Domestication of the Floating Fern Symbiosis *Azolla*

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Abstract

Ferns from the *Azolla* genus are highly productive without nitrogen fertilizer because filamentous cyanobacteria, *Nostoc azollae*, associated with the shoot stem cells, invade leaf cavities for N_2 fixation and reproductive structures for generational transfer. Previously used as nitrogen biofertilizer, their domestication is now considered for circular economy including the sustainable production of plant protein. The symbiosis recently transgressed into molecular research. Sequences from metagenomes of several species are available to study the contribution of the microbiome components to the symbiosis traits. A first assembly and annotation of the reference genome *A. filiculoides* was released; it allowed reconstruction of tannin biosynthesis, which determines *Azolla* biomass quality as a feed. Here, we begin with describing novel research areas required to integrate agrosystem development with domestication. We next describe first achievements to control the life cycle of the symbiosis in relation to dissemination, storage, and pre-breeding. We then identify key traits of the symbiosis that will need to be considered to achieve yield stability and discuss these traits with the little mechanistic insight available thus far. We conclude that for rapid breeding, the next vital development will be genome editing of fern host and cyanobacterial symbiont and describe our first steps toward this end.

Keywords

Azolla · Dinitrogen fixation · Ferns · Life cycle control · Neo-domestication · *Nostoc azollae* · Metagenome editing · Symbiosis

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Abbreviations

AP2	APETALA2
GA	Gibberellic Acid
GAMYB	Gibberellin- and Abscisic acid-regulated MYB
GFP	Green fluorescent protein
HiC	High-range interaction capture
Ipt	Isopentenyl synthase
MAG	Metagenome-Assembled Genome
$MIKC^C$	Transcription factor with MADS domain, I region, K domain, and C terminal domain of the C-type
RFP	Red fluorescent protein
STM	SHOOT MERISTEMLESS
WOX	WUSCHEL-Related Homeobox transcription factor

7.1 *Azolla*: A Model Aquatic Symbiosis that Requires Conceptually Novel Research in Ecology, Physiology, Development, and Genetics

Azolla ferns have been used as biofertilizer in diverse wetland agrosystems and have tremendous potential for sustainable plant protein or substrate production in tropical to temperate regions. They are wild strains, however, that require intensive labor input for their use, at times implying cheap labor, or child labor. A major challenge constitutes their domestication to reduce input labor: we currently lack industrialized methods to disseminate, store, and breed seed-free plants in general and more specifically these symbiotic heterosporous ferns. We also have a very scant understanding of fundamental processes relating to the development of heterosporous ferns or the physiology of phototrophic nitrogen-fixing symbionts.

We herewith aim to provide a timely review of what is needed for *Azolla* domestication based on recent advances in the control of the life cycle and the molecular genetics of these and other ferns. The recent advances are critically important for characterization of the biodiversity in the genus *Azolla*, for development of nursery practices and pre-breeding, which are required to eventually stabilize yield and tune biomass quality to specific applications by breeding. More advances will be needed, however, to achieve domestication; some will require conceptually novel research in biology and ecology, the latter because the ferns colonized the water surface. To begin with, we will introduce the topic of *Azolla* domestication by briefly presenting a few of these conceptually novel research areas to motivate readers from different fields to join the challenge.

7.1.1 Azolla Ferns Form Thick Floating Mats, Not Reefs, that Fixate CO_2 and N_2

Unlike most other floating plants, *Azolla* ferns are peculiar in that they form thick mats. Figure 7.1a shows 6-month-old cultures of *A. filiculoides*: the mats contain a significant amount of floating senescent plant and root biomass that does not degrade rapidly in the darkness the canopy generates. The potential of massive CO_2

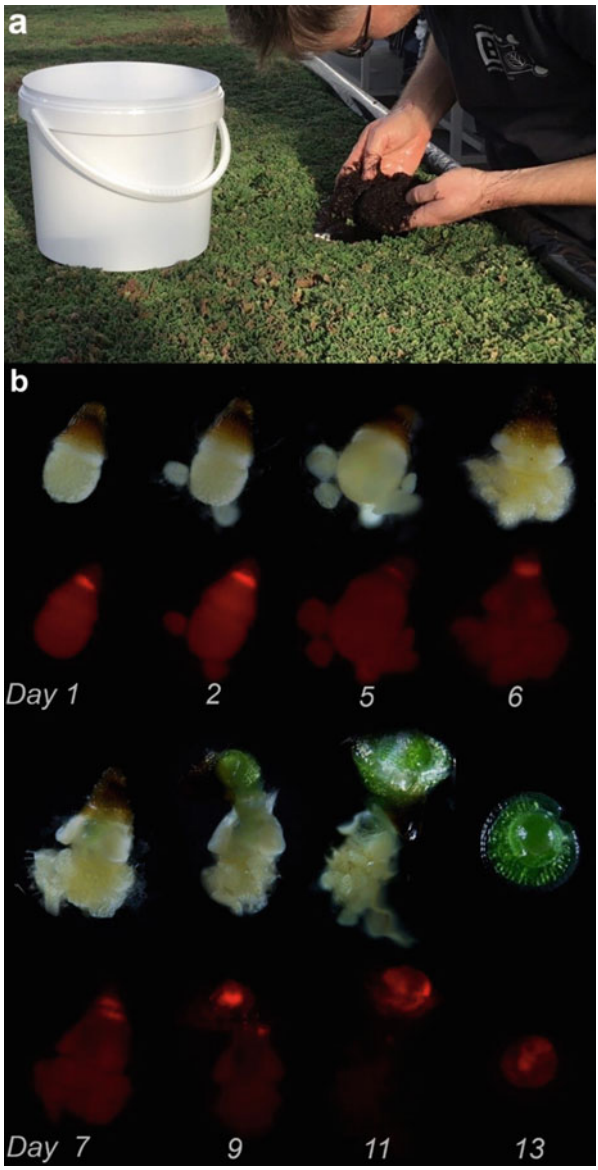

Fig. 7.1 Novel research areas important for integrated agrosystem development and domestication of *Azolla* ferns. (**a**) The true growth habit of *Azolla* ferns: mats with a substantial amount of floating senescent plant and root biomass that does not degrade rapidly in the darkness the canopy generates. Six-month-old cultures of *A. filiculoides* maintained in the glasshouses of Radboud University by Bas van de Riet from B-WARE in Nijmegen. (**b**) The coordinated development of symbionts and host for the establishment of the floating sporophyte. "Sporeling" and *N. azollae* akinetes germinate within 2 weeks after transfer to 20 °C and light. Images were taken using a Zeiss Axio Zoom V16 binocular with a 280× magnification, using white light to document megasporocarp development (above row), or using RFP filter fluorescence settings (bottom row) to reveal *N. azollae*. All images result from focus stacking (Helicon Focus)

drawdown when the mats sink is illustrated in sediments from the North Pole dating from the Eocene (Speelman et al. 2009). Drawdown over geological time periods may not be of much concern for the present realities. Nevertheless, the potential of *Azolla* mats has often been overlooked, or their development was deemed negative, even if nutrient remobilization was measured in mesocosms, as, for example, in Pinero-Rodríguez et al. (2021). Mats are of particular interest, however: they generate anoxia which in turn is required for the mobilization of phosphate precipitates (Gu et al. 2019; Wang et al. 2019); this represents an opportunity that should be exploited as phosphate will become a limiting resource for agriculture (Geissler et al. 2019). Mats causing anoxia in flooded wetlands further reduce CO_2 emissions from respiration of drained wetlands. Miller et al. (2008) pointed out that the mats also function to reverse soil subsidence in subsiding deltas such as the Sacramento-San Joaquin Delta. In ecology, therefore, research on the possible role of *Azolla* mats for biogeological engineering is needed to circularize the use of P-fertilizer before it runs out or reverse soil subsidence in subsiding deltas worldwide.

7.1.2 Azolla Ferns Exhibit Nitrogen Autotrophy at Astonishing Productivities

Thus far, the production of plant protein by nitrogen-fixating crops such as the legumes is N-fertilized because N_2 fixation rates that depend on carbon supply are limiting production (Salvagiotti et al. 2008). In contrast, *Azolla* fern biomass productivity, even at its peak, was not limited by N_2 fixation when tested under small-scale production conditions: it was driven by light energy while yielding some 50 ton/h/a dry weight biomass containing roughly 20% w/w protein (Brouwer et al. 2017, 2018). Research on the physiological mechanisms maintaining such high productivities and nitrogen fixation is needed to engineer staple crops with organelles that fixate N_2 using light energy; chloroplasts likely were derived from cyanobacteria that did not fixate N_2 (Nowack and Weber 2018). This idea is not novel, but molecular research on the specific case of the double photosynthesis in the leaves of *Azolla* is untouched, except for data collected some 40 years ago without molecular genetic insight (Peters et al. 1976; Ray et al. 1979; Tyagi et al. 1981; Calvert and Peters 1981).

7.1.3 Azolla Ferns and Symbionts Coordinate their Development So as to Colonize the Water Surface

Azolla ferns have adapted to the floating habit; they even evolved a mechanism to ditch their anchor, which in this case means to rapidly abscise their roots (Uheda and Kitoh 1994). They therefore are equipped to deal with sudden increases in water level by responding to submergence when respiration is inhibited. The developmental response does not require transcription and is mediated by oxidative stress

including reactive oxygen, nitrogen, and sulfur species; these species were proposed to trigger cell wall loosening, thus releasing the roots at the abscission zone in less than 1 h (Gurung et al. 2012; Cohen et al. 2015; Yamasaki et al. 2019).

The coordination of development between symbiont and fern is not understood at the mechanistic level. First insights on possible metabolites involved in the coordination were gained when testing the effects of deoxyanthocyanins synthesized by the fern on the development of the filamentous cyanobacteria *Nostoc punctiforme*, the facultative symbionts of cycads (Cohen et al. 2002).

Many ultrastructural observations lead to the conclusion that trichomes from the host could guide migration of the motile *N. azollae* into leaf cavities and sporocarps when the organs form in the shoot apical meristem (Calvert and Peters 1981; Calvert et al. 1985; Perkins and Peters 1993; Peters and Perkins 1993). Two types of trichomes seem to co-exist at the sites of attraction: the branched and unbranched type. Active membrane networks and other ultrastructure feature suggest that these trichomes are secreting substances. In a leaf cavity, only a single branched trichome was observed in contrast to the two-celled unbranched trichomes which are numerous. Sporophytes still form the trichomes inside the leaf cavity in the absence of the *N. azollae* (Forni et al. 1991). Glandular trichome metabolism in ferns is not studied; we expect differences in the types of trichomes as described for trichomes from the Solanaceae (Schuurink and Tissier 2020). To find out what substances may be secreted capitalizing on recent developments, we could begin with profiling gene expression and metabolites of cavity packets generated as in (Uheda 1986). More insight, however, would be gained from analysis of the thick glandular trichomes at the fern shoot apices, but these are more challenging to harvest for single cell profiling.

An understanding of the dormant stages in the life cycle of the symbiosis is lacking, yet these stages are critical for storage and dissemination of *Azolla* strains. It will be important, for example, to find out how the *N. azollae* (and possibly other low-abundance bacteria) are induced to form resting stages and how these resting stages are spurred into life again when the sporeling grows into the inoculation chamber under the indusium cap. *A. filiculoides* sporeling and *N. azollae* akinetes germinate within 2 weeks after transfer of megasporocarp/massulae clumps from 4 °C to 20 °C and to light (about 80 μmol/m^2/s photosynthetic active radiation; Fig. 7.1b). Under these conditions, the akinetes are seen as a red fluorescence under the indusium cap until day 6; at day 7, when the sporeling shoot apex reaches the indusium chamber, a clear separation of the red fluorescent signal is seen between indusium and shoot apex; from day 9 onward, when the shoot apex has emerged from the megaspore and the indusium cap is ditched, most of the red fluorescence from the *N. azollae* is seen at the sporeling shoot apex (Fig. 7.1b).

Understanding coordinated development of the host with the bacteria in the *Azolla* Ark will be key to engineering yield stability by efficient dissemination with inoculum that may also be stored long term. Such inoculum could be the fertilized megasporocarps, which in the case of *A. filiculoides* can be stored over several years at 4 °C as well as frozen (Brouwer et al. 2014).

7.1.4 Breeding Heterosporous Ferns and the Microbiome Associated with the Azolla Symbiosis

Combined molecular genetics and phylogenetic approaches will reveal mechanisms ruling the fern life cycle because of the likely conservation of at least some of the mechanism components with those found in well-studied plant lineages. Knowledge of these mechanisms will be required to control the production of dissemination and storage stages of ferns in general. The knowledge will further provide insight into the origins of life cycle regulators in seed plants, as the last common ancestor of ferns and seed plants had a homosporous life cycle.

The symbiosis is currently a wild plant with extremely promising yield potential, but we have yet to engineer yield stability. The most cost-effective approach to achieve yield stability in the case of seed plants has been by breeding (Cox et al. 1988; Bailey-Serres et al. 2019). Classical breeding by selection and inbreeding is not desirable, however, because it reduces the genetic diversity of the cultured populations (Fu 2015).

We know little of the genetic diversity of the symbiotic microflora and how to engineer the key traits associated with them including the further amelioration of N_2 fixation. The cyanobiont and fern genomes have co-evolved, and additional bacteria are persistently associated with the *Azolla* symbiosis (Dijkhuizen et al. 2018; Li et al. 2018). Co-evolution of the genomes implies that fitness of the symbiosis is determined by its metagenome and that, therefore, not only the fern but also associated microflora need to be considered when breeding from high yield potential to high yield. This is conceptually novel.

7.1.5 Engineering Sustainability and Resilience of Primary Production through Rapid Domestication of Botanic Diversity

Taken together, *Azolla* ferns represent a botanic oddity, but their applications are aligned with improving the sustainability of our primary production systems while increasing their resilience and yield. We lack much of the foundational knowledge to develop these applications. Nevertheless, recent advances in molecular genetics of cyanobacteria and ferns, including *Azolla* ferns, allow now to consider domestication of fern symbioses.

In the following, we will attempt at drafting the biotechnology approaches that already have been undertaken and those still needed toward *Azolla* fern domestication. These represent a change in perspective where, more generally, we aim to exploit botanic diversity in rapid breeding/domestication schemes of plant/microbe associations that are adapted to thrive in specialized environments. Proof-of-concept rapid breeding/domestication have recently been reported for a wild tomato, *Solanum pimpinellifolium*, for example (Zsögön et al. 2018). We have yet to embrace the concept for plant-microbe symbioses. When complemented with more rapid and systematic agrosystem engineering, including paludiculture, the method will extend

arable regions and widen crop diversity so as to achieve yield resilience (Massawe et al. 2016).

We will first review how the life cycle of *Azolla* ferns and the cyanobionts may be controlled as this is key to storage, dissemination, and breeding of strains for any one application. We secondly will describe what information is available to link traits with genes from the different components of the metagenome and provide a first impression of the pangenome diversity in the cyanobiont. Thirdly, we will concentrate on what traits could be tackled for engineering initially, given priorities for sustainable primary production with *Azolla* and commercial viability. Lastly, we will look at emerging advances in engineering the genomes of the fern and cyanobiont. In an outlook, we provide the rationale for the challenge of wanting to develop genetic tools for the heterosporous fern and the obligate cyanobacterial symbiont.

7.2 Domestication of the *Azolla* Symbiosis: What Bases Are Available?

7.2.1 Controlling the Symbiosis Life Cycle for Strain Storage and Dissemination and for Breeding

A sketch of the life cycle of *Azolla* ferns is provided in Fig. 7.2a using *A. filiculoides*, for example; *A. filiculoides* can complete its life cycle in 2 to 3 months. Sporophytes (Fig. 7.2a-*j*, *a*) transit into sexual reproduction when their shoot apical meristems form sporocarp initials in pairs (Dijkhuizen et al. 2020, Fig. 7.1). Such pair usually consists of a megasporocarp (mega) with a single megasporangium (Fig. 7.2a-*b*) and a microsporocarp (micro) with the degenerating megasporangium initial and many de-repressed microsporangia (Fig. 7.2a-*e*, arrow). In the meristem region of the shoot apex, *N. azollae* are recruited into the closing indusium cap (Fig. 7.2a-*b*, arrow). Pairs of sporocarps are found on the branch next to the branch point where roots are formed. When they mature, megasporocarps will form a megagametophyte fuelled by the megaspore (Fig. 7.2a-*d*). We do not know whether the gametophyte development occurs before or after the megasporocarp detaches from the sporophyte; eventually archegonia are formed by the megagametophyte (Fig. 7.2a-*d*, arrow). Mature microsporocarps rupture to release massulae containing several microspores (Fig. 7.2a-*f*); we do not know when the much reduced microgametophytes (Fig. 7.2a-*g*) develop from the microspores and when the microgametophytes generate the flagellate gametes, but this must happen before or when massulae attach to the megasporocarps with their glochidia (Fig. 7.2a-*h*). The flagellate gametes swim toward the archegonia and fertilize the egg cells which starts the outgrowth of the diploid "sporeling," still fuelled by the megaspore reserves (Fig. 7.2a-*i, j, k*).

The *Azolla* life cycle can be completed within 2 to 3 months for *A. filiculoides*. Sporocarps are the natural storage and dissemination stages which in the case of *A. filiculoides* may be stored up to 4 years at 4 °C or indefinitely, when first dried and then cryopreserved at −80 °C (Brouwer et al. 2014). In contrast, sporophytes cannot

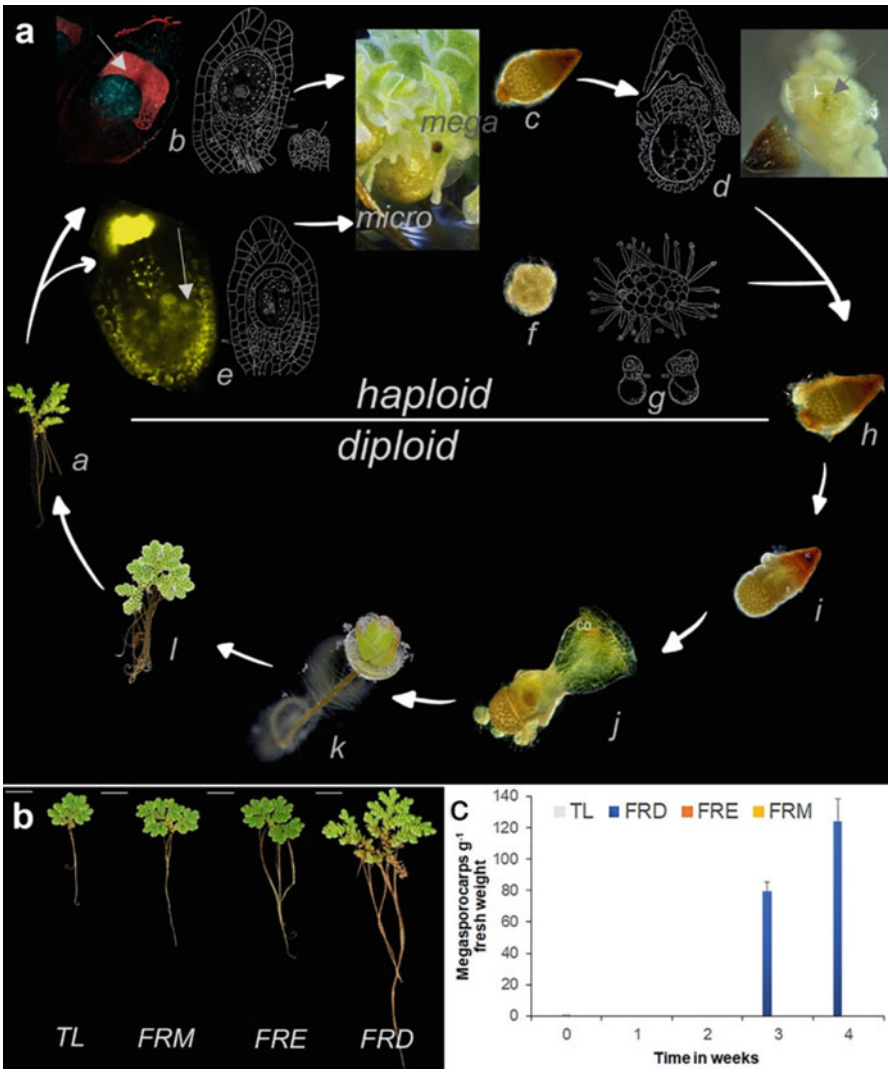

Fig. 7.2 The *A. filiculoides* symbiosis heterosporous life cycle and repression of sexual reproduction by red light. Graphic insets are from Coulter (1910). (**a)** Sporophytes (*i*−*a*) transit into sexual reproduction when they form sporocarp initials in pairs: a megasporocarp (mega) with a single megasporangium (*b*) and a microsporocarp (micro) with the degenerating megasporangium initial and many de-repressed microsporangia (*e*, arrow). *N. azollae* are recruited into the closing indusium cap (*b*, arrow). When they mature, megasporocarps will form a megagametophyte (*d*) which develops archegonia with egg cells (*d*, arrow). Mature microsporocarps rupture to release massulae (*f*) attaching to megasporocarps with their glochidia (*h*); microspores inside massulae germinate into microgametophytes, generating the flagellate gametes that fertilize the egg cells: this starts the outgrowth of the diploid "sporeling" (*i, j, k*). *haploid*, life stages with haploid tissues; *diploid*, life stages with diploid tissues. **(b)** Sporophyte habit after 4 weeks with a 16 h light period with tube light (*TL,* which contains no far-red light), tube light with far-red light during either the entire day length (*FRD*), 4 h in the morning (*FRM*), or 4 h in the afternoon (*FRE*). **(c)** Sporocarps produced under the conditions in (**b**). Shown are averages with standard deviation, from three replicate

be stored; they are comparatively fragile and heavy due to their high water content. Industrialized inoculation of production fields via sporocarps, therefore, needs to be considered in the future and will rely on methods to produce them in high quantity and quality.

The induction of the haploid phase change in fern sporophytes occurs when sporangia initials are formed. This phase change was shown to depend on photoperiod, temperature, or sugars in the medium in many ferns, including members of the heterosporous Salviniaceae (Labouriau 1958; Sussex and Steeves 1958; Harvey and Caponetti 1974). Analogies were made from the onset with the transition to flowering in seed plants. Unlike in seed plants, however, the formation of the meristems that developed sporangia was reported to be surprisingly plastic: it occurred at many locations in the sporophytes and even in gametophytes (Labouriau 1958). In the case of *A. filiculoides*, induction of sporocarps was dependent on far-red light; furthermore, numbers of sporocarps increased with the density of the fern mat (Dijkhuizen et al. 2020). Far-red light was required during the entire light period for complete elongation and three-dimensional growth of the sporophytes (Fig. 7.2b); it was also required for the induction of sporocarps (Fig. 7.2c); neither end of day nor morning or afternoon far-red light sufficed (Fig. 7.2b, c). We therefore conclude that the typically red-light-dominated spectrum of open fields represses entry into sexual reproduction in *A. filiculoides*. Light quality did not, in our hands, induce sexual reproduction in sporophytes from the species of the Anzali Lagoon or from *A. pinnata* (Dijkhuizen et al. 2020). Environmental cues inducing sporocarp formation, therefore, differed for other *Azolla* species. Environmental cues are, however, predicted to feed into a conserved phase transition signaling network with component regulators known from seed plants: the topologies of phylogenetic trees obtained with regulators such as the $MIKC^C$, AP2, or GAMYB and the conserved regulons such as the GAMYB/microRNA319 substantiate the prediction (Ambrose and Vasco 2016; Dijkhuizen et al. 2020). Furthermore, in situ hybridization with *$MIKC^C$* and *AP2* probes strongly light up the sporangia initials and show that these regulators are active during sporangia initiation (Hasebe et al. 1998; Ambrose and Vasco 2016). Together, the preliminary work suggests that control of flowering in seed plants may well have its origins in the diploid to haploid phase transition of the common ancestor of ferns and seed plants. If confirmed, much of the networks of control known from seed plants could be conserved, and this knowledge could be exploited to more quickly control the phase transitions in all *Azolla* fern species, as well as other ferns.

What induces spore germination and the development of gametophytes in *Azolla* is not known and difficult to study by comparison with homosporous ferns because the gametophytes are much reduced in size and develop inside closed structures (Fig. 7.2a-*c, f*). As a result, we expect regulation of spore germination and

Fig. 7.2 (continued) measurements of three independently grown cultures for each condition. The cultures were reset once per week to a fixed density

gametophyte development to be taken over by the sporocarp to some extent. Ripe megasporocarps, for example, exhibit a distinct break between indusium cap and body due to the growth of the mega-gametophyte (Fig. 7.2a-*h*). In *A. filiculoides* and *A. caroliniana*, these sporocarps will clump with massulae. This behavior was exploited to clean sporocarp clumps away from debris. In the case of *A. caroliniana*, the sporophyte biomass was dried before sieving of the clumps and setup of in vitro germination assays (Singh et al. 1990). Germination of dried sporocarps from *A. caroliniana* was improved with GA (Singh et al. 1990). Unlike *A. caroliniana*, mats of the temperate *A. filiculoides* did not yield viable sporocarps after drying. The *A. filiculoides* sporocarps were harvested by showering sporophytes over sieves instead. Then, gentle agitation of the harvest in water lead to clumps of megasporocarps and massulae which are picked up manually for storage or germination assays.

Mats of *A. filiculoides* grown with 0.3% w/v NaCl could be harvested for such sporocarp clumps, but when these clumps were germinated at RT with 16 h light/day in distilled water, they only yielded few viable sporelings, in contrast to clumps obtained from sporophytes grown without NaCl (Fig. 7.3a). Upon closer inspection, the clumps from sporophytes raised on NaCl had many very immature sporelings arrested in their development, presumably because of insufficient nutrient reserve in the megaspore (not shown). Germination rates of clumps were variable in our hands. They were, however, unaffected by dipping (12 h) into surfactant (Silvet L-77 at 0.02% w/v) or *Agrobacterium* suspensions. In contrast, dipping into sugars caused infections which affected germination rates, even if the clumps had been rinsed with abundant distilled water after exposure to the sugars and if the sporelings were transferred to IRRI medium as soon as they emerged (Fig. 7.3b).

Whether compounds in the part of the floating *Azolla* mat made of dead sporophytes and roots improve germination of sporelings is unknown. Given that this mat part floats, it retains the naturally sinking detached sporocarps close to the water surface, and thus to light, thereby increasing likelihood of germination and establishment of the sporelings. The mat structure and biochemical content, we expect therefore, have been selected for the ferns' after-life traits that facilitate re-establishment of generations of ferns. Analysis of the lipids and waxes in *Azolla* ferns reveals that they not only are characteristic but particularly abundant in hydrophobic very long chains (Nierop et al. 2018) which promotes buoyancy. Moreover, the very-long-chain lipids and waxes are recalcitrant to biodegradation since they are found in the fossil sediment layers dating from the Eocene *Azolla* (Speelman et al. 2009).

The industrialized collection of sporocarps still needs to be invented and may need genetic alteration of fern architecture and abscission of the megasporocarps for evenly induced development and detachment of many more megasporocarps, in analogy with seed shattering (Di Vittori et al. 2019).

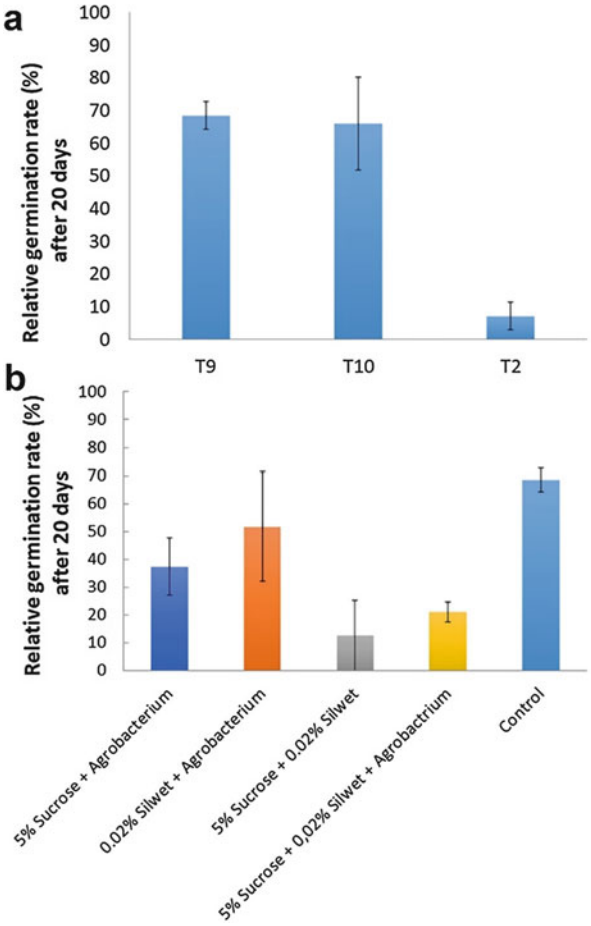

Fig. 7.3 *A. filiculoides* "sporeling" germination from clumped megasporocarps and massulae. **(a)** Effect of NaCl. Sporocarps were harvested from sporophyte mats grown with micronutrient and phosphate-fortified soil without (T9, T10) and with 0.3% w/v NaCl (T2). Germination of the clumps was scored after 20 days in triplicate replicate germination assays. **(b)** Effect of reagents used in the floral dip protocol (Clough and Bent 1998). Sporocarps (from T9 mats) were used to test overnight (12 h) dipping in combinations of sucrose (5% w/v), Silvet L-77 (0.02% v/v), and *Agrobacterium tumefaciens* (GV3101) compared to distilled water (control). After the dip, clumps were cleaned with distilled water and transferred to distilled water for sporulation at RT with 16 h light/day. Sporelings emerging to the surface were transferred to IRRI medium. Shown are averages with standard error of three replicate assays.

7.2.2 Life Cycle of Cyanobacteria inside the Fern: The Apical Colony and Germinating Akinetes Matter

Key to propagation of the *N. azollae* is its apical colony in the shoot apex and the consequent movement of its motile filaments, the hormogonia, to organ initials such

as the leaf cavities and the sporocarps. How the movement of the hormogonia and the differentiation of cell types are controlled by the fern host is not known. A role for host trichomes that are of the secretory style is likely and for deoxyanthocyanins which may regulate hormogonia formation (Cohen et al. 2002). A critical handicap for biotechnology is the fact that *N. azollae* cells may not be propagated outside the ferns, likely because of metabolic interdependence with the host, given the cyanobionts' eroded genome (Ran et al. 2010).

N. azollae, however, can be swapped between some *Azolla* fern species. A key stage for "swapping" *N. azollae* is the germinating sporeling of a "decapitated" megasporocarp, from which the indusium cap has been removed and, then, to which a new indusium cap from another species has been added. The germinating *N. azollae* resting stages, the akinetes, under the new indusium cap will apparently invade the developing sporeling shoot apex to generate hybrid fern cyanobacterial symbioses (Lin and Watanabe 1988; Plazinski et al. 1989). The hybrids are viable suggesting that the *N. azollae* functions and mechanisms of recognition/immunity are conserved between *Azolla* species. In contrast, hybrids of *Azolla* fern species (sexual fern hybrids) were not generally viable: for example, when crossing *A. microphylla* with *A. filiculoides*, only 8 crosses out of 114 gave viable fern sexual hybrids, of which only a single grew as well as either parent (Watanabe et al. 1993).

Synchronous akinete development under the indusium cap is a biotechnology opportunity not only for "swapping" the cyanobiont from different species but also for generating engineered bacteria using cyanobionts collected from under indusium caps. If we could induce the akinetes into cell divisions while they are still trapped in the mucilage under the indusium cap, we could attempt conjugation with *E. coli* by soaking indusium caps and then transfer the soaked indusium caps to "decapitated" megasporocarps. Alternatively, to engineer the *N. azollae*, *E. coli* conjugation must be carried out in situ using sporophytes so as to target the colony at the shoot apex.

7.2.3 Azolla *Pre-Breeding: Current Understanding of the Azolla Metagenome Diversity*

To exploit traits in natural populations, the ferns need to be preserved along with the wetland regions wherein they thrive. *A. nilotica* from the upper Nile regions was reported extinct (Birks 2002). The next-best resources of biodiversity are *Azolla* strain collections such as that from the International Rice Research Institute (IRRI) biofertilizer collection (Watanabe et al. 1992). The IRRI collection of *Azolla* strains was recently transferred to the University of the Philippines (Philippines) and the Dr. Cecilia Koo Botanic Conservation Center (Taiwan). The IRRI collection was conserved over several decades by sub-culturing which is labor-intensive and prone to mislabelling and could alter the microbial consortium associated with the original strains. Nevertheless, the first sample of sequenced accessions from the IRRI collection has shown that the ferns are indeed diverse and that also the diversity of their *N. azollae* symbiont was maintained along with other low-abundance symbionts (Dijkhuizen et al. 2018; Li et al. 2018). To improve on these collections,

recent knowhow of how to induce sporocarps and store them at −80 °C by cryopreservation should be extended beyond *A. filiculoides* and deployed for germplasm conservation (Brouwer et al. 2014; Dijkhuizen et al. 2020).

To make the most of the natural biodiversity of the *Azolla* symbioses, an improved characterization of the taxonomy inside the *Azolla* genus is required; it will likely uncover novel species or natural hybrids as recent studies have shown (Madeira et al. 2019; Dijkhuizen et al. 2020). Sequencing approaches that go beyond PCR amplifications between conserved regions in the chloroplast will allow more resolution of the analyses. Analyses of amplicons from the ITS gene regions are flawed by the intra-genomic variations in the many ITS repeats within the nuclear genome (Dijkhuizen et al. 2020). Nevertheless, the ITS1 region was used to infer that some *A. caroliniana* strains in China are hybrids: the PCR-amplified ITS1 sequence consensus from the "hybrid" when submitted to BLAST search of the NCBI database (Johnson et al. 2008) returned sequence regions more identical to *A. cristata* and to *A. caroliniana* (Madeira et al. 2019). This could be an artifact when assembling the PCR consensus, however. We suggest that whole-genome sequences should be used to reconstruct the diversity of ITS1 in each genome accurately, if we decide to continue using the ITS1 sequences to infer phylogeny for historical reasons.

7.2.4 Rapid Breeding Using Molecular Genetics

Accelerated breeding using molecular breeding approaches requires a high-quality inventory of genes in the symbiosis (Kole et al. 2015). Genome sequencing data is available for *Azolla* symbioses from six species as well as the non-symbiotic and related water fern *Salvinia cucullata* (Dijkhuizen et al. 2018; Li et al. 2018), mostly short-read technology. Assemblies of the reads from these species have not been released. In addition, genome assemblies from key species are missing including a species representing the second branch of the *Rhizosperma* section of the *Azolla* genus, for example, a (sub)tropical *A. pinnata*. The data has not been exploited beyond the figure of the co-evolution of fern host and cyanobiont (Li et al. 2018).

Gene expression for simultaneous profiling of all transcripts in the symbiosis was shown to be possible by rRNA depletion of both plant and gram-negative bacterial origins. At 20 M pair-end reads per sample sequencing depth, the assays were sensitive enough to extract differential gene expression in sporophytes for the fern host nucleus, the chloroplast, and the cyanobiont, but not for the associated bacteria (Dijkhuizen et al. 2020). Pooling six replicate samples of sporophyte revealed that increasing sequencing depth sixfold would only allow to profile the most highly expressed genes in the associated bacteria.

The *A. filiculoides* reference genome ($2n = 44$ chromosomes, predicted size 750 Mb) is not yet a chromosome-scale assembly. Long-read sequencing (PacBio RS II N50 14 kb) led to the first assembly of the *A. filiculoides* genome with some 4700 scaffolds covering some 650 Mb non-haplotype resolved sequences assigned as belonging to "Streptophyta" (Li et al. 2018). Annotation yielded some 32 k genes

based on de novo prediction and sporophyte RNA sequencing. Consequently, many genes uniquely expressed in specialized reproductive tissues are missing or have an erroneous annotation. To date, the miRNA loci discovered in Dijkhuizen et al. (2020) have not been included in the annotation of the *A. filiculoides* genome on fernbase.org. Functional annotation was exclusively done using homology predictions. Key resources to improve annotations are intron/exon annotation predictors (Keilwagen et al. 2019), more diverse RNA sequencing data, and the One KP resource (Leebens-Mack et al. 2019). To improve contiguity, HiC and optical mapping have been carried out (unpublished); we will ideally want whole chromosome assemblies and haplotype resolution of at least one cross to predict segregation. More urgently, the future assembly and annotation quality should be such that it can be shared and uploaded onto the NCBI and other databases as a more accessible resource.

7.2.5 Metagenomes from Six Species Have Provided Insight into Microbial Assemblages Associated with the Symbiosis

Breeding of the *Azolla* symbioses needs to consider the important traits conveyed by its microbiome. Our initial analysis comparing rRNA sequences in short reads of DNA from six species of *Azolla* with those in scaffolds from *A. filiculoides* long-read assemblies revealed near-complete bacterial genomes persistently associated with the symbioses. The two Rhizobiales genomes persistent in several *Azolla* species were found enriched in leaf juice DNA from *A. filiculoides*. The two Rhizobiales when combined were capable of denitrification (Dijkhuizen et al. 2018); more recent gene expression data suggests that the bacteria express specific sugar transporters at high levels and therefore may be thriving on imported sugars (data not shown).

Metagenome-assembled genomes (MAGs) of the *N. azollae* from the six sequenced *Azolla* species had over 95% identity with the *N. azollae* assembly from the *A. filiculoides* strain Stockholm, suggesting that they represent a single operational taxonomic unit (Ran et al. 2010; Dijkhuizen et al. 2020). This is surprising given the relative isolation of the *N. azollae* inside the fern host: crosses between the different fern species did not generally lead to viable offspring (Watanabe et al. 1993). The observation further suggests that the genome erosion caused by active transposons may have occurred early on during the evolution of the symbiosis. Alignment of the *N. azollae* MAGs from the different species suggested that assembly breaks often aligned (Fig. 7.4); manual inspection revealed that more than half of the breaks (Fig. 7.4 *red bars*) coincide with transposase genes as annotated in the reference *N. azollae 0708* genome (NCBI microbial genomes database). The data suggest that some 60% of the transposon coordinates date back to a common ancestor of the *N. azollae* strains inside the diverse fern species tested. We conclude, firstly, that the *N. azollae* are monophyletic and, secondly, that the evolution of their genomes might be constrained by the symbiosis. Early erosion would be consistent with theory proposing that the loss of functions no longer required presents benefits (McCutcheon and Moran 2010).

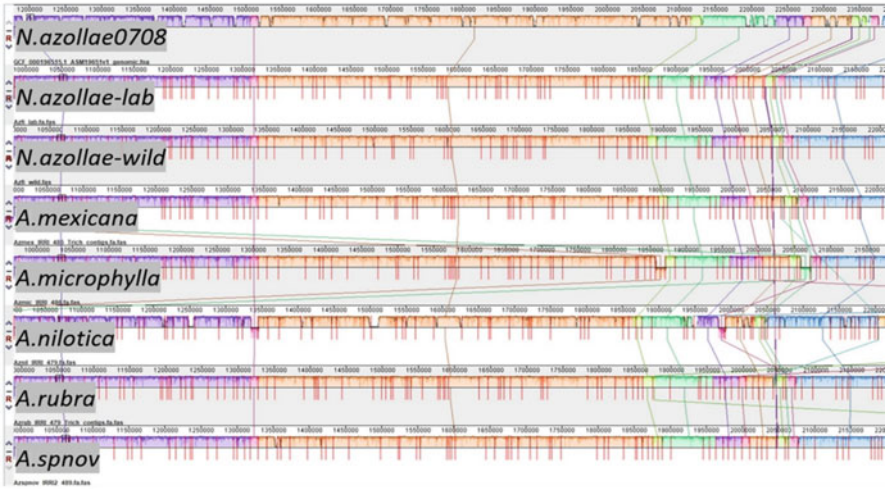

Fig. 7.4 Alignment of contig breaks in metagenome-assembled genomes (MAG) of *N. azollae* from different species of *Azolla*. MAG were sorted and aligned with MAUVE (Rissman et al. 2009). Sequences were assembled as described in Dijkhuizen et al. (2020) which yielded 341–366 contigs in the section *Azolla* assemblies. Sequences from *N. azollae* in *A. filiculoides* were from either ferns originating in Stockholm (*N. azollae* 0708) or ferns originating from Utrecht, laboratory cultured or collected in the wild (*N. azollae*_Lab and *N. azollae_wild*, respectively). Mauve colored "syntenic" regions, the *red bars* represent contig breaks in the MAG

Genome erosion by transposon mutagenesis is more generally found in the vertically transmitted species of *Burkholderia* bacteria associated with leaf nodules of angiosperm plants from the Primulaceae and Rubiaceae, yet these symbioses are of polyphyletic origin (Pinto-Carbó et al. 2018). Given erosion in all *N. azollae* genomes and presence of associated Rhizobiales bacteria in all *Azolla* ferns, we further suspect that, as in the case of the aphid/*Buchnera aphidicola* symbiosis, some tasks within the symbiosis have been transferred to associated bacteria in the leaf cavity (Dijkhuizen et al. 2018). This could have occurred by horizontal gene transfer as in the case of the aphid di-symbiontic systems (Manzano-Marín et al. 2020). A more in-depth analysis of the bacteria within the leaf cavity could shed light on as of yet undiscovered functions these Rhizobiales bacteria have for the *Azolla* host, for *N. azollae* cyanobionts, and for each other.

7.3 Biotechnology for Sustainable Development with *Azolla* Ferns: What Traits Could be Tackled in Priority?

Methods in rapid domestication require that we identify the relationships between traits and gene expression in a broader sense. This requires insight in molecular mechanisms underpinning key traits of the *Azolla* symbiosis. Traits of particular relevance toward the use of *Azolla* ferns for sustainable primary production include

high productivity in the absence of nitrogen fertilizer, high protein biomass, high tannin content, phosphate accumulation, and resistance to abiotic and biotic stresses.

We only have come as far as an inventory of genes: from next to zero genes known in 2013 to combined pro- and eukaryote RNA sequencing information backed by metagenome annotation in 2020. The annotation is based on homologies with characterized proteins in the databases; therefore, they are not definite predictions of gene function. Nevertheless, such inventory allows for deep phylogenetic comparisons with gene inventories from many species covering all lineages of land plants such as those from the One KP database (Ka-Shu Wong et al. 2020); these comparisons have strong predictive value because they capitalize on information gathered in many lineages. We therefore discuss how the present inventory and knowhow could be used to tackle the key traits listed above.

7.3.1 Transfer of Nitrogen Autotrophy into Staple Crops

Transfer of nitrogen autotrophy into staple crops requires knowing which processes in the fern regulate interactions with the N_2-fixating phototrophic symbionts so as to eventually align light-driven nitrogen fixation of the endophyte with high growth. This feature is unique for *Azolla*. In most other documented symbioses, including those with facultative symbionts from the Nostocales, N_2 fixation is fuelled with photosynthate derived from the plant and therefore presumed less efficient.

Engineering chloroplasts for N_2 fixation may be the first alternative to consider (nitroplast) when engineering nitrogen autotrophy of a crop plant since the coordinated development of a bacterium and vertical transmission during the life cycle of the crop plant are not needed. The nitroplast idea has been around for quite some time, but the endeavor is possibly still out of experimental reach, possibly because of the complex regulation needed to obtain the nitrogenase assembled. Very recent studies report a breakthrough with the expression of a whole recombinant *Nif*-operon from filamentous cyanobacteria: this led to nitrogenase complex formation. More work is needed to obtain functional complexes (Thiel 2019). Key for the engineering of a nitroplast will be the protection of nitrogenase from O_2 as well as the metabolic integration of N_2 fixation with reducing power and ATP production; this may now become feasible with improved efficiencies for chloroplast transformation and synthetic biology tools (Kwak et al. 2019).

Obviously, the common ancestor of the Archaeplastida (red algae, glaucophyte, green algae, and land plants) already possessed the machinery to phagocyte and maintain cyanobacteria (Nowack and Weber 2018); the machinery for phagocytosis likely was lost in the plant lineage when thicker cell walls became predominant. Genomic approaches indicate that chloroplasts are derived from an early-branching cyanobacterium related to *Gloeomargarita lithophora*; *G. lithophora* thrives in fresh water, is coccal, and lacks N_2 fixation (Ponce-Toledo et al. 2017). We conclude, therefore, that a machinery to specifically maintain N_2-fixating filamentous cyanobacteria, for example, may have evolved after chloroplasts were acquired; this occurred in hornworts, mosses, the *Azolla* ferns, the gymnosperm cycads, or

plants from the *Gunnera* genus (Angiosperm). Nevertheless, functions of the many nuclear genes unique to all Archaeplastida (Karpowicz et al. 2011) should be taken into account when engineering chloroplasts with N_2-fixating capability. Alternatively, the specific metabolism of the recently discovered UCYN cyanobacteria from the *Braarudosphaera bigelowii* marine haptophyte algae should be used as a basis of understanding (Landa et al. 2021). Given the slow progress on turning chloroplasts into nitroplasts, stimulation of crop symbioses with phototrophic N_2-fixating bacteria remains an attractive alternative.

The conserved set of symbiosis genes from the plants in symbioses with Rhizobiales or arbuscular mycorrhiza has been invoked for the facultative interactions of Nostocaceae with many plants, including rice and *Gunnera* species, but not in the case of *Azolla* (Li et al. 2018; Alvarez et al. 2020). These symbioses are thus very likely polyphyletic. This is corroborated by a phylogenetic analysis of the Nostocales engaging in plant symbioses (Warshan et al. 2018). The later study revealed a list of genes common to all the *Nostoc* strains able to enter a symbiotic relation with plants. Once we understand how facultative phototrophic N_2-fixating bacteria may be recruited into crop plants, we can move on to reconstituting obligate symbioses such as those from *Azolla* and the UCYN-A/haptophyte; the latter showed that N_2 fixation is viable even in N-rich coastal or high-latitude water (Mills et al. 2020). In the meantime, we are using the genomes of the different *N. azollae* in the species of *Azolla* ferns to understand which genes were inactivated first, given that these genomes eroded mostly by transposon activity (Ran et al. 2010).

7.3.2 Controlling Formation of Megasporocarps

The number of megasporocarps limits sexual reproduction of *Azolla* ferns in a nursery setting or when reproductive structures, the megasporocarps/massulae clumps, are used for dissemination. Sporocarps are made as a result of the *Azolla* fern transition from the diploid to the haploid phase; these structures are likely homologous to sori of the homosporous ferns. The process of sori induction in the homosporous fern *Ceratopteris richardii* is characterized by specific and high expression of the *Cr*MADS1 transcription factor (TF), from a TF clade sister to that of the floral homeotic genes and AtSOC1 (Hasebe et al. 1998; Dijkhuizen et al. 2020). *Azolla* ferns contain two homologues of *Cr*MADS1, consistent with the whole-genome duplication at the base of the *Azolla* fern evolution, which are induced upon sporocarp induction; we have yet to find out whether these *Azolla* homologues are responsible for sporocarp specification. Sporangia in ferns further exhibit specific EuAP2 expression as shown in the case of *C. richardii* (Zumajo-Cardona et al. 2021).

In *Azolla*, once sporocarp initials are induced, sporangia initials develop. In megasporocaprs, the most advanced sporangium will develop into a megasporangium, while the remaining sporangia are stopped in their development. Alternatively in microsporocarps, the most advanced sporangium initial degenerates which

permits development if many microsporangia inside the growing microsporocarp (Coulter 1910; Fig. 7.2a). To regulate the number of mega- to microsporocarps generated on the sporophytes, we need more insight into the mechanisms triggering megasporangium degeneration in the early development of *Azolla* sporangia. The mechanisms may be related to those of the nucellus (megasporangium) degradation in seed plants. In some seed plants, these mechanisms were linked to ethylene signalling and de-repression by the polycomb protein FERTILIZATION-INDEPENDENT SEED (Ingram 2017).

7.3.3 Quality of the Protein-Rich Biomass for Applications in the Food/Feed Sector

Azolla biomass is a protein-rich feed without any further modification; it is suited for the on-farm recycling of mineral nutrients where it does not need to be stored since it can be produced continuously (Leterme et al. 2009, 2010; Brouwer et al. 2018). *Azolla* biomass used in feed mixes has maximum inclusion rates that are somewhat lower than those for soymeal. This is likely due to the fact that *Azolla* fern sporophytes accumulate proanthocyanidins (PA, also known as condensed tannins; Güngör et al. 2021). PA likely function as antifeedants blocking enzymes of the digestive system and thus protecting sporophytes from grazers. PA extract together with proteins and they cause an unappetizing brown color upon oxidation. PA are regarded as the main bottleneck of *Azolla* protein extraction. On the other hand, PA could be beneficial anti-bloating agents in feed formulations (Kelln et al. 2020).

Manipulating the PA biosynthesis pathway would, therefore, be a first possible target for *Azolla* pre-breeding. PA have been reported to result from spontaneous polymerizations of *trans*- and *cis*-flavan-3-ols such as catechin and epicatechin (Dixon et al. 2005). However, new insights show that the polymerization might still be regulated by enzymes such as leucoanthocyanidin reductase (LAR). LAR is traditionally known as the enzyme converting leucoanthocyanidins into *trans*-flavan-3-ols such as catechin (Fig. 7.5). Consistently, LAR from *A. filiculoides* was shown to produce catechin from leucocyanidin in vitro (Güngör et al. 2021). A knock-out of the *LAR* gene in *Medicago truncatula*, however, caused accumulation of PA with a high degree of polymerization (DP) (Liu et al. 2016). LAR, therefore, was proposed to regulate the ratio of starter units (epicatechin) and extension units (epicatechin carbocation): knocking out LAR leads to a significant reduction in starter units causing the extension units to polymerize onto the few remaining starter units forming very large PAs. If there are more starter units, more smaller PAs are formed. Knocking out *Azfi*LAR might thus not directly lead to a reduction of the PA content in *Azolla* but in longer PA polymers. To manipulate PA accumulation instead, a transcription factor (TF) should be targeted that regulates expression of the PA biosynthesis pathway enzymes coordinately. Such a TF has not been characterized yet in *Azolla*, but phylogenetic analyses of MYB TF indicate that fern class VIII MYB may be involved (Güngör et al. 2021).

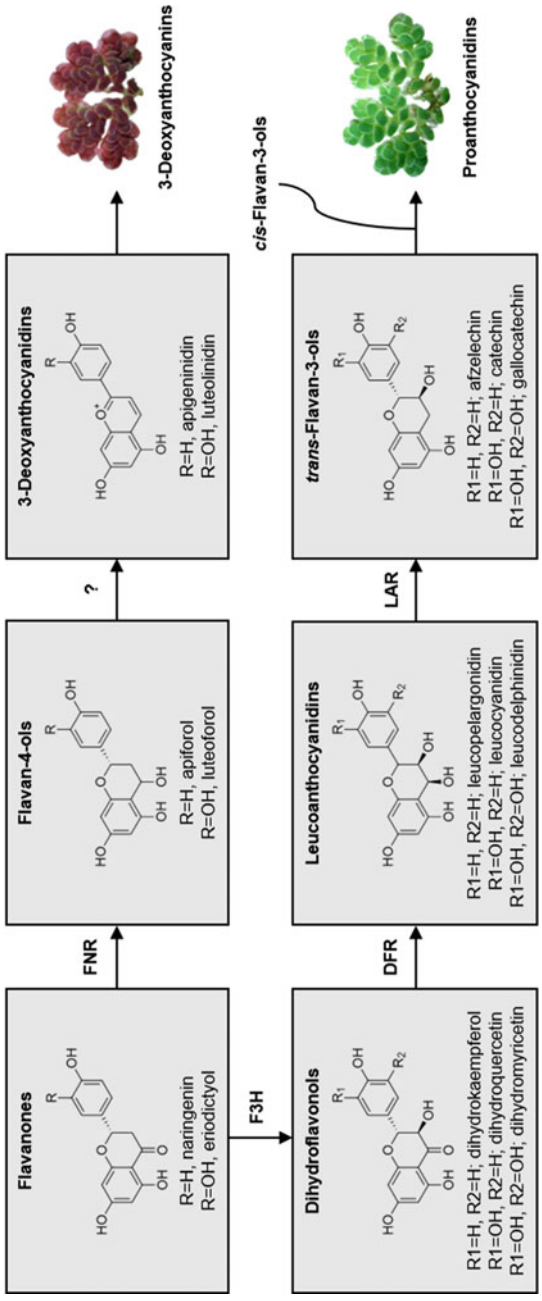

Fig. 7.5 Partial flavonoid biosynthesis pathway in *A. filiculoides* (Güngör et al. 2021). DFR, dihydroflavonol 4-reductase; F3H, flavanone 3-hydroxylase; FNR, flavanone 4-reductase; LAR, leucoanthocyanidin reductase. Proanthocyanidins accumulate in green ferns with a degree of polymerization consistent with a function as antifeed. The activity of the *Azolla* LAR was shown in vitro, yet LAR enzymes from some plants also control the extent to which proanthocyanidins condensate. Under abiotic stress, *Azolla* ferns are known to accumulate 3-deoxyanthocyanin which are made from the same precursor naringenin as proanthocyanidins. Enzymes for the biosynthesis of the 3-deoxyanthocyanins are unknown

Alternatively, reducing dihydroflavonol 4-reductase (DFR) activity may reduce the intermediates supplying the PA biosynthesis (Fig. 7.5). Knocking out DFR, however, could lead to accumulation of alternative products such as the red-colored deoxyanthocyanins, known to accumulate when sporophytes experience abiotic stress (Fig. 7.5). We have yet to find out whether deoxyanthocyanins are beneficial colorants. Stressed red sporophytes contain less protein and lipid than the green unstressed ones. Deoxyanthocyanins also are a feed deterrent for tadpoles and snails (Cohen et al. 2002; Nham Tran et al. 2020).

7.3.4 Breeding Phosphorus Accumulation for Circular Farming

Azolla ferns were reported to accumulate phosphate (P) which could be useful to circularize P nutrient on farm in addition to their role as in biological N_2 fixation (Temmink et al. 2018). Plants are known to store P in the form of phytate (inositol hexakisphosphate, InsP6), yet cyanobacteria store polyphosphate granules (poly-P bodies; Sanz-Luque et al. 2020). Non-ruminant livestock cannot access P nutrient from phytate because they lack phytase enzymes. As a result, phytate in their guts binds essential divalent cation mineral nutrients precluding their uptake. Phytate, therefore, is less preferred over polyphosphate in feed applications. *N. azollae* from the leaf pockets of the *Azolla* sporophyte depend on the fern cells to provide them with mineral nutrients because the leaf cavity is not in contact with the water surrounding the ferns; the leaf pore is adaxially oriented on the upper leaf lobe so as to promote gas exchange, particularly the poorly soluble N_2. A key research question, therefore, is how the flux of mineral nutrients may be controlled from the host to the cyanobacteria.

Recent studies on arbuscular mycorrhiza/host symbioses report specific accumulation of phospholipase C products at the interface of the symbionts, and these have been reported to cause movement of phosphate transporters to the membrane (Nakamura et al. 2005; Ivanov and Harrison 2019). Such compounds were also found to induce hormogonia (the motile filaments) of the facultative symbiotic Yaku-1 strain of *Nostoc punctiforme* isolated from cycad roots (Hashidoko et al. 2019). Taken together, improving the P accumulation of the *Azolla* symbiosis could be achieved by stimulating uptake into the cyanobiont without causing mineral deficiencies of the *Azolla* biomass due to increased phytate. Regulators of the Pho-regulon could be targeted because they control the expression of the polyphosphate kinase enzyme (Gao et al. 2018). This unlikely will suffice without the further increase of P transport from the fern cells surrounding the leaf cavities.

More generally, we should consider *Azolla* ferns as self-assembled bioreactors of cyanobacteria that are very easy to maintain and harvest compared cultures of cyanobacteria or algae.

7.3.5 Breeding Yield Stability of Floating Mats in Aquaculture to Counter Soil Subsidence, Grazing by Specialist Insects, and Tannase Activity

Populations of *Azolla* are effectively controlled by grazers, the most notorious being *Stenopelmus rufinasus* (McConnachie et al. 2004). *S. rufinasus* has become ubiquitous because it has been actively deployed in regions where *Azolla* ferns are deemed invasive weeds; the weevil prefers *Azolla* species with lower PA content, *A. filiculoides* and *A. cristata*, compared to *A. pinnata* (Madeira et al. 2016; Güngör et al. 2021). The populations are furthermore affected by aphids; aphid feeding precludes *A. filiculoides* from reaching reproductive maturity (most notably the water lily aphid *Rhopalosiphum nymphaeae* (Hance et al. 1994)). Fungal infections with *Sclerotium rolfsii*, *Rhizoctonia* sp., *Acremonium* sp., *Aspergillus* sp., *Curvularia* sp., *Botryodiplodia*, and *Fusarium thapsinum* have been described (Shahjahan et al. 1980; Natural and Mendoza 1991; Dey et al. 2017), but also unidentified fungi affect *Azolla* mat growth. Much work is needed in this area to obtain yield stability in an open aquaculture setting, for example, when flooding land on low-land regions.

We distinguish the rapid production mode from the slow growth mode that leads to the buildup of a thick mat complete with a large zone of dead fern/root material. In the former mode, fern mats are harvested to 60% of the surface weekly which avoids formation of senescing ferns and permits disease evasion. In the latter mode, disease and pests need to be controlled. Engineering resistance to biotic stress represents the biggest breeding/biotechnology challenge in the domestication of the *Azolla* ferns because of their aquatic habitat; in these habitats, spray applications of systemic insecticides or immune stimulating substances are restricted. To this date, we fail to understand how grazers evade the effects of tannins as well as other not yet discovered anti-grazing compounds from the *Azolla* biomass.

7.4 Development of *Azolla* Breeding Tools and Current Limitations

7.4.1 Breeding by Genome Editing to Counter Inbreeding and Maximize on-Farm Crop Diversity

Breeding by selection and inbreeding is not desirable because it reduces the genetic diversity of the cultured populations (Fu 2015). Genetic diversity of receptors is required, for example, to maintain crop resistance to a variety of biotic stresses (Wu et al. 2018). Molecular breeding and genome editing represent the better alternatives.

Technologies to edit genomes seamlessly exist today; they mostly employ RNA-guided DNA binding nickases such as Cas9 and result in a very high frequency of edited cells in spite of their dependence on the target cell repair mechanisms (Atkins and Voytas 2020). Recently, the RNA-guided insertion of a

transposon could be achieved in *E. coli* (Strecker et al. 2019): this means that editing may now become possible beyond a mere few bases even if the method is not yet seamless. The high efficacy of editing is key to its success: then only few meristem cells need to be targeted to engineer a new cell lineage. Alternatively, a genome-edited vegetatively growing lineage still needs to be induced into producing natural stem cells, including spores or gametes, for the production of homozygous gene-edited offspring.

Access to stem cells remains a critical bottleneck of all genome editing in practice, even with the latest genome editing techniques. Induction of shoot meristems from vegetative cells using WUSCHEL, SHOOT MERISTEMLESS (STM), and the bacterial isopentenyl synthase (ipt) for cytokinin biosynthesis proved an effective alternative to targeting natural or cultured stem cells in several Solanaceae, *Vitis vinifera*, and *Arabidopsis* (Maher et al. 2020). We know little of the components required for meristem induction in ferns (Ambrose and Vasco 2016). The only *Ceratopteris richardii* (Cer) WUSCHEL-related homeobox (WOX) from the modern WUSHEL clade of WOX proteins is not expressed in the shoot apical meristem. In contrast, *Cer*WOXB from the intermediate clade of WOX proteins is expressed, and its knockdown by RNAi causes a reduction of the sporophyte and meristem size (Youngstrom et al. 2019). Gene constructs that induced meristems in seed plants may thus not necessarily work in ferns. In addition, Maher et al. (2020) used constructs containing viral replicons such that the incoming T-DNA may be replicated and possibly trafficked between cells; viral replicons have never been tested in ferns.

7.4.2 Transient Transformation of the Fern Host and how to Progress from There

Tissue culture proved possible with the surface-sterilized *A. filiculoides* fern host devoid of cyanobacteria. When maintained in the dark, such sterilized sporophytes on C-medium stopped the outgrowth of leaves and branches; after 8 weeks, they produced cauliflower-like structures (Fig. 7.6a). C-medium was made of agar-solidified half-strength MS medium (Murashige and Skoog 1962) with 6% w/v sucrose, 1 mg/L 2,4-dichlorophenoxyacetic acid, and 1.5 mg/L 6-benzylaminopurine. Upon transfer to low light and R-medium for 2 weeks and then to liquid IRRI medium as in Brouwer et al. (2017), outgrowth resumed (Fig. 7.6b). R-medium was made of agar-solidified (0.7% w/v) half-strength MS medium with 150 mg/L ascorbic acid and 1 mg/L gibberellic acid at pH 6.0.

We have not yet learned how to re-inoculate ferns without *N. azollae* with their symbiont. Also we have not yet been able to induce sporocarp formation in ferns without *N. azollae*. Development of a transformation/genome editing method that does not require tissue culture, therefore, was the focus of initial studies.

For genome editing to be successful in *Azolla*, the following steps need to be achieved: (1) transfer of the DNA (RNA or ribozyme) into a cell, (2) movement to the nucleus, (3) transient expression of genes required for meristem activation and

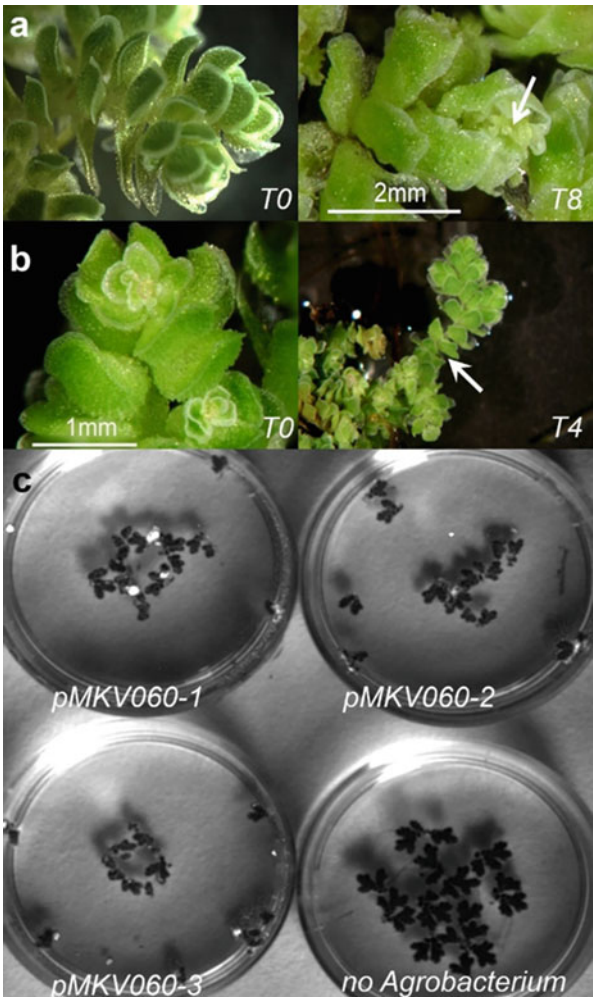

Fig. 7.6 Results toward developing transformation and genome editing for the *Azolla* symbiosis. **(a)** Growth of sterilized *Azolla* sporophytes on agar-solidified half-strength Murashige and Skoog (MS, Murashige and Skoog 1962) medium with 6% w/v sucrose, 1 mg/L 2,4-dichlorophenoxyacetic acid, and 1.5 mg/L 6-benzylaminopurine. T0, at the beginning; T8, after 8 weeks in the dark. Cauliflower-like structures at the shoot apex (arrow) are typically observed after 8 weeks of dark incubation. **(b)** Outgrowth of new shoot apices (arrow) from the cauliflower-like structures. Ferns with cauliflower-like structures, obtained as in (**a**), were transferred in low light and to agar-solidified (0.7% w/v) half-strength MS medium with 150 mg/L ascorbic acid and 1 mg/L gibberellic acid at pH 6.0. After 2 weeks, they were further transferred to liquid medium as in Brouwer et al. (2017). T0, shoot apex at the beginning; T4, representative situation after 4 weeks. **(c)** Transient expression of the luciferase gene 24 h after co-cultivation with *A. tumefaciens* GV3101 containing pMKV060 (Maher et al. 2020), compared to no *Agrobacterium* control. Luminescence is seen as white spots overlaid on the black and white image of the ferns. We tested that *Agrobacterium* with this construct do not express the luciferase unlike those with the pMM131 construct (Maher et al. 2020) which we also tested (data not shown)

catalytic insertion into the plant chromosome, (4) DNA insertion/editing in a meristematic cell, and (5) generation of reproductive organs/cells to obtain a homozygous offspring.

We first tested *Agrobacterium*-mediated transfer of DNA into cells of whole, cut, or dissected fern after dipping procedures. We used the previously tested plasmid pMKV060 (https://www.addgene.org/133315/) containing a transfer-DNA (T-DNA) encoding the Bean yellow dwarf viral (BeYDV) replicon and the firefly *LUCIFERASE* expression cassette and pMKV057 (https://www.addgene.org/133312/) containing the same T-DNA with in addition *STM* and *ipt* expression cassettes (Maher et al. 2020). The luciferase reporter was active in sporophyte leaf cells exposed to *Agrobacterium* with either of these plasmids, while it was inactive when the ferns were not exposed to the *Agrobacterium* (Fig. 7.6c). Moreover, the reporter was not active in these *Agrobacterium* strains, unlike those containing the pMM131 plasmid with the *LUCIFERASE* cloned behind the CaMV promoter (Maher et al. 2020). Luciferase reporter activity only lasted until 4 days after the co-cultivation, however. Also, the luminescence was never located in the shoot tips, unless we manually dissected these before co-cultivation (Fig. 7.6c). We conclude that *Agrobacterium* is able to transfer T-DNA into cells of *Azolla* ferns and that T-DNA is moved to the nucleus and then expressed. The T-DNA likely was silenced/degraded and unlikely incorporated into the fern genome. In addition, shoot meristem cells were not exposed to the *Agrobacterium* during dipping, regardless of whether Silvet L-77 and sucrose were added during the dipping procedure as in Clough and Bent (1998).

7.4.3 Transient Transformation for the *N. Azollae* and how to Progress from there

Shoot apices of the *A. filiculoides* sporophytes have proven impermeable to the outside solutions. It may be possible to dissect shoot apices to expose the apical *N. azollae* colony, apply a DNA transfer technique, and, possibly, then select for the engineered cyanobacteria by way of erythromycin resistance: the ferns but not wild-type *N. azollae* resist 60 mg/L erythromycin when grown on nitrogen medium (Forni et al. 1991).

Preliminary, tri-parental conjugation assays using leaf juice from sporophytes revealed that GFP was transiently expressed in *N. azollae* for up to 24 h after conjugation with a cargo containing the GFP expression cassette. This occurred at low frequencies, however, as the cells were not dividing and possibly no longer viable outside the fern leaf cavity (data not shown).

The process needs optimizing so as to achieve high rates of incorporation of the incoming cargo. One possible alternative to achieve this is to catalyze the insertion of the cargo into the chromosome or plasmids that are expected to be present in multiple copies per cyanobacterial cell. Recent characterization of CRISPR-associated transposases (CAST) and their optimization in *E. coli* reveal that this may be possible by way of RNA-guided transposition (Strecker et al. 2019). The

CAST were found in many filamentous cyanobacteria, including *Anabaena cylindrica* PCC 7122, and, therefore, they likely will function. The engineered version of CAST, however, should be tested in *Anabaena* first and then in *N. azollae*.

To move *Azolla* symbioses from high yield potential to high yield stability, we conclude, is a tall order that will require building a community of scientists able to communicate so as to transgress discipline boundaries and organization from molecular to ecosystem level. Many new developments fostering fundamental research on symbioses and their development as model organisms will facilitate developments with *Azolla* ferns; let us thus embrace the challenge together.

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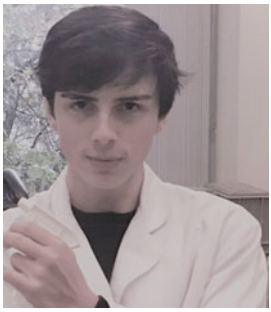
H. Schluepmann is a well-traveled plant biologist with a great respect for the achievements of young scientists. "After a Masters researching homologous recombination in the lab of Jerzy Paszkowski at ETH (Zürich, Switzerland), I completed my PhD on pollen tube cell wall biosynthesis in the laboratory of Tony Bacic (Melbourne, Australia). Since 2004, I am Assistant Professor at the Utrecht University Molecular Plant Physiology where I uncovered the regulatory role of trehalose metabolism in *Arabidopsis thaliana* before developing the innovation-driven *Azolla* research. I am very much hands-on, both in the laboratory and in bio-informatics, in spite of the visionary ambitions in sustainability of my research."



I. Bigot was a Master's Bio Inspired Innovation intern at the *Azolla* laboratory in 2019–2020 (Molecular Plant Physiology Department of Utrecht University). Ivan explored dissemination and storage of *Azolla filiculoides*, specifically the induction and the maturity of spores, fertilization, and sporeling establishment, and worked on T-DNA delivery in both sporophytes and spores. He is currently working at the INRA plum trees.

N. Rijken was a Master's Environmental and Plant Biology intern at the *Azolla* laboratory in 2018–2019 (Molecular Plant Physiology Department of Utrecht University); he currently is a research assistant at Koppert Biological Systems. Niels explored the effect of far-red light on, and analyzed miRNA loci of, *Azolla filiculoides*.

A. Correas Grifoll was a Master's Science and Business intern at the *Azolla* laboratory in 2018–2019 (Molecular Plant Physiology Department of Utrecht University). Using confocal microscopy, Anna explored the diversity of natural fluorophores synthesized during the development of reproductive structures of the *A. filiculoides* symbiosis. She currently is working as Marketing Associate at OPTI Medical Systems.

P. Gudde was a Master's Bio Inspired Innovation intern at the *Azolla* laboratory in 2019–2020 (Molecular Plant Physiology Department of Utrecht University). Peter analyzed the genomes of *Nostoc azollae* from different *Azolla* species. He is now working in the field of science communication.

L.W. Dijkhuizen is a Ph.D. candidate in the *Azolla* laboratory of Henriette Schluepmann. Laura studies the microbes associated with ferns of the *Azolla* genus using metagenomics techniques. In other *Azolla* projects, she supports colleagues with computational infrastructure and analyses.

E. Güngör is a Ph.D. candidate in the *Azolla* laboratory of Henriette Schluepmann. He obtained a bachelor in Medical Biology and a master in Bio Inspired Innovation. After practical research in methane microbiology, he moved on to investigating *Azolla* secondary metabolism in relation to biomass quality.