation; **b** metabolic reaction of BNF is highly expensive as it consumes 16 ATP to fix one molecule of N; **c** the key actor nitrogenase genes assembly—the genes are coloured by their function as blue (nitrogenase), green (cofactor biosynthesis, shading corresponds to operons), and yellow (e^- transport) and a horizontal bar indicates overlapping genes; **d** mature nitrogenase enzyme with electron-transport chain catalyses nitrogen fixation process (Temme et al. 2012; Laranjo et al. 2014)

in phylogeny and ecological niche and have a synchronized interaction with the host plant for N-fixation. These diazotrophic bacteria can be free-living or in symbiotic associations with crop plants. The BNF is an extremely sensitive process influenced by nutrient and environmental conditions. It enables to supply all or part of crop N requirements through endosymbiotic, associative and endophytic interactions (King and Purcell2005; Liu et al. 2011). Free-living diazotrophs constitute a small fraction of plant rhizosphere ecosystem including cyanobacteria like *Anabaena* and *Nostoc* and other genera such as *Azotobacter*, *Beijerinckia* and *Clostridium*. Many prokaryotes fix N symbiotically (*Rhizobium*, *Bradyrhizobium*, *Mesorhizobium* and *Sinorhizobium)* with the host where plants provide sugars to the microorganisms and in exchange microbes provide fixed N for host plant growth (Graham and Vance 2003). Symbiotic N-fixation is observed in water fern Azolla with Cyanobacteria *Anabaena azollae*, actinorhizal trees and shrubs with actinomycete *Frankia* (non-legume N fixers), and in legumes with *Rhizobium* and *Bradyrhizobium* bacteria. Several microorganisms also show the associative or endophytic symbioses such as *Azospirillum lipoferum, Azospirillum brasilense*, *Azoarcus evansii* and *Herbaspirillum seropedicae* in a wide variety of crop plants including cereals.

The BNF is a unique natural system under the partnership of plants and diazotrophs for capturing atmospheric N and processing it into a usable form of N through enzymatic reduction. The key enzyme that fixes atmospheric N, *i.e.* nitrogenase is highly conserved and complex present in free-living and symbiotic diazotrophs which enables them to participate in various types of associations/interactions with their host plants. The proteins and non-proteins involved in the constitution of the nitrogenase enzyme make it sensitive to the presence of oxygen (Burén and Rubio 2017). Thus, there are some obligate and facultative anaerobic bacteria also like *Clostridium pasteurianum* and *Klebsiella oxytoca*, respectively, which fix N in the complete absence of oxygen. Other microbes like *Azotobacter vinelandii*, being the obligate aerobes, carry out N-fixation activity by shielding nitrogenase and consuming oxygen through cytochrome oxidases (Mahmud et al. 2020). Endosymbiosis between legumes and rhizobia is the most conventional N-fixing system in agriculture. In legume nodule, rhizobia reside intracellularly and fix N symbiotically in cytoplasmic vesicles. Endosymbiosis is the most evolved, stable and efficient N-fixing mechanism as it creates reducing conditions for nitrogenase, a pathway to transfer N-fixation product to host and protect against oxygen and antagonistic bacteria (Quispel 1991). Other than nodular structures, there are other modes too for intracellular endosymbiotic N-fixation in plants likewise in non-legume *Gunnera* plants, cyanobacteria *Nostoc* fix N in mucilage-secreting glands of stem. It establishes similar symbiosis like legume-rhizobium by invading intracellularly in gland cells (Werner 1995). Rhizobium also fix N in many leguminous species (subfamilies Papilionoideae, Caesalpinioideae and Mimosoideae) without developing nodules (Bryan et al. 1996). In maize, an indigenous landrace Sierra Mixe grown in Oaxaca province of Mexico possess extensive aerial root system that secretes carbohydrate rich (mainly arabinose, fucose and galactose) mucilage and fix about 29–82% N (Van Deynze et al. 2018). Many other bacteria which colonize intracellularly for N-fixation have been detected in many plant species like banana (*Musa* sp.) (Thomas and Reddy 2013; Thomas and Sekhar 2014.), peach palm (*Bactris gasipaes)* (de Almeida et al. 2009), scotch pine (*Pinus sylvestris*) (Pirttilä et al. 2000), cactus and non-nodulating legumes (Sambukumar et al. 2015). This indicates that nodule formation is not a compulsory mode for intracellular N-fixation in crop plants. Several diazotrophic bacteria have been known for N-fixation in maize by establishing rhizospheric or endophytic associations like *Azospirillum*, *Klebsiella*, *Pantoea*, *Herbaspirillum*, *Bacillus*, *Rhizobium etli* and *Burkholderia* (Chelius and Triplett 2000; Dong et al. 2001; Gutiérrez-Zamora and Martinez 2001; Caballero-Mellado et al. 2004; Perin et al. 2006). These diazotrophs reside intercellularly in plant tissues without causing any disease and are part of so-called endophytic diazotrophs (Montañez et al. 2009). Various studies have been undertaken to estimate the effect of these diazotrophs on maize yield (Table 2). Estrada et al. (2002) isolated the most abundant N-fixing endophyte, *Burkholderia* associated with maize and further confirmed that these isolates densely colonize into maize tissues which subsequently enhances yield. Riggs et al. (2001) carried out a study to identify maize-endophyte associations and their effect on maize production under both laboratory and field conditions and a significant increase in yield was observed with the association of *Klebsiella pneumoniae* and *Herbaspirillum seropedicae* endophytes. Pandey et al. (1998) conducted an experiment using the strains of *Azotobacter chroococcum* and *Azospirillum brasilense* in local maize cultivars and found significant improvement of

Table 2 Diazotrophs for biological nitrogen fixation in maize

Diazotroph	Yield increase (%)	References
Burkholderia sp.	5.9–6.3*	Estrada et al. (2002)
Klebsiella pneumoniae	13–25†	Riggs et al. (2001)
Azospirillum	33*	Dobbelaere et al. (2001)
Azotobacteria	–	Pandey et al. (1998)
H. seropedicae	19.5*	Riggs et al. (2001)
Pseudomonas	11.7 (total biomass)†	Shaharoona et al. (2006)

Experiments in fields (*) or in controlled conditions (†). Adopted from (Santi et al., 2013)

1–1.5 fold in maize production in tropical conditions. Shaharoona et al. (2006) isolated several rhizo-bacterial strains from the maize field with a significant positive correlation between 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity of diazotrophs and maize root elongation. All these studies indicated that a number of endophytes are there to increase yield and biomass of maize, but the application of these inoculants is not reliable as they are highly environment dependent. Therefore, researchers are exploring to directly introgress *nif* genes in cereals through genetic engineering or breeding.

Potential genetic resources for assessing nitrogen fixation in maize

Screening or development of germplasm possessing N-fixation capability is the most crucial and prerequisite step to integrate BNF with the breeding process. Many important crops have been screened for BNF like wheat (Jain et al. 1986), maize (Von Bülow and Doebereiner 1975; Baldani et al. 1997; De-Polli et al. 1982), sugarcane (Reis et al. 2000), rice (Boddey et al. 1995) and other plants (Tapia-Hernandez et al. 2000). Several non-pathogenic diazotrophic bacteria have been detected in maize and there is a need to discover maize germplasm that would be benefitted in association with these endophytic bacteria (Triplett 1996). Estrada et al. (2002) screened maize varieties to isolate N fixer endophyte from the root tissues. The N-fixing genus *Burkholderia* isolates were identified in field-grown maize tissues and its wild relative, teosinte (*Z. mays* ssp. *mexicana*). The study speculated that the strains of *Burkholderia* species have a kind of primitive symbiosis with teosinte from the domestication period. The mucilage in the aerial root is also reported to be an important harbinger of such symbiotic associations (Dennis et al. 2010; Li et al. 2011). Teosinte, the wild relative of maize possesses an extensive aerial roots system with mucilage secretion (in mild quantity) and nitrogenase enzyme activity. The acetylene reduction assay (ARA) indicates that mucilage production with N-fixation ability is an ancient trait of maize.

Sierra Mixe, an indigenous maize landrace possessing similar mechanism with *Burkholderia* strains in its mucilage has been reported. This hypothesized that this trait has been potentially introgressed into the landrace from teosinte during the post domestication period (Van Deynze et al. 2018). Earlier also, Raju et al. (1972) observed N-fixing capacity in a hybrid Pioneer 3773 grown in Oregon, USA. They isolated a strain *Enterobacter cloacae* from plant rhizosphere and roots with the ability to fix N and producing gel, but the N-fixation rate was very low. It is speculated that the production of gel helps to protect against oxygen and facilitate N-fixation in roots. The similar feature of producing mucilage exudate as in aerial roots of Sierra Mixe have been detected in other cereal crops also like wheat (Sinha et al. 2002), sorghum (Werker and Kislev 1978; Li et al. 2014) and barley (Carter et al. 2019) indicating that it is an ancient common feature prevalent in cereal crops. Hence, screening of maize germplasm to identify new genotypes possessing different and unique mechanisms of N-fixation should be focused on to further utilize them in breeding programmes.

Genetic Basis of Nitrogen Fixation

The most evolved legume-rhizobia interaction is very complex at the genetic level. To develop such symbiosis in cereals deeper insight about the gene families involved in N-fixation process is needed. There are a number of gene sets from both host and diazotrophs responsible for BNF (Fig. 2c).

Host-specific genes

Nodulin genes: The host plant genes involved in a symbiotic association are nodulin genes. Nodulin genes are specifically expressed to develop nodule structure, morphogenesis, size, and number of nodules which together influence N-fixation rate. Nodulin genes are of two types: *C-Nodulins*, which are present in all legumes, and *S-Nodulins*, which are species specific (Lodha and Nainawatee 1993). Several genes for nodulin specific proteins (*S-Nodulins*) have been identified in many leguminous crops such as nodulin-35 of *Glycine max*, glutamine synthetase in *Phaseolus vulgaris*, apyrase *in Dolichos biflors* and Gs-50 in *Glycine soja* (Etzler et al. 1999; Stacey et al. 2000). The genetic dissection of nodulin genes is prerequisite for genetic manipulation of BNF by analysing their sequence, regulatory mechanism and function. Some early nodulins (like *ENOD2, ENOD40, ENOD12*) are activated by *Rhizobium* signalling molecules, while late nodulins like leghaemoglobin, sucrose synthase, glutamine synthetase and nodulin-26 are involved in the physiological adaptation of nodule development (Brewin 2001).

Bacterial Sym genes

In N-fixing bacteria, number of gene sets are responsible to develop the symbiotic association. These are *nod, nif* and *fix* genes, which are collectively called as *Sym* genes. In fast-growing rhizobial strains *Sym* genes are found in large plasmid, while in slow-growing strains these are present on bacterial chromosome only.

Nod genes

The bacterial *nod* gene set is responsible for nodule formation in interaction with host nodulin genes. Based on function, there are several *nod* genes present in bacterial strains (Table 3) involved to induce and control nodule formation. This major structural modification in infected host plant cells enclose the bacteroid inside providing the suitable conditions for nitrogenase expression (Verma et al. 1986).

Nif genes

The *nif* gene family is highly conserved among diazotrophs and encodes for MoFe-dependant nitrogenase complex enzyme, which is the key component to catalyse atmospheric N into reduced forms (NH_4^+) (Raymond et al. 2004). Various *nif* genes have been identified and characterized structurally and functionally (Galton and Smith 1993; Parvez and Wani 2007) (Table 3). Besides encoding nitrogenase, *nif* genes also play a role to encode several regulatory proteins needed in N-fixation. The expression of *nif* genes is sensitive to oxygen or fixed N concentrations. *Nif* genes vary in their regulatory mechanism as in *R. melilotus* and *R. leguminoserum* they expresses under single operon, while in *B. japonicum* and *R. phaseoli* reside on different operons (Ow et al. 1985). It has been observed that by direct cell contact only *nif* genes can be transferred in between bacteria so this seems a significant aspect to work on to transfer these genes in plants.

Fix genes

It is the other gene family which plays a critical role in regulation and metabolism of oxygen concentration for *nif* genes expression. *Nif* genes perform another important function of regulating these *fix* genes. Many *fix* genes have been identified and characterized. The *fix* genes are regulated under three core operon structures such as *fixABCX*, *fixGHIS* and *fixNOPQ*. The *fixABCX* operon regulates gene transcription under low oxygen concentration, while *fixNOPQ* operon encodes cytochrome *cbb3* oxidase complex that metabolizes oxygen by the bacteroid (Black et al. 2012).

Unlike conventional BNF process through nodulation, Van Deynze et al. (2018) identified sequence of six core *nif* genes (*nifH*, *nifD*, *nifE*, *nifK*, *nifN*, and *nifB*) from diazotroph associated with aerial roots of Sierra Mixe maize landrace. However, the genetic basis of N-fixation in aerial root associated with mucilage of landrace Sierra Mixe and its microbial inoculum is still unexplored. It can be hypothesized that this association is either environmental or seed-borne or other factors. It indicates that *nif* genes are key actors in N-fixation whether through forming nodules or in root mucilage. But as per current understanding, this novel way of BNF in maize has not yet been harnessed because Sierra Mixe has grown only in restricted areas. Further, the mucilage production largely depends on environmental conditions. Hence, currently this mechanism has been proposed as a model to study the association between cereal crops and diazotrophs to support N-fixation in cereals like maize, rice, sorghum and wheat. It will help to create a scaffold to understand diazotrophic activities and their functions in cereals which can be further utilized to increase their N-fixation potential by enhancing the mucilage production by practicing genetic selection or modifying the structural and regulatory pathways of the microbes associated with mucilage production. Further, there is need to understand at the molecular level that how these *nif* genes are able to express without creating anaerobic conditions in root mucilage or any other components are involved for their regulation. Thus, for future, it is important to study the genetic basis of BNF in Sierra Mixe, detection of the diazotrophs involved in its association, and the mechanism of their microbial recruitment which will

Table 3 Various identified and characterized *nod* and *nif* gene cluster in diazotrophs

nod genes		*nif* genes	
Gene	Function	Gene	Function
Roc	Root colonization	*nifH0*	FeMo biosynthesis
Roa	Root adhesion	*nifD*	Cofactor of holoprotein α2β2 tetramer
Hab	Hair branching	*nifK, B, E, X*	FeMo cofactor biosynthesis
Had	Hair deformation	*nifN*	Processing of Mo
Hac	Hair curling	*nifV*	Encode a homocitrate
Hsn	Host specificity	*nifA, L*	Positively and negatively regulate nif transcription, respectively
Inf	Infection	*nifF*	Reduction of Fe protein
Noi	Nodule initiation	*nifJ*	Reduction of flavodoxin
Inb	Infection thread branching	*NiT*	No need in diazotroph growth
Bar	Bacterial release	*nifW, Z*	Needed for full activity of MoFe protein
Bad	Bacterial development	*nifS, U, Y*	Processing of MoFe protein

open up new avenues for research into a potentially novel model of the BNF in maize.

Other genes

Various important accessory genes are also involved to fix N successfully, viz. *exo* gene that produces exopolysaccharide, *hup* gene that helps in hydrogen uptake, *gln* gene for glutamine synthase, *dct* genes to transport dicarboxylate, *nfe* genes to efficiently develop nodules and competitiveness, *ndv-1,2* gene for glucans synthesis and *lps* genes for lipopolysaccharide production (Laranjo et al. 2014).

Bacterial secretion systems

After induction of nodule formation the next step is an infection of bacteria in plants tissues and their maturation to N-fixing bacteroids. Many gene families are involved, but protein secretion systems are a crucial group of genes to infect plant with diazotrophs. There are seven groups of bacterial secretion systems, viz. Types I to VII in addition to the fimbrial chaperone–usher pathway (Black et al. 2012). These systems have diversified functions like assembling surface structures to contact target cells, deliver DNA or protein effectors, affect host range, produce nodulation outer proteins (Nops), virulence and conjugal transfer or communication between microsymbionts and host.

Exopolysaccharide production

In symbiotic association, production of exopolysaccharide (EPS) is critical for the initial bacterial invasion to form indeterminate types of nodules on legumes (Skorupska et al. 2006). EPS plays a role in nodules induction by facilitating both infection thread initiation and bacterial release in symbiosis with the host (Kelly et al. 2013). In *Sinorhizobium–Medicago* (alfalfa) model, *Sinorhizobium meliloti* invades and differentiates inside its host plant alfalfa by producing exopolysaccharides succinoglycan (EPSI) and galactoglucan (EPSII) which induce the formation of infection thread (Jones et al. 2007). In absence of EPS, the competition to nodulate host plant increases leading to a diminished capacity to infect host (López-Baena et al. 2016). The comparison among genome of various diazotrophs shows that there is genetic complexity in EPS regulation that is still needed to understand. During evolution, different species have adopted diverse sets of genes which indicates that for universal symbiome production of EPS is not a necessary feature (Black et al. 2012).

Different breeding approaches for introducing nitrogen fixation ability in maize

Breeding for symbiotic N-fixation is a promising area to research. The N-fixation is a very dynamic trait controlled by multiple genes in both diazotrophic bacteria and plant species in addition to affected by edaphic and abiotic factors. From last many years, huge efforts have been made to isolate, identify and test diverse diazotrophs from cereals with an inherent ability to fix N, but practically still it has a long way to go to turn it a reality. There are several factors/constraints that hinder it to incorporate in maize or any other cereal crop; for example (1) it requires the association and coordination of totally two different organisms (plants and microbes) to mutually function and fix N in a new environment (Rice et al. 2000), (2) N-fixation requires nearly 30 essential genes to function in a coordinated manner which is tightly regulated in their host under a very sensitive pathway (Rogers and Oldroyd 2014), (3) a large number of variables like plant genotype, diazotroph's strain, environmental factors (water, soil, O_2 permeability, salinity and nutrient cycle), etc., highly affect BNF, making it a very sophisticated and tricky process to manipulate (Rice et al. 2000; Serraj et al. 2001; Chaudhary et al. 2008; Hartmann et al. 2009; Chaparro et al. 2014; Pfeiffer et al. 2017), (4) several plant defence alkaloids (like salicylic acid, phytoalexins) show an inhibitory effect on host–microbes interaction (Lebeis et al. 2015), (5) from plant to plant, the quorum-sensing signal for diazotrophs varies (Venturi and Keel 2016), which also alters the bacterial genes expression, (6) nitrogenase expression is highly sensitive to oxygen and needs anaerobic conditions to function that makes it more challenging to implement this strategy, and (7) as N-fixation is a high energy demanding process so, it is also unclear whether cereal hosts can provide this much energy with reducing conditions to endure nitrogenase catalysis (Van Velzen et al. 2018).

To resolve all the issues, one methodology is to study a model system of any naturally existing non-rhizobial N-fixing bacterium like in sugarcane diazotroph that fix N intracellularly by colonizing in root systems (Cocking et al. 2006) or Sierra Mixe landrace producing mucilage with N-fixation ability. Following that different breeding approaches should be practiced to develop/identify maize germplasm with BNF traits. To identify a maize genotype with the ability to fix N and compatible with BNF fixers is a challenging and crucial task to accomplish. The traits that stimulate N-fixation in non-leguminous plants are called as N-fixation supportive (NFS) traits (like nodulation traits, fixed nitrogen, shoot dry weight, root traits) so

genetic variability and heritability of such traits should be traced to use in breeding programs. The genetics of mucilage production in aerial roots needs to dissect and the ways to exploit it for field level product development should be explored. In legume-rhizobium the defence alkaloids show an inhibitory effect on their interaction (Lebeis et al. 2015). Likewise in cereals also their effects on diazotrophs should be accessed. Correlation of N-fixation ability with the plant attributes like acid-tolerant plants (exude dicarboxylic acids) that shows higher N-fixation rate (Christiansen-Weniger et al. 1992) needs to be studied to develop BNF plants type. Mutagenesis can also be applied to develop novel diazotroph strains with improved N-fixation ability in cereal crops (Maier and Brill 1978). Ling et al. (2013) showed that by applying reversible mutation it is possible to manipulate both the nodulation and N_fixation genes without affecting host–microbes association in cereals.

Molecular breeding approach

Molecular breeding approaches provide opportunities for mapping and introgression of the essential genes for nodulin, nitrogenase, lectins and malate dehydrogenase into recipient plants. However, till now in any crop optimized expression of all N-fixation genes have not been worked out. One of the conventional approaches in maize will be the mapping of the genes/QTLs for NFS traits from the screened maize cultivars measuring total mucilage production, amount of aerial roots, total N-fixation and N use efficiency. Application of molecular markers is the primary approach to validate the expression of nitrogenase biosynthesis and N-fixation (Schmid and Hartmall 2007). Initially, the RFLP (Restriction fragment length polymorphism) markers with *nif* gene probe and PCR (Polymerase chain reaction) fingerprinting were used in cyanobacterium to identify the gene diversity (Plazinski et al. 1985). Ueda et al. (1995) also detected diazotrophic bacteria in rice using PCR amplified *nifH* sequences. Rai et al. (2014) isolated different restriction fragments using *nifH* RFLP markers analysis from the soil samples. Hence, the construction of a molecular markers library will be an efficient way to divulge the genetic diversity of uncharacterized diazotrophs in the maize rhizosphere.

There are many root architectural traits like root length, surface area, diameter, volume, root hairs and other nodulation traits involved in BNF. These traits are genetically controlled by polygenes or quantitative trait loci (QTLs). Therefore, identification of major QTLs for these traits will be a vital objective of maize breeding to enhance N-fixation. Akiyoshi et al. (2012) mapped 34 QTLs in *Lotus japonica* for controlling BNF traits like nodule number, nodule weight, acetylene reduction activity (ARA) per plant, ARA per nodule number and ARA per nodule weight etc. Similarly, Santos et al. (2013) identified two QTLs for shoot dry weight, three QTLs for nodule number, and one QTL for nodule dry weight with a phenotypic variance of 15.4, 13.8, and 6.5%, respectively, from a soybean RIL population. Therefore, such studies should be extended to track QTLs for BNF traits in maize and cereal crops to identify the contrasting genotypes.

The direct transfer of *nif* genes into maize should be attempted through simultaneously or one by one introgression of vital genes required for the biosynthesis of FeMo-Co unit and nitrogenase function. Evolutionary genomics of N-fixation suggests that introgression of BNF ability into non-legumes require only a few genetic elements by concatemerizing bacterial genetic constitution (Bailey-Serres et al. 2019). Thus, to introgress nitrogenase requires minimum three genes (*nifH*, *nifD*, and *nifK)* of bacterial genetic units (Yang et al. 2018) (Fig. 2d). A novel model for N-fixation as identified in Sierra Mixe landrace of maize (Van Deynze et al. 2018) also has its own limitations like it is long in duration with taller plants and is not directly suitable for cultivation purposes. Hence, the transfer of BNF traits from this landrace through marker-assisted breeding to adopted accessions of maize will be a fruitful venture (Fig. 3). Meanwhile, further understanding the mechanisms of symbiosis in plants and diazotrophs with high-throughput genotyping will also be beneficial to implement the innovative breeding approaches to introgress N-fixation traits into maize.

Omics Approaches

To elaborate the molecular basis of BNF traits, different functional genomics approaches like transcriptomics, proteomics and metabolomics can also be performed on rhizobial symbiosis. The integration of different omics data will help in better understanding of rhizobial cellular activities, their adaptation and homology with other functional diazotrophs. Metabolic databases like KEGG, MetaCyc and BioCyc (Caspi et al. 2008) provides huge data to study differentially regulated pathways. It will further be useful in selection and prioritization of candidate genes suitable for mutagenesis and validation experiments (Lardi and Pessi 2018). Recently omics approaches have been utilized as a viable tool to identify and isolate diazotrophs from cereal crops such as in rice (Bao et al. 2014), sugarcane (Fischer et al. 2012), sorghum (Kiwamu et al. 2019) and sweet potato (*Ipomoea batatas*) (Terakado-Tonooka et al. 2013). From obtained omics results microbes with nitrogenase proteins should be isolated and phylogenetic markers can be predicted. From the metagenome sequence, the nitrogenase gene sequences can be computed using nitrogenase databases. Kiwamu et al. (2019) carried out an omics study to identify functional N-fixing diazotrophs (*Bradyrhizobium*) associated with sorghum. Proteome analysis revealed that

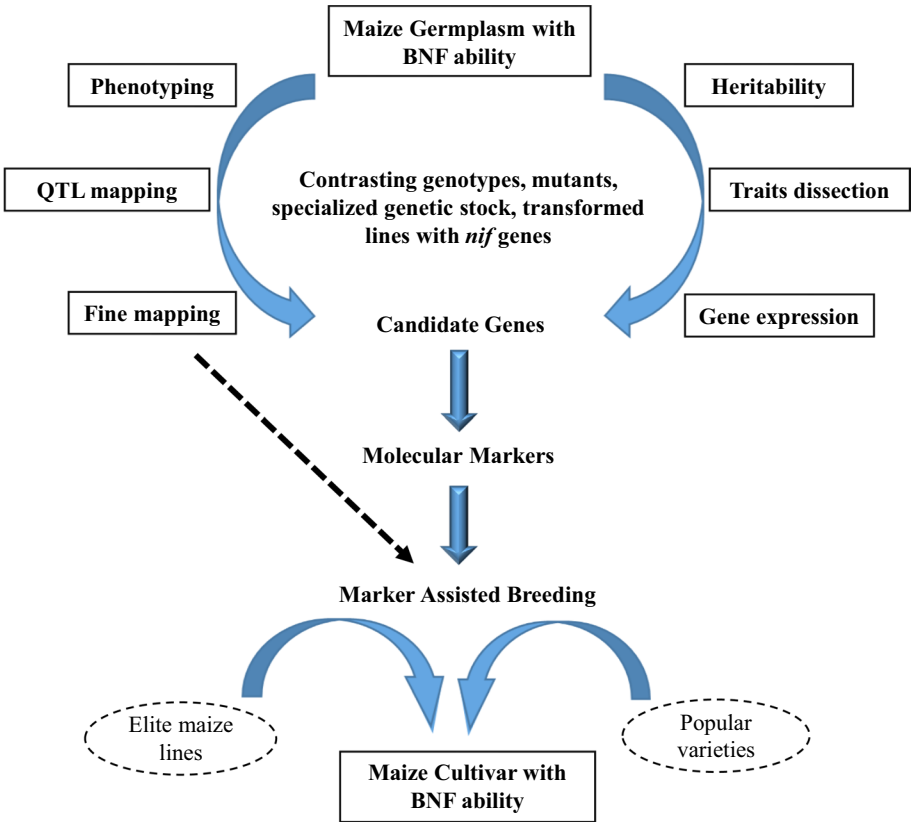

Fig. 3 Outline of a breeding strategy to introduce BNF in maize varieties

three *NifHDK* proteins of *Bradyrhizobium* species were consistently found across sample replicates. Thus, omics data will be used to identify and isolate different functional diazotrophic strains in maize with better understanding their regulatory mechanism. Metagenome and proteome data can be further used to study the genetic basis of N-fixation in mucilaginous aerial roots of landrace Sierra Mixe and its microbial inoculum.

Genome Editing

Genome editing, a novel approach facilitates modification in the genome of any living organism by inserting, deleting, modifying, or replacing any DNA sequence at site-specific locations. It allows remodelling of N-fixation regulation by diazotrophs without the inclusion of any trans-elements. After identification of efficient diazotrophs closely associated with the crop, gene editing will allow disrupting the regulatory pathways linked to N-fixation, sensing and assimilation. It can replace native promoters of *nif* genes from already characterized promoters of different strains permitting N-independent expression of *nif* genes. Such remodelling via promoters allows microbes to adapt to different niche and environmental conditions. Bloch et al. (2020) isolated a strain of *Kosakonia sacchari* from maize plants and through gene editing decoupled the nitrogenase enzyme biosynthesis from the regulatory networks that respond to exogenous cellular N. The edited non-transgenic strains were capable to express nitrogenase genes in the corn rhizosphere under various levels of applied N fertilizers in the greenhouse as well as field conditions. In comparison with initial field count, the overall colonization was significantly higher, but all the remodelled strains show a decrease in colonization which suggests that higher expression of nitrogenase activity led to a fitness cost so we have to optimize its expression. Hence, through gene editing by optimum modification of the regulatory pathways of diazotrophs can make this process accessible to cereal crops too to boost crop production sustainably.

Genetic engineering

Genetic engineering is one of the most viable approach to harness symbiotic N-fixation by modulating the mechanisms and signalling system (Oldroyd and Dixon 2014). Molecular and genetic dissection of diazotrophs with their interaction to non-leguminous crops opens a new door to engineer nodule development or other mode of symbiosis (Oldroyd et al. 2011; Oldroyd 2013). For genetic manipulation of multiple gene complexes (*nodulin*, *nod* and *nif* genes) there is need of a series of events to engineer BNF in maize. Hence, by thoroughly analysing all the feasible engineering approaches

Table 4 Different genetic engineering strategies, tools and challenges

Genetic engineering Strategies	Biotechnological tools	Challenges
I. Efficient mitochondria or chloroplast transformation with *nif* (*nifHDK*) genes	To constitute or control BNF genes expression design promoters, ribosome binding sites (RBS), untranslated region [UTR], insulators, terminators, and broad-host-range plasmids	Complex coding sequences (regulatory pathways and overlapping genes)
II. Engineering of synthetic *nif* clusters with optimized N-fixation		Control of multigenes expression
		Construction of large multigene synthetic cassettes
III. Engineering of respiratory protection and O_2-binding proteins for aerobic N-fixation by diazotrophs	To build multigene synthetic regulation-Nascent computer-aided design algorithms and computational	Very sensitive process to small changes in gene expression
IV. Optimization of the diazotrophs colonization process	DNA synthesis and DNA assembly techniques (Golden Gate and Gibson assembly)	Availability of characterized wide range promoters and their regulatory control in many organisms
V. Conditional suppression of ammonium (NH_4^+) assimilation by diazotrophs to deliver N to plants		Question on the release of genetically modified organisms
VI. Refactoring of *nif* genes cluster by combinatorial design and assembly for functional optimization		
VII. Optimization of carbon supply to endosymbiotic bacteria from host root cells		
VIII. Designing and characterization of a wide range of promoters to drive an equivalent expression of several transgenes in the same cells		

we should strategies our objectives by utilizing the different advanced biotechnological tools to develop BNF in maize (Table 4; Fig. 4).

1. *Introgression of nitrogenase genes into maize*

To introduce BNF in maize one needs to construct the bacterial genes assembly encoding the nitrogenase enzyme with optimized N-fixation capability. As genetics of both mitochondria and chloroplast resembles to diazotroph plasmid constitution so these will be the perfect site for *nif* genes expression as well as will provide the sufficient energy (ATP) required for nitrogenase expression (Biswas and Gressho 2014; Curatti and Rubio 2014) (Fig. 4a). In chloroplast, the transcription and translation signals also match more closely with the microbes. But still, organelle transformation also has some challenges to encounter as in chloroplast for optimum transcription of *nif* genes, there is a need to replace *nif* gene promoter with suitable chloroplast promoter. Further, oxygen generated during photosynthesis will be detrimental for the nitrogenase enzyme. To address these challenges, we need to separate both the photosynthesis and N-fixation processes by regulating the expression of *nif* genes in the night when photosynthesis is absent or in non-photosynthetic parts such as roots (Peoples et al. 2009). López-Torrejón et al. (2016) engineered *nifH, nifM, nifU and nifS* from *Azotobacter vinelandii* into yeast cell and depicts that functional nitrogenase protein can be formed if only *NifH* and *NifM* polypeptide is targeted into the mitochondrial background jointly. In another study, Buren et al. (2017) targeted nine *nif* genes from *A. vinelandii* (*nifH, nifD, nifU, nifK, nifM, nifS, nifE*, *nifB* and *nifN*) into mitochondria and generate an active *NifDK* tetramer that is an essential component for nitrogenase expression in a eukaryotic cell. In tobacco (*Nicotiana* spp.) *NifH* and *NifM* genes have been transferred in chloroplast generating an active *NifH* protein with low expression (Ivleva et al. 2016). Therefore, for stable expression, efficient transformation methodology with the ideal *nif* genes set is needed, and their amino acid sequence need to be optimized to enhance stability in eukaryotic cells without negotiating with nitrogenase activity (Allen et al. 2017; Buren and Rubio 2017).

2. *Development of maize root-nodule symbiosis (RNS) as in legume*

To develop root-nodule symbiosis, the arbuscular mycorrhizal (AM) associations in maize should be approached where the crop plant develops endosymbiotic associations with endomycorrhizal fungi (Fig. 4b). In legumes and cereals, symbiotic signalling (SYM) pathways with similar genetic constituents play a role to promote AM symbiosis (Gutjahr et al. 2008). Similar genetic components are

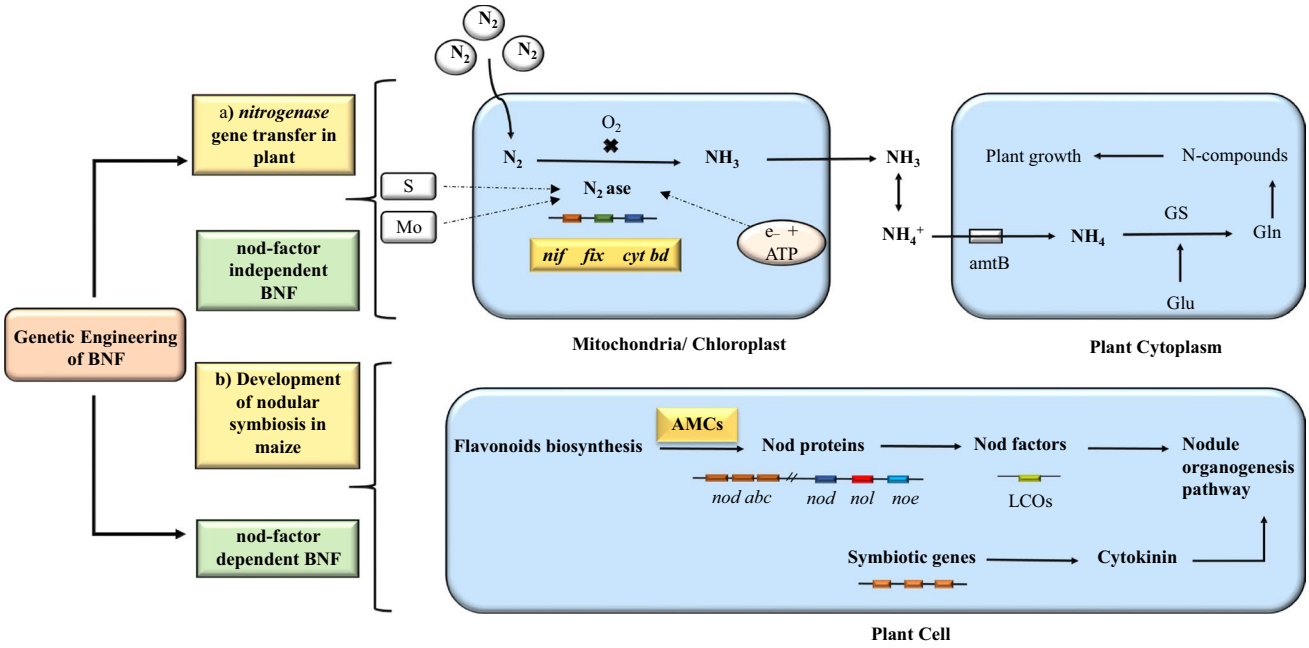

Fig. 4 Engineering strategies for BNF: **a** introgression of nitrogenase gene into maize mitochondria or chloroplast, **b** development of maize root-nodule symbiosis (RNS) as in legume. AmtB—ammonia transporter; AMCs—arbuscular mycorrhizal components; *cyt bd*—cytochrome *bd*; Glu—glutamate; Gln—glutamine; GS—glutamine synthetase; LCOs—lipochitooligosaccharides; Mo—molybdenum; NH_3—ammonia; N_2ase—nitrogenase; Nod factors—nodulation factors; S—sulphur

involved in AM and RNS development in legumes making it a "common symbiosis pathway" (CSP) (Markmann and Parniske 2008). Recently, phylogenomic studies also indicate that any species with AM associations can be turned into symbiont N fixer by introgressing small sets of genes (Griesmann et al. 2018). Hence, this association should be focused to research on developing a functional signalling pathway to support RNS in maize (Reddy et al. 2013; Delaux et al. 2015; Mus et al. 2016).

3. *Maize rhizosphere engineering for enhanced diazotrophs growth and colonization*

Mostly the diazotrophs population density is way too low in maize tissues to fix adequate N. Hence, it is important to develop a strategy to enhance diazotrophs colonization in maize roots to increase N-fixation. Nutritional resources also influence the diazotrophs population in the rhizosphere (Oger et al. 1997; Savka and Farrand 1997). A specialized carbon source will encourage microbe colonization by establishing appropriate signals between diazotrophs and maize (Mus et al. 2016). Host plant roots secrete polysaccharide mucilage to stimulate microbe colonization by providing significant carbon source in the rhizosphere. In pea (*Pisum sativum*), root mucilage promotes 3–25% growth of diazotrophic bacteria like *Rhizobium leguminosarum*, *Burkholderia cepacia,* and *Pseudomonas fluorescens* (Knee et al. 2001). Opines are the chemical compounds produced by rhizobial strains in legume nodules and are found to be suitable for chemical signalling between plants and rhizosphere bacteria (Murphy et al. 1995; Gordon et al. 1996). In this direction, rhizosphere engineering could be tried in maize to produce a metabolite that favour diazotrophs growth (Rossbach et al. 1994). It has been attempted in barley where rhizopine biosynthesis genes were successfully transferred by expressing a synthetic pathway for the production of rhizopine *scyllo*-inosamine (Geddes et al. 2019). It helps to produce synthetic signalling networks in between plant rhizosphere and microbes to regulate the targeted bacterial genes expression. In analogous to natural signalling in legume-rhizobium symbioses, rhizopine engineered transkingdom signalling controls the synthetic symbioses in cereal crops for N delivery and fixation. So overall engineering the rhizosphere regions, different genes and signalling system at the perfect site will enable the BNF a success in maize.

Amidst all these promises, genetic engineering has many challenges to work upon. Most importantly efficient control of the multigene expression is the biggest challenge. Modification of the complex regulatory pathways, construction of large synthetic multigene cassettes, characterization of wide range promoters and their regulatory control in many organisms are some of the major challenges. Finally, even if all these challenges are overcame release of such genetically

modified plant need to pass regulatory barriers, which may become a challenge.

Synthetic biology

Synthetic biology, a field of science, facilitates the redesigning of useful organisms/microbes with new and valuable abilities by engineering. Due to large *nif* genes machinery the transfer of nitrogenase in crop plants is complicated. There is need to simplify this complex process and develop robust genetic components that perform nitrogenase related functions in diverse cellular environments. Thus, reduction in nitrogenase genetic components is prerequisite to transfer in eukaryotic hosts. Synthetic biology will facilitate to overcome nitrogenase related challenges by regrouping genes into synthetic gene products by harnessing the knowledge of nitrogenase biosynthesis and catalysis steps. A few studies have been carried out in this direction where considering *Klebsiella pneumoniae* or *K. oxytoca* as *nif* genes sources, genes structures were reconstituted and reorganized by splicing nonessential genetic components to transfer in *E. coli* (Temme et al. 2012; Yang et al. 2014). The finding depicts that many genes are not required for artificial N-fixation (ANF) system in *E. coli*. This enables to engineer a minimal nitrogenase system in organelles comprising the structural and metallocluster biosynthesis genes. Wang et al. (2013) and Smanski et al. (2014) replaced the complex regulatory elements of *nif* gene clusters with a simple expression system which enables the expression of nitrogenase enzyme under different genetic and physiological conditions in eukaryotic organelles. Yang et al. (2017) utilized synthetic biology to reform electron-transport components (ETCs) of ferredoxin-NADPH oxidoreductases (FNRs) and ferredoxins molecules derived from plant organelles (chloroplast and mitochondria) which enable a reduction in microbial gene number required for nitrogenase engineering to generate a putative N-fixing plant. The results showed that the FNR–ferredoxin module from organelles is active in both natural and engineered nitrogenase enzyme depicting the potential for future engineering of diazotrophs in maize crop. Hence, with a detailed understanding of all the components required for BNF, nitrogenase can be engineered to simplify the complexity of the process by reducing targeted gene number into cereal crops.

Future perspectives

Cereal crops with N-fixation ability have been a dream of researchers since many decades. Maize is N exhaustive crop as it requires a millions tonne of N for high production, while heavy dose of N with low (20–30%) nitrogen use efficiency creates a number of side effects on the soil, water, and environmental pollution with millions dollar investment. Therefore, to transfer N-fixation traits in maize is the need of hour to solve the above issues for advance sustainability. Breeding or engineering for BNF in maize is a cumbersome task to develop the N-fixation ability. Hence, exploration of natural and phylogenetic diversity is needed to promote the BNF potential in maize. Technological advancements in next-generation sequencing, gene editing and synthetic biology will enable the dissection and manipulation of diazotrophs and plants at an unprecedented scale. For establishment of efficient symbiotic N-fixation, functional genomics will elucidate the molecular basis underlying this process for the maize. Large datasets can be generated through transcriptomics, proteomics and metabolomics by using faster and sensitive instruments to monitor the end products of gene expression in between host and diazotrophs and assess the physiological changes in symbiosis-specific pathways. The amalgamation of high-throughput and innovative technologies such as genome sequencing, transposon sequencing, and proteomics will enable to identify novel essential rhizobial genes, undetected coding genes, monitoring gene expression at different stages of symbiotic interaction and elucidate genetic elements required for an efficient symbiosis. Synthetic biology will open up the scope to shorten functional gene clusters so that the *nif* gene machinery can be successfully introgressed into heterologous hosts. There is need to study the non-leguminous model systems likewise in Sierra Mixe to harness it through breeding or engineering to enhance N-fixation rate. Development of large omics datasets for integrative analysis and mining of symbiotic genes will enable to identify the candidate genes to be used in breeding programs. Metagenomic studies are likely to identify microbiome at rhizosphere, non-rhizosphere, endosphere, phyllosphere and spermosphere, which may potentially be involved in BNF. However, this requires extensive computational facilities and precise sample handling, which is a limiting factor in low resource infrastructure and with untrained manpower. Directed investment in this direction is prerequisite to make BNF a reality in non-leguminous plants with the potential significant benefits. Integrating the prospects of plant and microbial diversity with genetic engineering will facilitate short- or long-term solutions to increase agricultural production for advance sustainability.

Glossary

Associative	Such bacteria are present in close association of plant roots and rarely observedin plant tissues. There is high competition

	with other soil microbes, so these are present in lowdensity, for example *Azospirillum and Azotobacter*.
Biological nitrogen fixation	It is a process to convert atmospheric nitrogen (N2) into ammoniaby nitrogenase enzyme through a specific group of microorganisms. In this process atmosphericnitrogen (N2) is incorporated into the tissue of certain plants.
Diazotrophs	These are prokaryotic bacteria that are able to fix atmospheric nitrogen (N2) intomore usable form for plants such as in ammonia.
Endophytics	These bacteria colonizes intercellular plant roots without inducing any extrastructures, but these bacteria do not survive well in soil. Their interactions with plants appear tobe less complex and provide more appropriate conditions for effective nitrogen fixation,for example *Azoarcus* spp., *Herbaspirillum seropedicae* and *Gluconobacter*.
Endosymbionts	These are bacteria which reside intracellularly of roots inducingdifferentiated structure like nodules. These display complex interaction between microbes andhost with facilitating the best appropriate conditions for effective nitrogen fixation, for example*Rhizobium, Frankia, and Anabaena azollae*.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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