Biochem. J. (2014) **460**, 1–11 (Printed in Great Britain) doi:10.1042/BJ20130271

REVIEW ARTICLE

Symbiosomes: temporary moonlighting organelles

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Symbiosomes are a unique structural entity that performs the role of biological nitrogen fixation, an energy-demanding process that is the primary entryway of fixed nitrogen into the biosphere. Symbiosomes result from the infection of specific rhizobial strains into the roots of an appropriate leguminous host plant forming an organ referred to as a nodule. Within the infected plant cells of the nodule, the rhizobia are encased within membrane-bounded structures that develop into symbiosomes. Mature symbiosomes create an environment that allows the rhizobia to differentiate into a nitrogen-fixing form called bacteroids. The bacteroids are surrounded by the symbiosome space, which is populated by proteins from both eukaryotic and prokaryotic symbionts, suggesting this space is the quintessential component of symbiosis: an inter-kingdom environment with the single purpose of symbiotic nitrogen fixation. Proteins associated with the symbiosome membrane are largely plant-derived proteins and are non-metabolic in nature. The proteins of the symbiosome space are mostly derived from the bacteroid with annotated functions of carbon metabolism, whereas relatively few are involved in nitrogen metabolism. An appreciable portion of both the eukaryotic and prokaryotic proteins in the symbiosome are also 'moonlighting' proteins, which are defined as proteins that perform roles unrelated to their annotated activities when found in an unexpected physiological environment. The essential functions of symbiotic nitrogen fixation of the symbiosome are performed by co-operative interactions of proteins from both symbionts some of which may be performing unexpected roles.

Key words: legume, moonlighting protein, nitrogen fixation, nodule, rhizobia, symbiosome.

INTRODUCTION AND OVERVIEW

Nitrogen fixation is the most important biological process on earth, second only to photosynthesis. The enzyme nitrogenase, which catalyses the reduction of atmospheric dinitrogen to ammonium, is encoded into the genomes of a few members of the Alpha-, Beta- and Gamma-proteobacteria. The primary route of fixed nitrogen into the biosphere occurs via the symbiosis of nitrogen-fixing Alphaproteobacteria with leguminous plants. The symbiosis forms a novel organ on the roots and in a few cases on stems of the plant, referred to as a nodule, that contains specialized compartments affording spatial separation of the symbionts within modified plant cells. Upon infection and throughout symbiosis the bacteria are encased within a plant-derived membrane compartment called the symbiosome. Within the symbiosome, the bacteria differentiate into bacteroids, which express nitrogenase. The intervening space between the symbiosome membrane (also called the peri-bacteroid membrane) and the bacteroid is the symbiosome space. All nutrients and signals must traverse the symbiosome space, but there are few reports attempting to elucidate the functional purpose of this space. The majority of our knowledge of the symbiosome is derived from microbial mutants that affect symbiosome development and the cytolocalization of plant gene products. It is known that this space is populated by proteins contributed by both symbionts making it a unique confluent inter-kingdom domain. The function of these proteins and the metabolic activities of the symbiosome remain largely unknown. Symbiosomes are temporary organelle-like structures that contain 'moonlighting proteins', which are defined as proteins that perform roles unrelated to their annotated activities when found in an unexpected physiological environment [1]. Understanding the functional attributes of the symbiosome will lead to enhancing nitrogen fixation capacity and ultimately to extending the range of plant species capable of hosting symbiotic nitrogen-fixing bacteria.

NITROGEN-FIXING SYMBIONTS

The most commonly studied nitrogen-fixing symbioses are those between rhizobia and leguminous plants, primarily *Bradyrhizobium japonicum–Glycine max* (soya bean), *Sinorhizobium meliloti–Medicago sativa* (alfalfa), *Rhizobium leguminosarum–Pisum sativum* (pea), *S. meliloti–Medicago truncatula* and *Mesorhizobium loti–Lotus japonicus*. Nitrogenase, the prokaryotic-encoded enzyme complex, requires a minimum of 16 MgATPs per reaction:

$$N_2 + 8e^- + 16\ MgATP + 8H^+ \rightarrow 2NH_3 + H_2 + 16\ MgADP + 16P_i$$

The eight high-energy electrons are provided in the form of ferredoxins which also add to the energy requirement of the reaction. The required energy is derived from cellular metabolism, primarily from metabolism of dicarboxylates. Nitrogenase is oxygen-labile and thus the energy yield from anaerobic metabolism is reduced relative to aerobic metabolism, i.e. the Pasteur Effect. Symbiosis with plants provides direct access to a wealth of photosynthetically derived carbon compounds that can be used to provide the energy to reduce atmospheric dinitrogen [2].

Abbreviations: Hsp, heat-shock protein; IRLC, inverted repeat-lacking clade; PQQ, periplasmic pyrroloquinoline quinine; SNARE, soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptor; ZIP, Zrt-, Irt-like protein.

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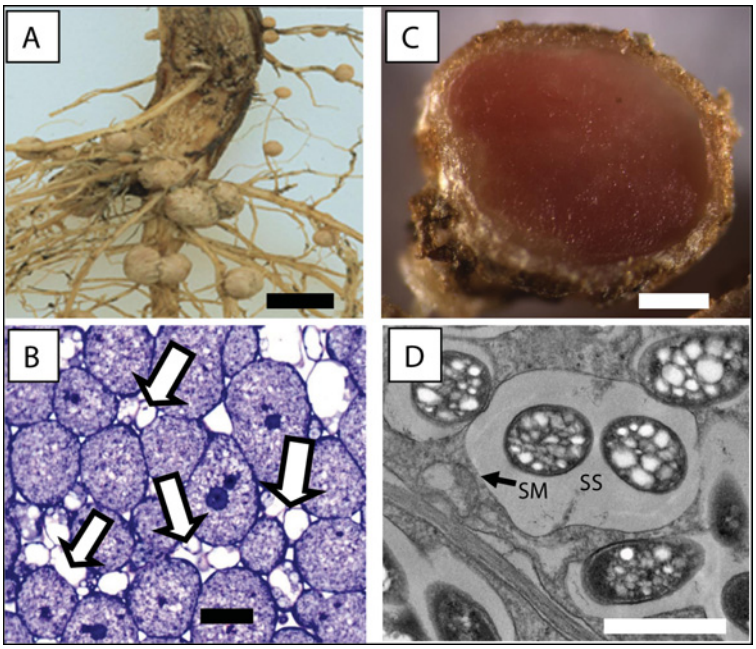

Figure 1 Morphology of soya bean nodules

(**A**) A nodulated soya bean root system (courtesy of H.J. Evans and D.G. Blevins). The scale bar represents 1 cm. (**B**) A light micrograph of the infected (stained blue) and uninfected (unstained, white) plant cells. The scale bar represents 25 μm. The arrows identify three separate regions containing uninfected cells. (**C**) The interior of a soya bean root nodule revealing the red colour of leghaemoglobin. The scale bar represents 1 mm. (**D**) An electron micrograph of bacteroids within symbiosomes. SS, symbiosome space; SM, symbiosome membrane. The scale bar represents 1 μm.

INFECTION EVENTS

The molecular signals of rhizobia–leguminous plant interactions are initiated in the soil when the appropriate host plant secretes specific flavonoids which are recognized by a rhizobial receptor protein. The binding of the specific plant flavonoid(s) transcriptionally activates rhizobial *nod* genes [3,4] producing Nod factors, which are lipochitin-oligosaccharide molecules, replaced with sulfate, acetate or fucosyl residues in a species-specific manner [5,6]. For example, the Nod factor produced by *B. japonicum* and *Sinorhizobium fredii* have a unique 2-*O*-methylfucose substituent on the reducing end of an *N*-acetylglucosamine residue [7–10]. The tips of the newly emerging plant root hairs are the preferred sites of attachment by rhizobia [11]. Soon after the rhizobia binds, the root hairs curl and the bacteria invade the root hair encased within an infection thread. Infection threads are Nod factor-induced plant-derived structures that invaginate into the root cells forming a tube-like conduit allowing the bacteria to move, and proliferate, from the root surface to the cortical region of the root [12,13]. The infection thread elongates into the central cortical region of the root, ramifies in the plant root cortex and deposits bacteria within membrane sacs called symbiosomes. Endocytosis of the bacteria from the unwalled infection threads into the cytoplasm of the host cell requires physical contact between the bacterial membrane and the glycocalyx of the plant [14]. In soya bean, nodule primordia, referred to as 'foci', are visible as early as 2 days after inoculation [15]. Beginning at 4 days after inoculation, cell expansion of the foci towards the outer root surface results in the formation of 'domes'. Nodules emerge as early as 8 days after infection (Figure 1A). The bacteria within the symbiosomes of the infected cells differentiate morphologically and biochemically into bacteroids that can express nitrogenase, the enzyme complex that reduces atmospheric N_2 to ammonium. Nitrogen fixation activity can first be detected as early as 10 days after inoculation [15].

NODULE MORPHOLOGY AND DEVELOPMENT

Legume nodules are classified either as determinate or indeterminate based on the site of initial cell divisions, presence or absence of persistent meristems and shape of the mature nodule. Legumes which form determinate nodules (soya bean, bean and lotus) are mostly found in subtropical and tropical regions, whereas those with indeterminate nodules (alfalfa, clover and pea) occur in temperate regions, although exceptions can be found to this broad classification [16].

The nodule is a complex structure with different cell types in the central infected zone and with different physiological roles. Plant cells in the infected zone differentiate into infected and uninfected cells, which multiply and expand to form a nodule (Figure 1B). The mitotic phase of soya bean nodule development, a period of rapid cell division, continues for 12–18 days after inoculation, and is characterized by declining soluble protein content and slowly rising total protein content per nodule [17,18]. A period of cell expansion follows, which is characterized by relatively constant soluble protein content [17]. Interspersed between the larger infected plant cells are smaller uninfected plant cells occupying approximately 20 % of the central nodule zone [18]. There are approximately 1.5 uninfected nodule cells for each infected cell. Plasmodesmata connect infected and uninfected cells within the central core and to cortical cells surrounding the central region and have been proposed to participate in the distribution of sugars [19–21]. The infected and uninfected cells have different metabolic roles as well as different morphologies and spatial orientations.

Oxygen concentration plays a seminal role in the differentiation and function of the plant nodule cells and the bacteroids [22,23].

Surrounding the central infected nodule zone in the cortex is an endodermis layer that has suberized walls. This layer acts as a gas diffusion barrier thereby reducing the partial pressure of oxygen within the infected region of the nodule to approximately 10 nM O_2. Leghaemoglobin, the most abundant protein in the nodules, is a myoglobin-like protein that acts as an oxygen buffer, found in the cytoplasm of nodule cells in the central portion of the nodule, but not within the organelles nor within the symbiosome space [24] (Figure 1C). Elimination of leghaemoglobin synthesis in the model legume *L. japonicas* by RNAi results in an increase in nodule-free oxygen and loss of symbiotic nitrogen fixation, demonstrating the crucial role of leghaemoglobin in oxygen transport and nitrogen fixation [25]. Leghaemoglobin is the most obvious example of nodulins, which are plant-derived, nodule-specific or highly expressed proteins associated with symbiosis [26,27]. The bacteroids also produce symbiosis-specific proteins with nitrogenase as the principal example. Leghaemoglobin delivers oxygen to the symbiosome surface, but there is no known symbiosome space protein for the transport of oxygen from the symbiosome membrane to the bacteroid inner membrane. Dissolved oxygen released by oxyleghaemoglobin at the symbiosome membrane presumably diffuses through the symbiosome space to the bacteroid membrane. A low partial pressure of oxygen is mandatory for the functioning of the oxygen-labile nitrogenase component proteins within the bacteroids [22]. To compensate for the low partial pressure of oxygen, bacteroids express high-affinity cytochrome oxidases to facilitate respiration and provide the energy needed by nitrogenase [28].

SYMBIOSOMES

In most rhizobial-infected nodules, the bacteria invade the host cell cytoplasm via an endocytotic-like process creating transient organelle-like structures referred to as symbiosomes [11]. Unlike true organelles, symbiosomes must be regenerated *de novo* during each infection cycle. Nodule cells maintain their ability to divide for several cycles after rhizobial infection. The dividing infected cells exhibit the normal microtubule and actin patterns of dividing plant cells [29]. The microtubules and actin microfilaments in infected lupin and pea nodules appear to be in direct contact with the symbiosomes [29,30]. The clustered symbiosomes are tethered to the spindle-pole regions and move to the cell poles during spindle elongation. In metaphase, anaphase and early telophase the symbiosomes are found at opposite cell poles where they do not interfere with the spindle filaments or phragmoplasts, demonstrating a symbiosome positioning similar to that of organelles [29].

The bacteroids never come into direct contact with the host cell cytoplasm (Figure 1D). Each infected plant cell may contain up to 20000 bacteroids that occur as single bacteroids to groups of up to 20 within a single symbiosome [31]. The tremendous increase in bacterial symbionts can be viewed as a method of gene amplification to alter the intercellular environment of the infected plant cell and redirect metabolic functioning to nitrogen fixation [32]. Symbiosomes can occupy up to 80 % of the infected cell volume [31], therefore an enormous amount of membrane material must be delivered to the symbiosome membrane for its development and maintenance. The complex symbiosome membrane system plays a pivotal role in nutrient and signal exchange [33].

Symbiosome development is dependent on a nodule-specific protein secretory pathway [34–36]. A symbiosis-defective mutant of *M. truncatula* (*dnf1*) was found to be defective in the signal peptidase complex, which is responsible for cleavage of signal peptides of nasant polypeptides targeted for intracellular compartments such as symbiosomes [35]. In *Medicago*, *Pisum* and *Trifolium*, legumes belonging to the IRLC (inverted repeat-lacking clade), the bacteroids undergo terminal differentiation which is coupled with genome amplification. In contrast, non-IRLC legumes, such as *L. japonicus*, the bacteroids maintain their normal size, genome size and maintain their reproductive capacity [37]. It has been speculated that the terminal differentiation of bacteria is mediated by specific plant-derived factors. A study has identified these factors as NCR (nodule-specific cysteine-rich) peptides which occur only in IRLC legumes [34].

The mature symbiosome consists of three separate components: the symbiosome membrane, the symbiosome space and the bacteroids. Each of these components progresses through a series of developmentally programmed phases. The most thoroughly defined component is the bacteroids which enter the infection thread as soil saprophytes that differentiate into nitrogen-fixing non-growing forms within the symbiosome [38]. Transcriptomic analysis indicates that approximately one-third of the rhizobial genome participates in establishing and maintaining the symbiosis [39,40]. Among the genes up-regulated in the bacteroid during symbiosis are nitrogenase and supporting metabolic activities, but the largest number of genes and proteins identified in global analyses are classified as unknown or hypothetical which demonstrates that a great deal is yet to be determined about symbiotic functioning [39–42].

The symbiosome is the interface between the plant and the bacteroids. The plant provides carbon substrates in the form of glucose and sucrose photosynthesized in the leaves and transported to the nodule (Figure 2). Bacteroids utilize the plant-derived carbon substrates and produce reduced nitrogen that is transported to the plant. The carbon substrates and reduced nitrogen must travel through the symbiosome and may be transformed enzymatically as well.

PLANT PROTEINS IN THE SYMBIOSOME

Between 1985 and 1990, direct measurement of enzyme activities in symbiosome preparations identified acid phosphatase, α-mannosidase II, proteases, protease inhibitors, α-glucosidase, aspartate aminotransferase and trehalase [43]. The fragile nature of symbiosomes and the possible contamination of proteins from each symbiont are inherent experimental limitations making unambiguous localization to the symbiosome difficult. Confirmation of these enzyme activities by localization studies have not been reported. In 1996, Kardailsky et al. [44] reported that PsNlec1, a lectin which possesses a hydrophobic N-terminal signal peptide, was preferentially localized to the symbiosomes of pea nodules. The expression of PsNlec1 was strongest in the late symbiotic zone. In 1999, Simonsen and Rosendahl [45] demonstrated that isolated pea symbiosomes can import a number of plant-translated nodule proteins and the bacteroid can also provide proteins to this symbiotic compartment. The authors concluded that the majority of the proteins in the symbiosome space were of plant-origin. Their results clearly demonstrated that the transport of multiple plant and bacteroid proteins to the symbiosome space is an intrinsic feature of symbiosomes. Wang et al. [35] confirmed the earlier results of Simonsen and Roesendahl [45] and Coque et al. [46] by demonstrating that symbiosome development in the *M. truncatula–S. meliloti* symbiosis requires the co-ordinated secretion of proteins via a nodule-specific transport system.

A number of transporters for inorganic anions, calcium and iron have been reported for the symbiosome membrane [2,47]. Iron,

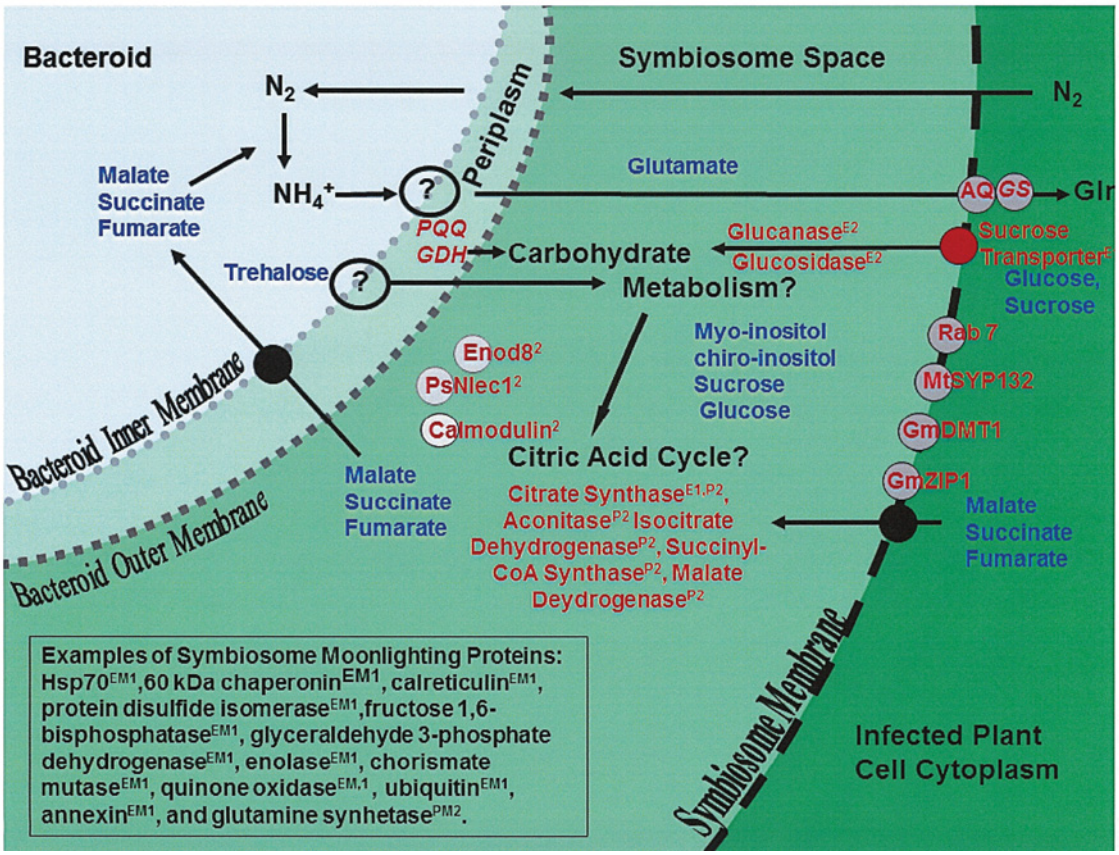

Figure 2 Organization and metabolic processes of symbiosomes

The diagram represents the structural organization of the infected cell cytoplasm, the symbiosome membrane, the symbiosome space, the inner and outer bacteroid membrane, the periplasm and the bacteroid cytoplasm. Metabolites are shown in blue, enzymes are shown in red and metabolic processes (pathways and transport) are shown in black. Known transporters are represented by solid circles and unknown or diffusion processes are shown by open circles enclosing question marks. POQ GDH, pyrroloquinoline quinine-dependent glucose dehydrogenase. Examples of moonlighting proteins found in symbiosomes are placed within the box. '1' refers to being identified in symbiosome membrane preparations; '2' refers to being identified in the symbiosome space.

which is required for synthesis of leghaemoglobin, nitrogenase and cytochromes, plays a crucial role in maintaining the nodule environment for the symbiosis [48]. In order to enter the bacteriods, iron must be transported across the symbiosome membrane to enter the symbiosome space. Interestingly, several proteins belonging to transport families such as NRAMP (natural resistance-associated macrophage protein), YSL (yellow stripe-like), VIT (vacuolar iron transporter) and ZIP (Zrt-, Irt-like protein) have been identified from the genomes of *M. truncatula* and *G. max*. Some of these proteins reveal enhanced expression in nodules, suggesting that they may be involved in the transport of iron and other metals across symbiotic membranes [48]. A divalent metal transporter from soya bean (GmDMT1) is the only Fe(II) transporter that has been identified in the symbiosome membrane [49]. GmZIP1, a zinc transporter belonging to the ZIP family of transporters, has also been detected on the symbiosome membrane [50]. The availability of specific antibodies against the above-mentioned transport proteins would facilitate their subcellular localization. Subsequently, many reports have demonstrated the localization of specific proteins, usually plant-derived nodulins, localized to the symbiosome. ENOD8 is an early nodulin of approximately 8 kDa and possesses a signal sequence and no predicted transmembrane domains and may be a peripheral membrane protein integral to membrane functioning [46,51,52]. Hohnjec et al. [53] demonstrated that the targeting of *M. truncatula* nodulin, MtNOD25, requires only an N-terminal signal peptide consisting of 24 amino acids. Liu et al. [54] identified that six calmodulin-like proteins expressed specifically in root nodules of *M. truncatula* were localized in the symbiosome space. Each of six was shown to bind calcium, but with different affinities. This suggests the need for calcium binding during symbiosome functioning.

Catalano et al. [55] found that SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptor) MtSYP132, a *M. truncatula* homologue of *Arabidopsis thaliana* synthaxin, was preferentially localized to the plasma membrane surrounding infection threads and persisted in the symbiosome membrane throughout nodule development [55]. However, the vacuolar SNAREs SYP22 and VTI11 do not appear until the onset of senescence. Symbiosome formation is considered to be an endocytosis-like process, yet symbiosomes of *M. truncatula* do not acquire Rab5 at any stage during their development [56]. However, the endosomal marker Rab7 does appear on symbiosomes at an early stage of development when they have stopped dividing and remain until senescence. Rab5 and Rab7, which are small GTPases, are endosomal membrane markers, denoting different, but not discrete, endosome populations. *M. truncatula* symbiosomes do not display the *trans*-Golgi network identity marker SYP4, which is presumed to mark early endosomes in plants. This suggests that symbiosomes

remain in a unique SYP132- and Rab7-positive endosome stage during the period of active nitrogen fixation and acquire a lytic or vacuolar identity at the beginning of senescence [55,56].

GLOBAL ANALYSIS OF PROTEINS

The proteins of bacteroids and the nodule plant cells have been actively investigated both from enzymological and proteomic analyses, whereas the proteins of the symbiosome space and membrane have been investigated to a lesser extent. Several research groups have demonstrated that symbiosis requires the co-ordinated secretion of plant proteins into the symbiosome [35,44,45,46,51,54]. Analysis of the symbiosome membrane and space have found a mixture of proteins of plant- and bacteroid-origin [57–60], suggesting directed transport and sequester of proteins from each symbiont. Distinction of proteins associated with the symbiosome membrane compared with the symbiosome space should be made with caution as peripheral membrane proteins can be partitioned to either location depending on the particular chemical and physical properties of the separation technique. Among proteins found in the global analysis were three proteins identified previously by localization studies: pea lectin PsNlec1 [44,59], calmodulin in *L. japonicus* [53,60] and Rab7 in *Medicago* [56,60].

Proteomic analyses revealed that the majority of the approximately 150 proteins associated with the symbiosome membrane are annotated with plant origins and are non-metabolic in nature [57–60]. It has been suggested that the symbiosome membrane proteins would participate in the transport of carbon, nitrogen and other nutrients [28]. In contrast, the symbiosome space proteins identified are mostly metabolic enzymes annotated with bacterial origins [59]. The results support the concept that the symbiosome is an inter-kingdom domain populated by proteins secreted from each symbiont. Saalbach et al. [59] used proteomic methods to identify 46 proteins in the symbiosome space of pea nodules. Among the proteins found were chaperonins^{P2}, Hsps (heat-shock proteins)^{P2}, protein-disulfide isomerase^{EM2} and an ATP synthase component^{EM2}. (Proteins identified associated with symbiosomse fractions are described as follows: ^{E} denotes symbiosome proteins annotated as eukaryotic, ^{P} denotes symbiosome proteins annotated as prokaryotic, ^{M} denotes symbiosome proteins identified as having a moonlighting function, ^{1} denotes symbiosome membrane and ^{2} denotes symbiosome space.) But the symbiosome space contained a number of enzymes of the tricarboxylic acid cycle (citric acid cycle), such as aconitase^{PM2}, succinyl-CoA synthetase^{PM2} and malate dehydrogenase^{PM2}. Succinate semi-aldehyde dehydrogenase^{P2} was also found, which, together with the identified tricarboxylic acid cycle enzymes, suggests that the symbiosome space may possess a functional citric acid-like cycle. In contrast with Simonsen and Rosendahl [45], Saalbach et al. [59] found the majority of the identified proteins were of bacterial origin, primarily *R. leguminosarum*, the endophyte of pea. Weinkoop and Saalbach [60] reported a greater number of eukaryotic proteins, whereas Catalano et al. found similar numbers of prokaryotic and eukaryotic proteins [57]. Proteins identified in the symbiosome membrane include nodulin 25^{E1} [57], Hsp70^{E1M,P1M,2M} [57,60], galactose-binding lectin^{E1} [57], glutamine synthetase^{P1M} [57,60], lysyl-tRNA synthetase^{P1M} [57], sucrose transporter^{E1} [60], hexose transporter^{E1} [60], enolase^{E1M} [60], citrate synthase^{E1M} [59], 14-3-3 proteins^{E1M} [60], β-1,3-glucanase^{E1} [60], chorismate mutase^{E1M} [60], phytoene dehydrogenase^{P1} [60], 3-isopropylmalate dehydrogenase^{P1} [60] and leghaemoglobin^{E1} [60]. All of these analyses suggest that the symbiosome is an integrated metabolic compartment possessing proteins both of plant and bacteroid origin. However, this assortment of proteins does not suggest a concerted metabolic outcome.

The largest group of symbiosome proteins can be broadly classified as chaperones and protein-folding catalysts, ATP synthase activities, protein destination/storage, transporters, pathogen response-related, plasma and endomembrane-related, and metabolic functions [57–60]. There is a numerical and mass over-abundance of the non-metabolic/maintenance proteins relative to the metabolic proteins, which could reflect the importance of these functions to the symbiosome. The identification of similar, if not identical, proteins in each proteomic analysis confirms a similarity of functional attributes of the symbiosome in these various rhizobial–leguminous plant symbioses [57–60]. Unfortunately, the data do not conclusively reveal these functional systems and pathways.

The glycolytic enzymes 1,6-bisphosphate aldolase^{E1M}, glyceraldehyde-3-phosphate dehydrogenase^{E1M} and enolase^{E1M} were found associated with the symbiosome membrane [60]. These enzymes represent a partial glycolytic pathway and interestingly glyceraldehyde-3-phosphate dehydrogenase^{E1M} and enolase^{E1M} precede the only two energy-yielding steps of glycolysis. The missing enzymes of glycolysis may have escaped identification, or may exist as distant homologues hidden among the many hypothetical or unknown proteins present within the symbiosome.

Both the symbiosome membrane and the bacteroid membrane possess transporters for organic acids [47,61]. The bacteroid dicarboxylate transporter has been shown to be essential for symbiosis [62–64]. Exogenously supplied succinate, fumarate and malate provide the greatest rates of nitrogen fixation in suspensions of *ex planta* bacteroids [65,66]. Malate is the most abundant dicarboxylic acid in soya bean nodules [67] and can be rapidly accumulated by bacteroids [47].

The tricarboxylic acid cycle enzymes identified from the symbiosome membrane, citrate synthase^{E1M} [59], and the symbiosome space, aconitase^{P2M} [59], succinyl-CoA synthetase (β subunit)^{P2M} [59] and malate dehydrogenase^{E1,P2M} [59,60], constitute a partial tricarboxylic acid cycle consisting of enzymes contributed by both symbionts. Citrate, the product of citrate synthase^{P1M}, and α-oxoglutaric acid, the product of isocitrate dehydrogenase^{P1}, has been identified in the symbiosome space [68]. As mentioned above for glycolysis, the missing enzymes of the tricarboxylic acid cycle may have escaped identification, or remain encrypted among the many hypothetical or unknown proteins present within the symbiosome.

Unlike the carbon metabolic proteins, very few of the proteins identified in the symbiosome membrane and space are associated with amino acid or nitrogen metabolism. Not a single unambiguous amino acid transport protein has been identified via proteomic analysis [57–60]. Uptake of phenylalanine, methionine, leucine, glycine and proline into both symbiosomes and bacteroids was very slow with rates less than one-tenth of the rate of succinate uptake [69]. In comparison, isolated soya bean nodule bacteroids are able to rapidly transport glutamate, aspartate and alanine with kinetics of uptake comparable with that of the dicarboxylic acid uptake system (Dct) [70,71]. The Dct transporter may also transport aspartate even in the presence of succinate, malate or fumarate [47]. In *R. leguminosarum* bacteroids, amino acids enter via a general amino acid permease^{P}, first characterized biochemically [72] and later identified proteomically [59], which transports a wide range of amino acids. A branched chain ABC transporter^{P} was also identified in *R. leguminosarum*. Mutation of both transporters,

each capable of exchanging amino acids, prevented all high-affinity amino acid uptake [73].

Aspartate aminotransferase, an acid phosphatase, a pyrophosphatase, proteases and protease inhibitors in symbiosome preparations were identified by direct enzyme measurement [43,74–76]. Two asparagine synthetases[E1] [60], two cysteine synthases[E1M] [60], 4-aminobutyrate aminotransferase[P2] [59] and lysyl-tRNA synthetase[P1M] [57] have been found via proteomic analysis. Glutamine synthetase[E], a principal ammonium assimilatory enzyme, was found associated with the symbiosome membrane of *L. japonicus*. Masalkar et al. [77] demonstrated that glutamine synthetase[E] binds to the conserved C-terminal domain of Nod26, a transporter of ammonium [77]. This shows the peripheral proteins and metabolic complexity of the symbiosome membrane. This complex would promote efficient assimilation of fixed nitrogen, as well as prevent potential ammonium toxicity, by localizing the enzyme to the plant cytosolic side of the symbiosome membrane.

METABOLITES OF THE SYMBIOSOME

Sugars are the predominate metabolites (∼95 % sugars, ∼4 % organic acids and ∼2 % amino acids) of soya bean nodule symbiosome space preparations [68]. *Myo*-inositol accounts for >60 % and D-chiro-inositol for >15 % of the total sugars of the symbiosome from soya bean nodules [68,78]. Some of the metabolites play an important role in efficient nitrogen fixation. For example it has been shown that a functional *myo*-inositol dehydrogenase of *S. fredii*, a soya bean symbiont, is required for nitrogen fixation [79]. Other metabolites may also have a protective role. Biosynthesis of cyclitols by direct cyclization of glucose and their accumulation in plants is a widespread response that provides protection against various environmental stresses [80].

Glucose and sucrose comprises ∼3 % and ∼6 % respectively of the symbiosome space metabolites [68]. Similar to the symbiosome, the bacteroid does not transport sucrose or glucose [47]. Notably absent in the symbiosome space was trehalose and fructose. Although fructose was not transported by the symbiosome membrane, it was shown to be rapidly taken up by bacteroids [69]. Reibach and Streeter [81] noted that when whole nodules are pulsed with $^{14}CO_2$, fructose was the only carbohydrate to decline during the chase period [81]. Fructose appears to have a high flux rate, but occurs at a low concentration in the symbiosome space.

As mentioned above, malate is the most abundant dicarboxylic acid in soya bean nodules [67] and can be rapidly accumulated by bacteroids [47], but organic acids only constitute 2–4 % of the total metabolites of the symbiosome space [68]. The organic acids in the most abundant group of the symbiosome space were oxalic, citric, tartaric, pyruvic and α-oxoglutaric acids.

Amino acids constitute only ∼2 % of the total small metabolite pool, with glutamate accounting for 37 % of the total amino acids found in the symbiosome space [68]. Proline, histidine, arginine, alanine and threonine were the next most abundant amino acids. Bacteroids had a more restricted number of measurable amino acids, which were glutamate, glycine, proline, aspartate, arginine and alanine [68]. The small amino acid pools would be consistent with ammonium serving as the primary fixed nitrogen metabolite released from the bacteroid [47]. In addition, pea (*P. sativum*) root nodules exposed to $^{15}N_2$ and monitored by ^{15}N-NMR had a substantial pool of free ammonium located in the bacteroids [82]. The observed ^{15}N-labelled amino acids, glutamine/glutamate and asparagine, were assigned to the plant cytoplasm. The symbiosome space was not apparent. In pea nodules the symbiosome membrane is closely wrapped around each bacteroid yielding a smaller symbiosome space when compared with other rhizobial–leguminous plant symbioses. The rate of transfer of fixed nitrogen compounds through the symbiosome space combined with its relatively small volume could have occluded measurement [82].

BACTEROID PERIPLASMIC SPACE

The periplasmic space is the compartment between the plasma membrane and the outer membrane of Gram-negative bacteria, which may constitute up to 40 % of the total cell volume [83]. The periplasm actively acquires and transports nutrients, synthesizes peptidoglycan, promotes electron transport, inactivates substances toxic to the cell, and senses the environment [83]. The periplasm of non-symbiotic bacteria retains its integrity from the extracellular environment, but the symbiosome represents a unique 'extracellular' environment. One-dimensional SDS/PAGE of the periplasm and symbiosome space proteins revealed similar patterns for these two fractions, but some proteins were quantitatively enhanced [76,84]. Kinnback and Werner [76] measured enzymes in the symbiosome space and the periplasm of soya bean nodules and found that the same enzyme activities were present in both the periplasm and the symbiosome space, but several enzymes had much higher specific activities in one of the two spaces. Three *R. leguminosarum* periplasmic proteins were identified as components of the symbiosome space of pea: a general L-amino acid-binding protein[P2]; DppA protein[P2] (dipeptide transporter) and an unspecified periplasmic binding protein[P2] [59]. Discrete isolation of the periplasmic and symbiosome spaces is inherently difficult, thereby hindering assessment of their contents and metabolic activities.

Bernardelli et al. [85] have shown that glucose metabolism occurs extracellularly via a PQQ (periplasmic pyrroloquinoline quinine)-dependent glucose dehydrogenase[P] that oxidizes glucose to gluconate in *S. meliloti* bacteroids [85]. Gluconate can serve as a substrate for the pentose phosphate pathway or the Entner–Doudoroff pathway.

MOONLIGHTING PROTEINS

The secreted proteins of each symbiont are performing tasks in a novel 'extracellular' environment and thus in a sense are moonlighting, which can be defined as working at another job in addition to one's full-time job. Jeffery [1] has defined moonlighting proteins as proteins that perform roles unrelated to their annotated activities which are displayed in different physiological environments [1]. Many of the bacterial moonlighting proteins have been shown to perform roles in virulence with many well-documented roles in human diseases [86]. Moonlighting proteins are considered to be ancient gene products [86], as is nitrogenase and the nitrogen fixation process [87–89].

Although the annotated roles of the proteins identified in the symbiosome generally fit the symbiotic model, notable gaps remain in the anticipated metabolic pathways. The proteins encoding these enzymatic steps could simply have escaped detection or their function fulfilled by unknown or hypothetical proteins. Curiously, about one-fourth of the approximately 150 proteins that have been identified occupying the symbiosome membrane and symbiosome space [57–60] are also recognized as moonlighting proteins [1,86,90,91] (Table 1).

Table 1 Moonlighting proteins in symbiosomes

The proteins identified in proteomic analysis which have also been identified as moonlighting proteins. The listed proteins do not include proteins annotated with putative or possible functions. [E], eukaryotic; [P], prokaryotic; [M], moonlighting; SM, symbiosome membrane; SS, symbiosome space.

Protein	Location	Symbiosome reference(s)	Moonlighting reference(s)
10 kDa Chaperonin^{PM}	SS	[59]	[90]
14-3-3 Proteins^{EM}	SM	[60]	[116]
30S Ribosomal protein S1^{PM}	SM	[57]	[117,118]
60 kDa Chaperonin^{EM}	SM	[58]	[119]
60 kDa Chaperonin^{PM}	SM	[57]	[90,119]
Aconitase^{PM}	SS	[59]	[85,120]
Annexin^{EM}	SM	[60]	[100]
ATP-synthase, β-chain^{EM}	SS	[59]	[121]
Calmodulin^{EM}	SS	[54,60]	[99]
Calreticulin^{EM}	SM	[60]	[119]
Chorismate mutase^{EM}	SM	[60]	[90]
Citrate synthase^{EM}	SM	[59]	[90]
Cysteine synthase^{EM}	SM	[60]	[86]
Cytochrome P450^{EM}	SM	[60]	[122]
Elongation factor G^{PM}	SS	[59]	[90]
Elongation factor Tu^{PM}	SS	[59]	[90]
Enolase^{EM}	SM	[60]	[120]
F_1-ATPase^{EM}	SM	[59]	[90]
Fructose-1,6-bisphosphate aldolase^{EM}	SM	[60]	[120]
Glutamine synthetase^{PM}	SM	[57,60]	[104]
Glyceraldehyde-3-phosphate dehydrogenase^{EM}	SM	[60]	[86,90]
GroEL^{PM}	SM	[57]	[90]
Hsp70^{EM}	SM	[57,60]	[119]
Hsp70^{PM}	SM	[60]	[90,119]
Hsp70^{PM}	SS	[57,59]	[90,119]
Luminal binding protein BIP^{EM}	SM	[58]	[90]
Lysyl-tRNA synthetase^{PM}	SM	[57]	[123]
Malate dehydrogenase^{PM}	SS	[59]	[117]
PEP carboxylase^{EM}	SM	[60]	[124]
Peroxidase^{EM}	SM	[60]	[94]
Protein-disulfide isomerase^{EM}	SM	[57,58,60]	[119]
Protein-disulfide isomerase^{EM}	SS	[59]	[90]
Quinone oxidase^{EM}	SM	[60]	[90]
Succinyl-CoA synthetase, β-subunit	SS	[59]	[120]
Ubiquitin^{EM}	SM	[60]	[90]

Many bacterial moonlighting proteins are highly conserved cytoplasmic proteins endowed with adhesive functions [86,92]. For example, most of the enzymes of the glycolytic pathway have moonlighting functions as external adhesive molecules [86]. The active sites of moonlighting proteins have only been identified conclusively in a few bacterial proteins. As expected they map to the surface of the protein [86]. Levy et al. [93] has found a propensity for particular amino acids located in the interfaces between proteins and derived a 'stickiness' scale for each amino acid. A consequence of the analysis revealed that 'stickiness' constrains protein evolution. This may in part explain the repetitive occurrence of moonlighting proteins among diverse organisms. The spectrum of surface adhesion moonlighting proteins can change with environmental/growth conditions of the target host [86]. The adhesive property may serve as a mechanism to form molecular attachment points on the bacteroid membrane or the symbiosome membrane for metabolic pathways or signalling cascades or to provide scaffolds to assemble structural macromolecular units.

The 'rules' governing moonlighting protein expression, transport, function and turnover are not understood. Presently, more is known about moonlighting in eukaryotes, but many of the eukaryotic moonlighting proteins have homologues in bacteria. The preponderance of moonlighting analogues in the symbiosome strongly suggests that the symbiosome is a unique structure not only in terms of its primary function, symbiotic nitrogen fixation, but in terms of its protein composition and molecular architecture.

Jeffery [94] proposed that proteomic analysis of discovering proteins in 'unusual' locations was one way of identifying moonlighting proteins. The large majority of the plant proteins identified in the symbiosome membrane were chaperones and protein-folding catalysts and ATP synthase activities. To date, 18 eukaryotic and four prokaryotic chaperones and protein-folding catalyst moonlighting proteins have been reported to have the ability to signal to human cells [86,90,95]. Chaperones and protein-folding catalysts associated with the symbiosome membrane that have analogues identified as moonlighting proteins include: Hsp70^{E1M}, 60 kDa chaperonin^{E1M}, luminal binding protein BIP^{E1M}, calreticulin^{E1M} and protein-disulfide isomerase^{E1M}, and those identified in the symbiosome space include Hsp70^{P2M}, 60 kDa chaperonin^{P2M}, 10 kDa chaperonin^{P2M}, elongation factor Tu^{P2M} and protein-disulfide isomerase^{P2M} [57–60,86].

Bacterial molecular chaperones and protein-folding catalysts moonlight as virulence determinants [86]. *R. leguminosarum* chaperonin 60.3, but not the more abundant chaperonin 60.1 induces cytokine production by human monocytes [96]. Saalbach et al. [59] found chaperonin 60.1, not chaperonin 60.3, in their proteomic analysis [59]. Chaperonins 60.2 and 60.3 are expressed only in low amounts and are not required for protein folding. In *Xenorhabdus nematophila*, a gammaproteobacterium, the 60 kDa chaperonin moonlights producing insecticidal activity allowing the host nematode to reproduce efficiently [97]. These examples demonstrate that homologous proteins can exhibit different moonlighting activities [1].

ATP synthase components are common examples of moonlighting proteins. The prokaryotic analogue of the ATPase α subunit^{P} has been shown to complex with bacterial lysyl-tRNA^{P1M}, which then participates in the transport of cytosolic tRNAs into mitochondria in *Leishmania* [98]. The ATPase F6 subunit has been shown to be present in human blood plasma and involved in hypertension through its binding on ecto-F_1-ATPase expressed on endothelial cells. The whole ATPase complex was expressed at the endothelial cell surface and has been implicated in many physiological roles, including ATP synthesis. Multiple eukaryotic and several prokaryotic ATPase components were found associated with the symbiosome membrane and space, which may assemble to provide the symbiosome space with a source of ATP. Several calcium-binding proteins were identified in the proteomic analyses with moonlighting activities, calmodulin^{E1M} [60,99], calreticulin^{E1M} [60,95] and annexin^{E1M} [60,100]. Calreticulin is a lectin chaperone that recognizes proteins retaining one or more monoglycosylated N-glycan side chains promoting folding in an ATP-independent manner [95]. Pea lectin, PsNlec1^{E2}, was found in the proteomic analysis of the pea symbiosome space [59] and a galactose-binding lectin was found in *Medicago* nodules [57]. The complex of calreticulin with protein-disulfide isomerase forms a unique cage-like structure, similar to that of Hsp60 [101]. Annexin is a moonlighting protein in human blood also known as lipocortin, which suppresses phospholipase A2 [100]. Wienkoop and Saalbach [60] found annexin^{E1M} in the symbiosome membrane of *L. japonicus* nodules.

The metabolic plant proteins in the symbiosome membrane identified as moonlighting proteins are fructose 1,6-bisphosphate aldolase^{E1M}, glyceraldehyde-3-phosphate dehydrogenase^{E1M}, enolase^{E1M}, citrate synthase^{E1M}, malate dehydrogenase^{E}, cysteine synthase^{E1M}, chorismate mutase^{E1M} and quinone oxidase^{E1M} [57–60,86]. Enolase is a prototypic moonlighting protein in both prokaryotes and eukaryotes, with putative roles in a variety of

human diseases [102] and is involved in vacuole fusion in yeast [103].

In contrast with the symbiosome membrane, proteins identified from the symbiosome space [59] were mostly metabolic enzymes annotated with bacterial origins. Among the prokaryotic proteins identified in the symbiosome space that have also been identified as moonlighting proteins include aconitase^{P2M} [92] and glutamine synthetase^{P1M} [104]. Aconitase has the established moonlighting role as an iron-responsive-element-binding protein, which would have the obvious role of supplying the multiple iron atoms for the nitrogenase complex and its supporting ferredoxins. As described previously, eukaryotic glutamine synthetase^{E} located in the plant cytosol is complexed with the ammonium transporter presumably for the assimilation of ammonium from the bacteroid. The prokaryotic glutamine synthetase^{PM}, which has been reported as a moonlighting protein, would not be required to perform its primary function if ammonium assimilation occurs as it exits the symbiosome. Glutamine was only a minor component of the symbiosome space [68]. Curiously, the nitrogenase component proteins have been found in the symbiosome in two of the proteomic analyses [59,60]. The redox capacity of these iron–sulfur proteins would render them valuable components for a multiplicity of moonlighting functions.

A METABOLIC MODEL OF THE SYMBIOSOME

Our knowledge of cytolocalization of metabolic enzymes in infected and uninfected plant nodule cells originated with Saganuma et al. [105] and Kouchi et al. [106]. However, in their work the entire infected cell cytoplasm, including the symbiosome space, was used. Since the symbiosome space occupies a significant portion of the infected cell volume, up to 80 % in infected soya bean nodule cells, their measurements include enzymes within the infected plant cell cytoplasm and those within the symbiosome. Their results do not preclude differentiation of metabolic activity between the infected plant cell cytoplasm and the symbiosome, but the proteomic identification of carbon metabolic enzymes within the symbiosome do not exclude this possibility. A working model of symbiosome metabolism is that the symbiosome metabolizes carbohydrates to organic acids for bacteroid energy metabolism for atmospheric dinitrogen reduction.

The plant provides carbon substrates in the form of glucose and sucrose photosynthesized in the leaves and transported to the nodule (Figure 2). Bacteroids prefer organic acids, in particular succinate, fumarate and malate [65]. The identified metabolic proteins support this overall model. The symbiosome membrane of *L. japonicus* has a sucrose transporter^{E1}, a hexose transporter^{E1}, a putative mannitol transporter^{E1} and an oligosaccharyltransferase-like^{E1} protein, which were identified proteomically [60], but soya bean symbiosomes take up sucrose, glucose and fructose only slowly [47,69].

Although proteomic analysis has identified glucanase^{E1} and glucosidase-like^{E1} proteins [57,60], these are the only metabolic proteins that have actually been measured from symbiosome space preparations [43,74–76]. α-Mannosidase II, β-glucosidase and α-trehalase were measured in soya bean root nodule symbiosome preparations, but the genomic origin of these activities was not determined [43,74–76]. An α-glucosidase was also reported but could not be confirmed [107]. Golgi α-mannosidase II is a key enzyme involved in N-linked glycan processing. β-Glucosidases are exocellulases and have been found in the apoplast [108], which emphasizes that the symbiosome represents an ‘external space’ relative to the infected plant cytoplasm. Soya bean nodule bacteroids have the ability to synthesize trehalose [109] and the symbiosome space was shown to contain trehalase [74], thus perhaps the prokaryote provides a source of glucose since the symbiosome membrane is impermeable to plant-derived glucose [47].

Gluconate, produced by the PQQ-dependent glucose dehydrogenase^{P} [85] can serve as a substrate for the pentose phosphate pathway or the Entner–Doudoroff pathway, the later pathway requiring glyceraldehyde-3-phosphate dehydrogenase^{EM} and enolase^{EM}, and producing pyruvate. Pyruvate can support nitrogen fixation by *ex planta* bacteroids but only at ∼20 % of the rate of succinate [43] and can be converted into alanine [110]. A potential Entner–Doudoroff metabolic pathway would consist of both eukaryotic and prokaryotic enzymes. A subsequent tricarboxylic acid cycle would feature primarily prokaryotic enzymes; succinate dehydrogenase and fumarase have yet to be identified. Glycolysis and the tricarboxylic acid cycle form metabolons, which are structural–functional complexes formed between sequential enzymes of a metabolic pathway [111,112]. The symbiosome is populated by proteins from each symbiont and in order to achieve the metabolic efficiency of metabolons would require interdigitation of eukaryotic and prokaryotic enzymes to form a complete functioning metabolon.

The absence of amino acid metabolic enzymes in the symbiosome, the low relative amounts of amino acids combined with the relative abundance of ammonium and the Nod26–glutamine synthetase complex question the proposed metabolic cycles involving amino acid export from bacteroids coupled with carbon metabolite uptake [61,64,113–115]. Nitrogen transport is the most fundamental activity of the symbiosome and appears to proceed without appreciable nitrogen metabolism within the symbiosome space.

SUMMARY

The symbiosome is a novel temporary organelle populated by proteins from both eukaryotic and prokaryotic symbionts. Although our current knowledge of the symbiosome supports the overall function of a carbon and nitrogen exchange, the lack of a complete or nearly complete set of metabolic pathway enzymes leaves much to be discovered. The presence of many unexpected proteins, a surprising number of which are moonlighting proteins, suggests there are more functional aspects to symbiosomes than previously thought. Whether some of the proteins identified in the symbiosome actually perform other functions (moonlighting) or are artefacts of the isolation procedure needs further investigation.

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Received 21 February 2013/10 March 2014; accepted 13 March 2014
Published on the Internet 25 April 2014, doi:10.1042/BJ20130271