Commentary

Ectomycorrhizal fungi – fairy rings and the wood-wide web

Many of us will have seen fairy rings, the mysterious circles of fungal fruiting bodies that occur in open grassy places or in forests. The secrecy about these rings is expressed in their naming: in English folklore, the rings were said to be caused by fairies dancing in a circle (Fig. 1) whereas in German-speaking Europe they are known as ‘Hexenringe’, stemming from an old mediaeval belief that they represented places where witches would have their gatherings. An old belief says that if you run around a fairy ring nine times on the first night of the new moon, you will hear sounds of music and laughter coming up from the underground home of the elves. Unlike this sorcerous practice, Lian et al. (this issue; pp. 825–836) used sturdy molecular techniques to provide insights into the below-ground fungal world of fairy rings formed by the ectomycorrhizal (ECM) basidiomycete *Tricholoma matsutake*. Using microsatellite markers they revealed the genetic composition of this species within and between fairy rings based on fruiting bodies as well as ECM tips, and they provide new information about the composition of other ECM species inside, beneath and outside of the rings using ITS polymorphism analysis. Another interesting aspect of this precise work is the use of microsatellite markers on ECM root tips to identify the genotypes of both the fungus matsutake and host *Pinus densiflora*, which allowed the authors to provide the first direct evidence that each fungal genotype colonized multiple pine trees and vice versa.

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Fairy rings and shiros

In grass, the best-known fairy ring fungus is *Marasmius oreades*, commonly known as the fairy ring mushroom, which forms distinct rings or arch-like structures that result in stimulation or suppression of the surrounding plant growth and the seasonal production of fruiting bodies. The mycelium, which is found in the soil beneath the ring, interferes with plant–water relationships and produces metabolites capable of damaging grass roots. As in this species, most of the fairy-ring forming fungi are saprotrophs, but similar structures are not uncommonly produced by ectomycorrhizal fungi which sometimes but not always form these rings around a tree, as described by Last et al. (1984). Fairy rings are thought to originate from one individual, whereby the mycelium grows in a radial fashion away from a central point. Growth can continue for several years and, depending on the species, occurs at a rate of up to 100 cm yr\(^{-1}\). As the mycelial front advances the mycelium behind

the advancing front, within the ring, decays. However, whether the rings originate from a fragmented vegetative mycelium or by radial growth from a single spore has been a matter for debate. For example, a study of Matsunia oreades showed that almost all of the 30 fairy rings studied, in two sand dune areas, represented a separate genotype and that the fruiting bodies from within rings were formed by one individual (Abesha et al., 2003). This shows that the establishment of new rings was generally mediated by basidiospore dispersal and outgrowth. For Tricholoma matsutake, Lian et al. detected several genets in each study site from pre-existing and newly produced fairy rings over the last 10 yr. They therefore concluded that sexual reproduction, previously thought to play a minor role, is important for the establishment of matsutake rings. This has important implications for the conservation strategies of this economically important edible fungus in Japan, the harvests of which have markedly decreased in recent years. Unlike studies of other fairy-ring forming fungal basidiomycetes, the authors found that about half of the fairy rings (the development and expansion of which has previously been documented for the last 10 yr) consisted of two to four genets. From this interesting result it can be inferred that there must be some form of coordinated growth present between these genets, which were aggregated and not intermingled within a ring. Such a genetic mosaic pattern of matsutake rings was suggested more than 30 yr ago by Ogawa (1975), and was also described more recently by Murata et al. (2005) who proposed that such a behavior is only possible because of the fact that T. matsutake lacks a somatic incompatibility system.

A special feature of Tricholoma matsutake is the formation of a solid and tight white aggregate of mycelia and mycorrhizas below the litter layer – this has been termed a ‘shiro’, and in Japanese means white color, castle or place. In many cases it has been shown that the above and below ground occurrences of ECM species do not overlap (e.g. Gardes & Bruns, 1996). However, Lian et al. now show that in the case of T. matsutake, the above and below-ground genet locations correspond closely and that the matsutake ECM tips were distributed in a very limited area, consisting of about 30–50 cm of shiro beneath the fruiting bodies of the fairy ring. This therefore substantiates the conclusions drawn by Murata et al. (2005) who inferred from the genetic composition of the fruiting bodies that the shiros consist of multiple genotypes.

Ectomycorrhizal community shifts around shiros

Lian and colleagues reveal that beneath the fairy rings the matsutake shiro dominates the ECM community. At least in the soil volume of the upper 10 cm horizon, sampled by these authors and in which the shiro is usually located (approx. upper 20 cm; cf. Ogawa, 1975), only a small number of other ECM fungal species with low abundance were detected on root tips of pine trees. Within and outside of the fairy ring however, the ECM communities were similar in terms of species richness, diversity and species composition. Ogawa (1975) accurately described the development and expansion of shiros over a period of years in both horizontal and vertical directions and showed that there was a zone starting about 15–20 cm behind the advancing front in which ECM tips and mycelia were decomposed, leaving black dead root tips. Unlike other ECM fungi, T. matsutake forms a thin and undifferentiated fungal sheath and carbonized root tips that resemble general plant necrotic reactions, indeed it was only shown recently that it promotes plant growth as a symbiont (Guerin-Laguette et al., 2004; Yamada et al., 2006). The soil within this decomposing zone was described as desiccated, probably resulting from an impermeable layer formation. Behind this zone, around 50 cm from the advancing front, Ogawa (1975) described that the root and soil condition had recovered. Lian and colleagues now confirm that the ECM species within the fairy ring returned to its previous composition, concluding that although ECM fungi are suppressed during the expansion of matsutake mycelia, they are able to recolonize after the passage of the fairy ring-forming shiro. In addition, the shiro does no seem to be formed over the entire soil horizon where roots are present, but is usually limited to the upper 20 cm of the B-horizon. Since the authors did not sample over the whole horizon, it is possible that additional species were present above and below the shiro.

Ectomycorrhiza fungi – host connections

Microsatellite markers have proved powerful tools for population genetic studies on both ECM fungi and plants. Here, Lian et al. combined such genetic studies to analyze both the ECM fungi and that of their tree hosts at the intraspecific level, thus providing new insights into ECM–host connections. By studying the genotype composition of Tricholoma matsutake and that of the associated host pine trees, Lian and colleagues were able to show that each matsutake genet was associated with more than one host pine tree. An existing common mycorrhizal network (CMN) in the forest, a so called wood-wide-web, in which plant species are interlinked via hyphae of mycorrhizal fungi, has been suggested previously (see review in Simard & Durall, 2004). Direct physical and functional evidence for such a network, in which nutrients and carbon are exchanged between plant individuals via the CMN was demonstrated in laboratory experiments using macroscopic, microscopic and autoradiographic tracings within transparent microcosms. Further to this, isotope labeling studies have been used in the field to demonstrate that carbon (C) transfer occurs between plant species sharing ectomycorrhizal fungi, with negligible transfer to incompatible hosts, strongly suggesting that the C transfer occurred predominantly through a CMN pathway (Simard et al., 2004).
1997). However, direct observations of interplant hyphal linkages in the field are rare because of technical difficulties; as the mycorrhizal pathway could not be visually quantified using autoradiography, the identity of the transfer pathway remains in question. Although there is still no guarantee that the CMN is intact and functional, the work of Lian et al. provides new evidence, by showing for the first time in the field that an ectomycorrhizal genotype is associated with several host genotypes. Since densely packed ectomycorrhizas, which consisted of multiple hosts, and extraradical mycelia of a single matsutake genotype were found within a small location, the integrity of the CMN is most likely.

Another important methodological aspect of Lian et al.’s work is the possibility of determining both fungal and host genotypes on the same ECM tip which allows the study of genotype relations in terms of interaction, specificity and coevolution. A recent study by Korkama et al. (also in this issue; pp. 815–824) showed that the ECM community not only significantly varied between different phenotypes (i.e. slow vs. fast growing) of even aged spruce tree clones, but also within phenotypically similar spruce clones. This indicates that the host genotype can directly or indirectly affect the ECM community. It would be very interesting to see whether this is also true at the intraspecific level of ECM species. Are there differences in host genotype specificity for certain fungal genotypes or vice versa? Are certain genotypes better adapted to each other, as has been presumed for native vs. nonnative host genotypes to a local ECM community (Newton & Haigh, 1998; Korkama et al., 2006)? Regarding specialist ECM fungal species that associate with one or only a few host species, do phylogeographic patterns of ECM fungi and their hosts correspond, as has been suggested for the Perigord truffle and its hosts oak and hazelnut (Murata et al., 2004)? The coevolution and specificity of mutualistic and antagonistic systems have been studied at the population level for a variety of species interactions (Thompson, 1998); however, knowledge for the ECM symbiosis is scarce, probably because of the practical difficulties of studying it in the field but now we have a tool at hand to start these investigations.

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References

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Supercharging rice photosynthesis to increase yield

Rice (Oryza sativa and Oryza glaberrima are the cultivated species) is a plant with C₃ photosynthesis that is mostly grown in climates where photorespiration rates are high. The fact that rice is a C₃ crop is often a surprise, given the prevalence in the tropics and subtropics of C₄ photosynthesis in crops (maize, sorghum, sugar cane), fodder grasses (e.g. Cynodon dactylon), grass weeds (e.g. Imperata cylindrica) and sedges (e.g. Cyperus papyrus). Although C₄ photosynthesis has evolved independently at least 45 times in 19 families of angiosperms (Sage, 2004), it is not known in the genus Oryza or in any close relatives or anywhere in the Bambusoideae subfamily of grasses, many species of which are tropical.

Rice is the most important crop in the world for human food. Similar amounts of rice, wheat and maize are produced annually but a large proportion of wheat and maize goes for livestock feed or industrial uses (Rice Almanac, 2002). Asia accounts for 90% of the rice grown and consumed and the poorest people spend up to half their wages on rice (Dawe, 2000). Over the last 40 yr the production of rice in Asia has kept pace with the increase in population (Fig. 1) as more land has been brought into cultivation and the Green Revolution (better cultivars, use of irrigation, fertilizers and pesticides) has increased yields per hectare. As populations continue to grow, rice consumption must increase (currently half the population of south-east Asia has a calorie intake inadequate for an active life) and the area for cultivation will remain constant or decrease as land is taken for urban and industrial use. Ensuring food security and protecting the environment for the world is a continuing challenge (Evans, 1998) and requires a second Green Revolution.

It is therefore generally agreed that rice yields must increase but without proportionate increases in the use of water or fertilizer, and within the context of climate change (Evans, 1998; Hossain & Pingali, 1998; Dobermann, 2000; McCarthy et al., 2001; Tilman et al., 2001; Depledge, 2002). Improving rice productivity is the key to a better life for rice farmers and consumers, and contributes to economic development and the route out of poverty for less developed countries (Dawe, 2000). However, yields in some Asian countries may have reached a plateau (Cassman, 1999) and yield potentials in breeders’ trials at the International Rice Research Institute (IRRI; www.irri.org) have not increased for 30 yr (Sheehy, 2001a). Yield potential (Evans & Fischer, 1999) is the yield in an optimal physical environment (solar radiation, temperature, mineral nutrients) and with complete protection from weeds, pests and diseases. In general, farm yields can be pushed to c. 80% of yield potential: the gap is inevitable and allows for the physical environment being suboptimal on most farms, and for moderate use of fertilizer and crop protection measures in environmentally sensitive farming. A substantial increase in yield potential is required, along with better use of water and fertilizer, and we propose that only rice with supercharged, C₄ photosynthesis is likely to provide this.

How could a C₄ rice be constructed? Are there any traces of ‘C₄-ness’ in rice or its near relatives that could be used in a breeding programme? What are the features of productive C₄ plants that would need to be transferred to rice? These are questions for a workshop in July 2006 at IRRI in the Philippines. The aim is to form a consortium, led by IRRI, to co-ordinate and conduct the research necessary to construct a C₄ rice. Scientists with the relevant skills and experience from institutions all round the world will need to be included in this long-term project. Rice with C₄ photosynthesis could make a major contribution to the second Green Revolution, and may indeed ‘involve the most audacious feat of genetic engineering yet attempted’ (Surridge, 2002). In this paper we explain why we concentrate on photosynthesis to increase yield potential, why C₄ photosynthesis, and why now, and suggest how the endeavour could be started.

Fig. 1 Rice production and the population of Asia for the years 1961–2004. As an indication of the trend, the line is the regression of production on population (y = 191.1x – 98.3; P < 0.001, r² = 0.98), and the dotted line is an extrapolation to the population predicted for 2050 (square dot).
Why improve photosynthesis to increase yield potential?

Yield potential depends on the proportion (harvest index) of crop biomass that is directed towards the harvested part of the plant. Biomass is accumulated during the life of the crop as photosynthetically active radiation (PAR, 400–700 nm) is intercepted and used in photosynthesis; the effectiveness of the use and the production of biomass is quantified as the radiation use efficiency (RUE), the amount of dry matter per unit of PAR intercepted (Monteith, 1977). Summarizing these ideas in an equation (simplified from Mitchell & Sheehy, 2000) allows the components to be examined in turn.

\[ Y = H \varepsilon \sum_{i=1}^{n} Q_i f_i, \]

Eqn 1

where \( Y \) is the grain yield as dry matter (g m\(^{-2}\)); divide by 100 for t ha\(^{-1}\) and divide by 0.86 for weight at 14% moisture content); \( H \) is the harvest index; \( \varepsilon \) is the radiation conversion factor, so-called RUE (g MJ\(^{-1}\); above-ground dry matter, intercepted PAR); \( n \) is the growth duration (days); \( Q_i \) is the PAR incident on the crop on the \( i \)th day (MJ m\(^{-2}\); and \( f_i \) is the fraction of incident PAR that is intercepted, averaged over the \( i \)th day.

Yield potential can be improved by increasing the value of any one or more of the terms in the equation. It is unlikely that harvest index can be increased much from its current value for modern cultivars in best conditions of \( c \), 0.5, and it may have to decrease slightly to provide stronger stems that are more resistant to lodging. There is little interest in prolonging the growth duration (\( n \)) because the current durations coincide with suitable seasons or allow multiple cropping in a year. The incident PAR cannot be increased except by selecting sunnier locations or growing seasons. Intensively grown rice crops are probably close to intercepting the largest fraction of incident PAR that is possible, given that rice is an annual crop and so starts on bare ground. That leaves RUE as the only term for which a substantial increase might be available. The current general values are for rice 2.2 g MJ\(^{-1}\) (above-ground dry matter, intercepted PAR) and for maize 3.3 g MJ\(^{-1}\) (Kiniry et al., 1989); the higher value for maize is associated with C\(_4\) photosynthesis, which is especially effective in hot, sunny environments. If a C\(_4\) rice can be constructed by genetic engineering, with RUE increased by 50% to the value for maize, then yields should be increased by 50%. High values of RUE for rice (2.6 g MJ\(^{-1}\)) in well-fertilized experimental crops are associated with higher yields (Sheehy et al., 2000a).

Taking RUE as constant, on a given day it can be calculated from this equation (Mitchell et al., 1998):

\[ \varepsilon_{\text{total}} = \frac{P_{\varepsilon} - m W - 0.25P_{\varepsilon}}{Q_{\text{int}}}, \]

Eqn 2

where \( \varepsilon_{\text{total}} \) is the RUE (g MJ\(^{-1}\); for total dry matter including roots, and intercepted PAR); \( P_{\varepsilon} \) is gross photosynthesis (g m\(^{-2}\); dry matter, ground) for the day; \( m \) is the coefficient for maintenance respiration (g g\(^{-1}\); dry matter, dry matter) for the day; \( W \) is the total dry matter (g m\(^{-2}\); dry matter, ground); and \( Q_{\text{int}} \) is the amount of intercepted PAR for the day (MJ m\(^{-2}\)).

The top line of the equation is the increase in total biomass as the balance between gross photosynthesis and respiration (neglecting shedding of plant parts during crop growth). Respiration is maintenance respiration (mW, proportional to total biomass) and synthetic respiration (0.25 P, a fraction of fixed carbon used to provide energy for synthesis). Large reductions in respiration are unlikely (Byrd et al., 1992) so increasing photosynthesis is probably the only option for raising the value of RUE. A 50% increase in RUE requires at least a 50% increase in the rate of gross photosynthesis for leaves (Sheehy, 2001b). Evans and von Caemmerer (2000) showed that maize leaves had substantially higher rates of photosynthesis than rice at the same nitrogen content. Rice leaves can double their maximum rate of photosynthesis when provided with an atmosphere of 900 ppm carbon dioxide (Murchie et al., 1999).

Harvest index is an empirical way of relating grain yield to biomass: grain yield (dry matter) is expressed as a proportion of the above-ground dry matter. Part of the actual mechanism is the formation of spikelets (florets), their pollination and then grain filling. It would be little use increasing photosynthesis if there were not the spikelets available for filling. In rice a much larger number of spikelets develop on the panicle when it is initiated than are harvested as filled grains, so there is unused sink capacity (Sheehy et al., 2001). If the output of the source, i.e. photosynthesis, is increased then a larger grain yield should be formed, a conclusion supported by results from experiments using elevated concentrations of carbon dioxide (Baker & Allen, 1993; Ziska et al., 1997; Kobayashi et al., 2005).

Why C\(_4\) photosynthesis?

A 50% increase in canopy photosynthesis will require both higher leaf quantum yield (initial slope) and higher rates of leaf photosynthesis when saturated by PAR (plateau, on the curve of leaf photosynthesis vs. PAR). This allows all leaves in the canopy, in the range of conditions during the day and during the growing season, to contribute to increased photosynthesis overall. Higher quantum yield is important for leaves receiving low PAR, in canopies with PAR well distributed through the canopy or when incident PAR is low. Higher saturated rates of photosynthesis are required for leaves receiving high PAR, high in the canopy, at appropriate angles, when the incident PAR is high.

Ultimately, improved photosynthesis requires better performance from Rubisco (ribulose 1,5-bisphosphate
carboxylase–oxygenase) because there is no alternative to this enzyme (and the Calvin cycle) for continuous net fixation of carbon dioxide into carbohydrate (Sage, 2004). In modern atmospheres some oxygenase activity by Rubisco is inevitable, hence the occurrence of the pathways of photorespiration to minimize the loss of carbon skeletons from the Calvin cycle.

The options are more Rubisco, better Rubisco or make Rubisco work harder. Rubisco already accounts for up to 50% of soluble protein in leaves and increased amounts would require a proportionate increase in nitrogen; this is not an attractive choice. Moreover, Rubisco only exerts low control on rates of photosynthesis in many environments (Quick et al., 1991). Rubisco with a higher specificity for carbon dioxide is certainly of interest but is almost always accompanied by lower rates of catalysis. This option has been explored by Zhu et al. (2004) who concluded that worthwhile increases in canopy photosynthesis (up to 27%) could be obtained if Rubisco with the properties found in some nongreen algae could be incorporated into leaves. The success of the best versions of C4 photosynthesis, supercharging the basic C3 system, arises from an absence of photorespiration and more effective use of Rubisco in higher concentrations of carbon dioxide – Rubisco simply works harder, in conditions more like the primitive atmosphere in which it evolved. Several systems of C4 photosynthesis in single cells are now known (Reiskind et al., 1997; Edwards et al., 2004; Sage, 2004) but none are associated with high productivity (von Caemmerer, 2003); instead they allow some photosynthetic gain at high energy cost in conditions conducive to high photorespiration. Consequently it is productive C4 photosynthesis with Kranz anatomy, as occurs in maize, that will be required in rice.

What makes the C4 system even more attractive is its economical use of water and nitrogen. The water-use efficiencies (transpiration) of crops in the field are notoriously variable, but the values for C4 crops are about double those of C3 crops (Brown, 1999; Mortlock, 2003). Data from various sources are given in Table 1 to compare the efficiencies of water use (WUE), photosynthetic nitrogen use (PNUE) and radiation use (RUE) in crops of rice and maize. The ratio of the maize to rice values suggests that changing from C3 to C4 rice would increase WUE by 89%, PNUE by 180% and RUE by 50%. In addition, Greenwood et al. (1990) showed that C4 crops contained 60% of the nitrogen used by C3 crops for maximum yield.

### Why attempt this project now?

The question of supercharging rice photosynthesis by constructing a C4 rice was examined in a workshop at IRRI at the end of 1999 (Sheehy et al., 2000b). The conversion of C3 plants to C4 photosynthesis has been covered in several later reviews (Häusler et al., 2002; Leegood, 2002; Miyao, 2003; von Caemmerer, 2003; Raines, 2006). Nearly 7 yr after the workshop, a number of developments make the task appear feasible. There is an increased understanding of C4 photosynthesis and of molecular biology, and techniques in genetic engineering continue to improve rapidly.

The C4 pathway is an addition to the basic C3 system of photosynthesis and plants are classified as C3 or C4, apart from a few intermediate species (and plants with crassulacean acid metabolism – CAM). It is now clear that the C4 and C3 syndromes are not as rigidly separated as was first thought. The enzymes that are prominent in the C4 pathway also exist in C3 leaves, although with very low activity (Matsuoka et al., 2001). More surprisingly, there is a well developed C4 pathway in certain locations in C3 plants: in the green tissue around vascular bundles (Hibberd & Quick, 2002), and probably in rice spikelets (Imaizumi et al., 1997). In the opposite direction, maize, an archetypal C4 plant, has patches of C3 tissue wherever a mesophyll cell is not adjacent to a bundle sheath cell, particularly in leaf sheaths and husk leaves (Langdale & Nelson, 1991). These observations help in understanding why the C4 system has evolved repeatedly: apparently there is much preconditioning and whenever there are testing conditions of high photorespiration the C4 system readily emerges (Sage, 2004).

In molecular biology, Brown et al. (2005) suggested that study of Cleome gynandra, the C4 plant most closely related

<table>
<thead>
<tr>
<th>Attribute and source</th>
<th>Rice (C3)</th>
<th>Maize (C4)</th>
<th>C4 to C3 advantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (transpiration) use efficiency, WUE; adjusted for relative humidity of the atmosphere (g DW kg⁻¹ water) (Loomis &amp; Connor, 1992)</td>
<td>76</td>
<td>144</td>
<td>1.9</td>
</tr>
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<td>Photosynthetic nitrogen use efficiency, PNUE (µmole CO₂ s⁻¹ mmole⁻¹ N) (Evans &amp; von Caemmerer, 2000)</td>
<td>0.26</td>
<td>0.74</td>
<td>2.8</td>
</tr>
<tr>
<td>Radiation (PAR) use efficiency, RUE (g DW MU⁻¹ intercepted PAR) (Környi et al., 1989)</td>
<td>2.2</td>
<td>3.3</td>
<td>1.5</td>
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to *Arabidopsis*, would accelerate understanding of the C₄ syndrome. The advantages of this approach are that; (a) all the knowledge of *Arabidopsis* leaf development can be used to identify key genes in *Cleome*, (b) *Arabidopsis* can be used as a test system into which to transfer C₄ genes from *Cleome*, and (c) *Cleome* has a short life cycle. Hall and Langdale (1996) reviewed molecular aspects of leaf development and further work has elucidated the roles of the genes associated with differentiation of mesophyll and bundle sheath cells in C₄ leaves (Cribb et al., 2001; Rossini et al., 2001). Insertion of genes for C₄ enzymes into rice and successful expression is now routine (Miyao, 2003).

In genetic engineering there is a wider choice of promoters and of transit peptides to control the compartment in which the product of the transgene occurs. The transformation of plastids is possible so that genes can be expressed in the chloroplast (Maliga, 2002). A large number of enhancer trap lines have been made in rice (Wu et al., 2003; Johnson et al., 2005; Liang et al., 2006) and these are a promising resource.

**How do we start?**

The construction of C₄ rice must be planned from the top downwards, starting with the ultimate objective (food security in rice) and then working out the steps required to achieve it (higher yields, improved photosynthesis, etc.), as outlined in this paper. The opposite approach, pushed by genetic engineering from the bottom upwards, is frequently disappointing. Sinclair et al. (2004) gave examples of how improvements made at the level of molecular biology are dissipated when scaled up through biochemical and physiological levels to the response of crops in the field.

In the short term, we see five lines of research (and these will no doubt be refined by the July 2006 workshop at IRRI).

1. Establish benchmark values of yield potential, physiology and anatomy for rice against which to measure the effect of C₄ additions. These values will include the yield potential of current best cultivars in typical environments, characteristics of leaf and canopy photosynthesis, carbon dioxide compensation point and carbon isotope discrimination as measures as C₄-ness, distance between minor veins in the leaf, characteristics of bundle sheath cells, and so on.
2. Evaluate material in the IRRI genebank (100 000 lines of *Oryza sativa*, 6000 accessions of other species of *Oryza*) for C₄-like phenotypes, from which a breeding programme could start. Yeo et al. (1997) reported carbon dioxide compensation points characteristic of C₃–C₄ intermediates in a few species of *Oryza*, and rice panicles show some aspects of C₄ photosynthesis (Imaizumi et al., 1997).
3. Produce and evaluate C₄ transgenics in the best current cultivars (high yield, pest and disease resistant) using maize genes involved in the C₄ biochemical pathway, to complement the initial work in other cultivars (Ku et al., 1999). Maize is the most productive C₄ grain crop, and many C₄ genes are available.
4. Use bioinformatics on the genomes of rice and maize to identify the key regulatory genes (e.g. transcription factors) that are involved in C₄ photosynthesis.
5. Use *Arabidopsis* and *Cleome* to find C₄ genes to understand how development of Kranz anatomy is controlled (Brown et al., 2005).

In the medium term, we would concentrate on producing model plants to enable a complete understanding of Kranz anatomy, and determine what regulates the expression of photosynthesis genes in the bundle sheaths of rice. Ultimately, it will be necessary to embed the full C₄ system in rice, to conduct field trials to determine its effectiveness, and to incorporate it in a range of cultivars for commercial use in various rice-growing systems.

**Conclusions**

Supercharging photosynthesis is the only way to improve yield potential substantially in rice whilst not increasing the demand for water and nitrogen. This means adding the C₄ biochemical pathway and modifying leaf anatomy so that the C₄ system works at its best. We are confident that now is a pivotal time for harnessing all current progress in understanding C₄ photosynthesis and in techniques of genetic engineering to try to construct a C₄ rice. To do this, partnerships will be required between institutions with the specialized expertise, and that is why IRRI is forming the C₄ Rice Consortium.

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**References**


Does the enhanced tolerance of arbuscular mycorrhizal plants to water deficit involve modulation of drought-induced plant genes?

Water deficit is one of the most common environmental stress factors experienced by land plants, having a major adverse effect on plant survival and productivity (Kramer & Boyer, 1997; Bray, 2004). Plants can respond to drought stress with modifications that allow the plant to avoid the stress or to increase its tolerance. Tolerance to drought stress in plants is a complex phenomenon and involves many changes at both biochemical and physiological levels. The cellular responses of plants to water deficit appear to be conserved in the plant kingdom. Among a diversity of responses, plants can adapt to water deficit by the induction of specific genes such as genes encoding late embryogenesis-abundant (LEA) proteins, or genes encoding proteins involved in the biosynthesis of osmoregulatory compounds, as well as by modulating the expression of genes encoding aquaporins (Zhu et al., 1997; Bray, 2004; Loo & Maurel, 2005). Most terrestrial plants can also establish a symbiotic association with arbuscular mycorrhizal (AM) fungi. A number of studies have demonstrated that the AM symbiosis can protect host plants against the detrimental effects of drought stress (for reviews see Augé, 2001; Ruiz-Lozano, 2003). It is accepted that the contribution of the AM symbiosis to plant drought tolerance results from a combination of physical, nutritional and cellular effects (Ruiz-Lozano, 2003).

Although in recent years there has been an increase in understanding of the water relations of AM plants and the physiological processes involved in the enhanced tolerance of mycorrhizal plants to water limitation, the molecular basis for the tolerance to water stress in AM plants remains far from being understood (Ruiz-Lozano, 2003). Thus our research group has initiated an investigation aimed at evaluating, at a molecular level, the possible participation of drought-induced genes in the enhanced tolerance of AM plants to drought stress. The most important results are discussed in the following sections.

Late embryogenesis-abundant proteins

The LEA proteins accumulate in plant seeds during their maturation phase, when they are developing tolerance to desiccation (Close, 1996). Nevertheless, a variety of studies have demonstrated that LEA proteins also accumulate in vegetative plant tissues during periods of water deficit, reinforcing a role for these proteins as desiccation protectant. It has been proposed that, during cellular dehydration, LEA proteins play an important role in maintenance of the structure of other proteins, vesicles or endomembrane structures; in the sequestration of ions such as calcium; in binding or replacement of water; and functioning as molecular chaperones (Close, 1996; Koag et al., 2003). The overexpression of LEA proteins in plants and yeast confers tolerance to osmotic stresses (Imai, 1996; Babu et al., 2004).

Dehydrins are an important group of LEA proteins (LEA group 2). They represent the most conspicuous soluble proteins induced by a dehydration stress (Close, 1996). It appears that dehydrins play a fundamental role in the dehydration response of plants to a range of environmental and developmental stimuli (Close, 1996). The multiple targets of dehydrins (euchromatin, cytosol, cytoskeleton) suggest that the direct consequences of dehydrin activity are biochemically diverse.

It is of interest to determine whether the AM symbiosis is able to alter the pattern of dehydrin accumulation under drought stress, and whether such possible alteration functions...
in protection of the host plants against drought. We cloned two dehydrin-encoding genes from *Glycine max* (*gmlea 8*, *gmlea 10*) and one from *Lactuca sativa* (*lslea 1*) and analysed their contribution to the response against drought in mycorrhizal soybean and lettuce plants.

The analysis of *gmlea* and *lslea* gene expression showed that, in general, these genes responded to drought and were expressed only in drought-stressed treatments (Fig. 1a,b), suggesting that these dehydrins are important for the plant response against drought stress (Giordani et al., 1999). In any case, a consistent effect observed both for soybean and lettuce plants is that the expression of *gmlea* and *lslea* genes was lower in drought-stressed AM plants than in noninoculated plants. To understand this effect, it must be considered that abscisic acid (ABA) induces the expression of water deficit-responsive genes such as *lea* (Giordani et al., 1999). It has been proposed that mycorrhization can alter the levels of ABA in the host plant, and that under drought stress levels of ABA are lower in AM than in nonAM plants (Goicoechea et al., 1997; Estrada-Luna & Davies, 2003); thus the level of *lea* gene expression may be lower in these plants. Additionally, AM plants can be less affected than nonAM plants by drought stress, and for that reason the expression of the *lea* genes studied is lower. It has been proposed that primary drought-avoidance mechanisms (direct water uptake by hyphae) or increased water uptake related to mycorrhizal changes in root morphology or soil structure (Augé, 2001) might have contributed to the AM protection of host plants against drought. This hypothesis was supported by data on relative water content and leaf water potential ($\Psi$), which were significantly higher in AM plants than in nonAM plants subjected to a similar level of drought stress (Porcel & Ruiz-Lozano, 2004; Porcel et al., 2005a).

In conclusion, our results demonstrate that the levels of *lea* transcript accumulation in soybean and lettuce plants colonized by either *Glomus mosseae* or *Glomus intraradices* were considerably lower than those of the corresponding nonmycorrhizal plants, suggesting that the accumulation of LEA proteins is not a mechanism by which the AM symbiosis protects the host plant.

**$\Delta^1$-pyrroline-5-carboxylate synthetase (P5CS)**

Maintenance of a favourable water flow gradient from soil into roots is a fundamental process for plants under conditions of water deficit, when soil water potential becomes more negative. The most important mechanism of plants to decrease their water potential is to decrease the osmotic potential in their tissues by active accumulation of organic ions or solutes, a phenomenon known as osmotic adjustment or osmoregulation (Morgan, 1984). Of these metabolites, proline is probably the most widespread in plants, although it is not the only one, and it has been shown that proline accumulates under conditions of water shortage, high salinity, chilling, heat and heavy metal exposure. It plays a major role in osmoregulation and osmotolerance (Yoshida et al., 1995; Armengaud et al., 2004).

Accumulation of proline is caused primarily by *de novo* synthesis, although a reduced rate of catabolism has also been observed. The first two steps of proline biosynthesis are catalysed by $\Delta^1$-pyrroline-5-carboxylate synthetase (P5CS) by means of its $\gamma$-glutamyl kinase and glutamic-$\gamma$-semialdehyde dehydrogenase activities. Subsequently, the $\Delta^1$-pyrroline-5-carboxylate (P5C) formed is reduced to proline by P5C reductase (P5CR) (Hu et al., 1992). The rate-limiting step in this pathway is represented by the $\gamma$-glutamyl kinase activity of P5CS. The overexpression of the P5CS-encoding gene in transgenic tobacco plants has been shown to increase proline production and to confer to such plants tolerance to osmotic stress. Hence the P5CS-encoding gene is of key importance for the biosynthesis of proline in plants (Ábrahám et al., 2003).

Investigations carried out so far on proline in the AM symbiosis are scarce and somewhat contradictory. While some
studies have shown an increase in proline accumulation in mycorrhizal plants subjected to drought (Ruiz-Lozano et al., 1995; Azcón et al., 1996; Goicoechea et al., 1998), the same studies also demonstrated that the increase in proline accumulation was quite variable depending on the AM fungus involved (Ruiz-Lozano et al., 1995). By contrast, other studies on drought (Ramakrishnan et al., 1988) or salt stress (Ruiz-Lozano et al., 1996) have shown a lower proline accumulation in AM plants than in nonAM plants.

The establishment of the expression pattern of genes such as p5cs in AM plants under osmotic stress conditions should provide new insights into the role of the AM symbiosis in the process of osmotic adjustment during drought stress. We cloned a P5CS-encoding gene from G. max (gmp5cs) and another from L. sativa (lsp5cs), and analysed their contribution to the response against drought in mycorrhizal soybean and lettuce plants.

The expression of gmp5cs and lsp5cs (Fig. 2a,b) genes responded to drought and was upregulated in drought-stressed treatments, suggesting that these genes are important for the plant response against water deficit (Parvanova et al., 2004). Results on proline accumulation paralleled those on p5cs gene expression in both soybean and lettuce (Fig. 2a,b). A contrasting result was obtained in soybean plants singly inoculated with Bradyrhizobium japonicum, where the gmp5cs gene showed little upregulation in roots under drought-stressed conditions, and there was also low proline accumulation (Fig. 2a). To explain this result, it must be considered that the expression of p5cs genes has two regulatory pathways, an ABA-dependent and an ABA-independent pathway, and that both can act simultaneously and with cumulative effects (Abrahám et al., 2003). Hence it may be possible that nodulation itself may affect one of these regulatory pathways, avoiding the accumulation of p5cs transcripts. By contrast, the mycorrhization of nodulated plants restores the normal p5cs transcripts accumulation pattern, at least in part, by compensating such ABA-dependent and ABA-independent pathways in some way.

In any case, as also happened with lea genes, the expression of gmp5cs and lsp5cs genes decreased in drought-stressed AM plants compared with noninoculated plants (Fig. 2a,b). This was probably caused by a decrease in the ABA level in AM plants, and by the fact that AM plants were less affected by drought stress than nonAM plants because of primary drought-avoidance mechanisms. The results suggest that the induction of p5cs genes does not appear to be a mechanism by which the AM symbiosis protects the host plant (Porcel et al., 2004).

Regulation of aquaporin abundance

Aquaporins are water channel proteins that facilitate and regulate the passive movement of water molecules down a water potential gradient. The discovery of aquaporins in plants has caused a significant change in the understanding of plant water relations. In recent years, much effort has been concentrated on investigating the function and regulation of plasma membrane aquaporins (plasma membrane intrinsic proteins, PIPs). These aquaporins appear to play a specifically important role in controlling transcellular water transport. For instance, they are abundantly expressed in roots, where they mediate most soil water uptake (Javot & Maurel, 2002); transgenic plants downregulating one or more PIP genes had lower root water-uptake capacity (Siefritz et al., 2002).

However, the relationship between aquaporins and plant responses to drought remains elusive and shows contradictory results (Aharon et al., 2003). Moreover, the contribution of aquaporin genes to the enhanced tolerance to drought in
AM plants had never been investigated. However, it has been shown that the impairment of a PIP gene in an antisense tobacco mutant reduced the symbiotic efficiency of two AM fungi under drought stress conditions (Porcel et al., 2005b).

Mechanisms of osmotic adjustment and modulation of tissue hydraulic conductivity are required to maintain tissue water potential. Such mechanisms, which regulate water flux, are likely to be mediated, in part, by aquaporins (Maurrel, 1997). It is of interest to study whether the expression of aquaporin-encoding genes in roots is altered by AM symbiosis as a mechanism to enhance host-plant tolerance to water deficit. To achieve this, genes encoding PIPs from soybean and lettuce were cloned and their expression pattern studied, in AM and nonAM plants cultivated under well watered or drought-stress conditions. If AM fungi can transfer water to the roots of host plants, it is expected that the plant must increase its permeability for water and that aquaporin genes should be upregulated in order to allow a higher rate of transcellular water flow (Javot & Maurrel, 2002).

In contrast to the above hypothesis, our results showed that the PIP genes studied were downregulated under drought stress in both soybean (Fig. 3a, harvest time, 35 d after inoculation (dai)) and lettuce plants (Fig. 3b), and that such downregulation was even more severe in plants colonized by G. mosseae than in nonAM plants. A similar result was obtained recently by Ouziad et al. (2006) regarding the expression of PIP and tonoplast intrinsic protein (TIP) genes in roots of AM tomato plants subjected to salt stress. When the expression of gmPIP2 was analysed in a time course (Fig. 3a), it was clearly visible that AM plants already downregulated that gene significantly at 5 and 12 dai, while both nonAM control plants still maintained gmPIP2 gene expression almost unaltered. At 20 dai, the more intense downregulation of that gene in AM plants than in both nonAM plants was still clearly visible. Finally, at 35 dai all treatments had the same level of gmPIP2 gene expression. This effect of the AM symbiosis anticipating the downregulation of the gmPIP2 gene may have a physiological importance in helping AM plants cope with drought stress (Aharon et al., 2003). The decreased expression of plasma membrane aquaporin genes during drought stress in AM plants may be a regulatory mechanism to limit the water lost from cells (Smart et al., 2001). In support of this hypothesis, data on leaf Ψ and relative water content show that AM plants (soybean and lettuce) had higher leaf Ψ and water content than nonAM plants.

Data obtained with lettuce plants also colonized by G. mosseae point in the same direction (Fig. 3b): under drought-stress conditions there is a higher downregulation of the PIP genes studied (and also at the protein level, as revealed by Western blot) in AM than in nonAM plants. In contrast to G. mosseae, plants colonized by G. intraradices do not exhibit such downregulation of PIP gene expression or protein accumulation. The expression of PIP genes under drought stress in these plants is similar to control nonAM plants.

The reason for the differing influence of G. mosseae and G. intraradices on lettuce PIP gene expression is not known. However, in a previous study, also with lettuce, we evaluated the ability of six AM fungal species, including G. mosseae and G. intraradices, to enhance the amount of soil water uptake by these plants (Marulanda et al., 2003). The study demonstrated that there were substantial differences among the six AM fungi used. One of the most efficient fungi stimulating water uptake by plants was G. intraradices, while G. mosseae showed a reduced ability to improve plant water uptake. This may suggest that the strategy of both fungi to protect the host plant against water deficit is different. Glomus intraradices appears to have an important capacity to enhance the rate of water uptake by lettuce roots. This means that water movement in these roots must be enhanced and thus root water permeability must also increase, maybe by maintaining high levels of PIP aquaporin gene expression, as observed in this study. By contrast, G. mosseae appears to direct its strategy for plant protection against water deficit toward the conservation of the water existing in the plant, and thus downregulates the expression of PIP genes. Such downregulation of PIP genes has been interpreted as a
mechanism to decrease membrane water permeability and to allow cellular water conservation (Smart et al., 2001). In any case, both strategies appear to protect the host plant in a similar way, as lettuce plants had similar relative water content and leaf Ψ regardless of the fungus colonizing their roots (Porcel et al., 2006).

In conclusion, the results suggest that AM plants respond to drought stress by downregulating the expression of the two PIP genes studied and anticipating downregulation, as compared with nonAM plants, rather than by maintaining high levels of expression of these PIP genes. This downregulation of PIP genes is likely to be a mechanism to decrease membrane water permeability and to allow cellular water conservation. It must be considered, however, that as PIP are members of a multigene family, other PIP isoforms in soybean and lettuce plants may be regulated differently and that, depending on the AM fungus implicated in the symbiosis, the pattern of aquaporin gene expression may also be different.

Perspectives for future investigation

Considering the overall results presented here, it is evident that some aspects are more promising than others for future research. It appears that there is no sense in investigating further the possible roles of lea and p5cs genes; however this must be considered with caution, as we analysed only a few of the lea genes belonging to the dehydrin group, while other lea genes in plants remain to be checked. The evidence obtained with these two genes suggests that it will be of interest to study the role of ABA in modulating host-plant responses to water deficit by AM symbiosis. Results obtained with aquaporin genes suggest that studies of the correlation between up- or downregulation of aquaporin gene expression, root hydraulic conductivity and plant water status may be of interest. The number of aquaporin genes analysed should also be enhanced as aquaporins constitute a multigenic family in plants, and it is likely that some genes not yet analysed may be regulated by AM symbiosis in relation to the alleviation of drought stress in the host plant.

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