Forisomes: calcium-powered protein complexes with potential as ‘smart’ biomaterials

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Sieve tubes in legumes contain forisomes, which are spindle-like bodies that are composed of ATP-independent, mechanically active proteins. Upon injury, forisomes occlude sieve tubes by dispersion and thus, help to prevent loss of nutrient-rich transport sap. Forisome enlargement by dispersion is brought about by Ca2+-induced conformational changes that confer radial expansion and longitudinal contraction. Forisomes react upon Ca2+ removal. In vitro, forisomes reversibly disperse and contract in the presence or absence of Ca2+, respectively, and at distinct pHs. Recently, forisomes have received renewed attention because of their unique capacity to convert chemical into mechanical energy independent of high-energy organic compounds. Forisome-based ‘smart’ materials can be used to produce self-powered monitoring and diagnostic systems. Here, we focus on physiological, chemical and physical aspects of forisomes and discuss their potential as biomimetic devices.

Introduction

Exploitation of the biological machinery of nature for technological applications offers an exciting opportunity for biologists and engineers to join forces in tracking and designing so-called ‘smart’ molecules. These are able to sense and respond to environmental changes, or function as the heart of nanoscale machines that might find application in diverse fields such as healthcare, engineering and textiles [1–3]. An absolute requirement for the design and use of ‘smart’ molecules (e.g. motor proteins) is a basic understanding of their structure and functioning under natural conditions [4]. Motor proteins usually convert chemical energy into mechanical energy in order to perform their action [5,6]. Most use energy released by ATP hydrolysis to perform their function, such as kinesins, which are responsible for cell dynamics and three-dimensional organization of living cells [7], and helicases, which are involved in DNA/RNA unwinding [8–10].

This review, however, deals with ATP-independent motor protein complexes, which are exclusively present in transport channels (sieve tubes) of legumes [11,12]. These spindle-like protein bodies (originally named crysalline P proteins) gained much attention in the 1970s [13–16]. They were suspected to undergo structural transformations, as globular configurations were also observed [13,16,17]. Visualization of dispersion [18] and reversible dispersion/contraction of these bodies [19,20] gave rise to the novel term ‘gate bodies’ or forisomes (latin foris: wing of a gate; greek soma: body) coined by Knoblauch et al. in 2003 [20].

When legumes experience mechanical injury, forisomes disperse and occlude sieve tubes to hinder leakage of photoassimilates or invasion of phytopathogens. Provided that the damage can be repaired, forisomes reversibly recontract [19,20]. Dispersion/contraction depends on elevation or decrease of free calcium (Ca2+) in sieve tubes [19]. It has been found that other bivalent cations, such as Sr2+ and Ba2+, weakly imitate the effect of Ca2+, whereas Mg2+ is unable to do this [19–21].

In vitro, the anisotropic contractility in forisomes is triggered by an increase of Ca2+ concentration [21,22], or by alterations in pH [20,21]. Taken together, forisomes are a class of protein complexes with interesting properties that could be exploited in biomimetics and in nanobiotechnological devices [12,23–25]. Possible applications of forisomes are in micro-valves and micro-actuators or, they might be utilized as self-powered smart biosensors. In this review, we highlight the physiological, chemical, genetic and physical aspects of forisomes that provide the basis for their potential use in biomimetic devices for sensory and mechanical nanosystems.

Sieve elements (SEs) – the natural forisome environment

Phloem tissue in angiosperms contains sieve tubes, which are composed of complexes between SEs and companion cells (CCs), which are arranged end-to-end to form modular tubes (Figure 1). After an unequal longitudinal division of their phloem mother cell, SEs and CCs remain connected via their common walls by specialized plasmodesmata (intercellular cytosolic connections) that are branched at the CC side. At their ends, (pore–plasmodesma units; PPUs) plasmodesmata between SEs are connected by sieve pores. SE plasma membranes are continuous through the branched plasmodesmata and sieve pores. The intimate association of mature SEs and CCs is necessary because...
SEs depend on CCs for most of their vital functions because of the absence of the nucleus, tonoplast and several other important organelles in SEs [26].

SEs contain only a few organelles, including mitochondria and SE plasts in a narrow margin of cytoplasm, the mitocplasm (Figure 1), but their function has not yet been established [25]. Several types of plastids have been described [26] that are classified according to their inclusions [27,28]. For example, Behnke distinguished starch bodies, protein crystals and protein filaments and these occur in different combinations [28]. In addition, SE plastsids and mitochondria are found tethered by nano-anchors to endoplasmic reticulum (ER) cisternae [29], which are often oriented perpendicularly to the plasma membrane. Similar anchors also link ER cisternae to each other and to the plasma membrane [29] as a means to keep the cytoplasmic components in place despite the turbulent mass flow.

**Phloem-specific proteins**

A broad diversity of structural and soluble proteins assembled under the general term P-proteins have been described to occur in phloem sap throughout the angiospermous plant families [30]. The composition is highly family-specific. Apart from phloem-specific proteins are unknown. They might be engaged in maintenance of SEs or long-distance signaling through Sieve tubes. According to their morphology, phloem-specific proteins can be classified into the following six categories [31] (Figure 2). (i) Soluble proteins: at least 150 soluble proteins have been described that cover several functional classes [32–35], including a strikingly complete set of antioxidative enzymes [36]. (ii) Mesh proteins; meshes of fine, filamentous proteins throughout SE lumina disclosed after careful fixation for electron microscopy [29,37]. (iii) Non-dispersive protein bodies: giant protein bodies with unknown functions in several plant families [11]. (iv) Protein inclusions in SE plastids [28]. (v) Parietal proteins: structural proteins mostly pressed against the SE plasma membrane. Among these are receptors and various ion channels, of which calcium channels are the most important in this context. In the lower part, the microscopic structure of the SE/CC complex in legume phloem is depicted. As they lack most of the usual cell organelles in the mature state, SEs remain in close association with CCs, which fulfill many vital functions for SEs. Therefore, SEs are connected to the cytoplasm of adjacent CCs by plasma channels. CCs have a large nucleus, active mitochondria and all other usual plant cell components. SEs posses a plasma membrane and contain SE plastsids, phloem-specific proteins, a marginal ER and a few mitochondria which all reside in a narrow, marginal zone of cytoplasm called the mitocplasm. In SEs of legumes, specific protein bodies (forisomes) occur, which disperse and contract reversibly in response to changes in Ca2+. Upon stress perception and subsequent Ca2+ influx, elevation of the Ca2+- concentration in the SE mictoplasm causes dispersion of forisomes with the result that sieve tubes become occluded. Abbreviations: SP, sieve plate; P; sieve pore; N, nucleus; M, mitochondria; SEP, SE plasma membrane; Pl, plastid; V, vacuole; CP, chloroplast CP).

**Dimensional shifts during conformation changes of forisomes**

As demonstrated by scanning electron microscopy, forisomes are composed of largely identical subunits [40], which we here propose to name ‘forisomettes’. As indicated by a striated appearance in transmission electron microscopy pictures [29], forisomettes consist of strictly ordered arrays of a number of forisome proteins (Figure 3a). Occasionally, forisomettes can be recognized microscopically as the forked ends of a forisome [39]. The number of forisomettes can be adapted to the dimensions required such that the final forisome size is tuned to the diameter of the sieve tube to guarantee optimal occlusion. Thus, the narrow sieve tubes in minor veins of leaves contain smaller forisomes than those in the broader sieve tubes in stems. The architecture and degree of expansion of forisomes differ between legume species. A species-specific feature is the presence of thin tails at forisome ends [16], which do not appear to sense changes in Ca2+ levels. The tails are unable to disperse [41,42], but might serve the immobilization of forisomes. There are doubts concerning the differential capability of forisomes to induce full occlusion between legume species. Canavalia forisomes can expand to their full sieve-tube diameter [41], whereas in vitro forisome expansion has been calculated to be insufficient to fully block sieve tubes in Vicia faba [43]. We surmise,
Protein bodies only present in legumes [30].

During dispersion, forisomes swell radially and contract longitudinally, which results in a threefold increase in volume of V. faba forisomes (Figure 3b–d). Their length decreases by 29±7%, whereas their diameter increases by 120.5±50% [20,21]. Electron-dense contracted forisomes become more translucent during dispersion as demonstrated by electron microscopy (Figure 3e–g). Continuing structural coherence during expansion and contraction [44] indicates that forisome expansion is not caused by random dispersion, but rather by controlled unfolding of the protein complex.

The terminology used in the literature regarding the forisome conformation appears contradictory as contraction is used for both conformation states (Figure 3). Plant biologists tend to use terms that match microscopic observations. Hence, they describe an increase in forisome volume as dispersion or expansion and a decrease as contraction or condensation (Figure 3c and d). On the other hand, because the longitudinal axis of forisomes shortens as the volume increases (Figure 3b), nanoengineers prefer to use the term contraction or contractile for the large-volume state, which better matches the technological goals. In this review, we stick to the terms dispersion” and expansion for the large-volume state, and contraction” and condensation” for the small-volume state of the forisome.

Forisome genes and proteins
Forisomes contain at least three highly ordered forisome proteins. Genes expressing forisome proteins initially have been named For [45], which appears to be a misnomer and is now replaced by the acronym SEO (sieve-element occlusion) [42,46]. Nevertheless, we will use For, when reference is made to the original study [45]. Thus far, four genes (For1 and SEO 1,2 and 3) that encode forisome proteins have been reported for Medicago truncatula (Mt). The sequence of MtFor1 is virtually identical to that of MtSEO1. They differ in only four residues, therefore, For1 and SEO1 are regarded as the products of two alleles of the same gene [42]. To date, seven sequences of genes for forisome proteins have been submitted to the National Center for Biotechnology Information (NCBI) database, which include four genes (SEO1, 2 and 3 and For1) from M. truncatula (accession numbers: EU938018, EU938017, EU938016 and EU016204); [42,45]) and one each from V. faba ([VfFor1: EU016203; [45]), Canavalia gladiata ([CgFor1: EU016202; [45]) and from Pisum sativum (PsSEO1: GQ478228) [submitted by Tuteja et al to the NCBI databank]. The genes are approximately 2 kb in size and unique for Fabaceae because orthologous genes have not yet been reported for other plant families. The sequence alignments for For1 and SEO1, 2 and 3 proteins show highly conserved regions along the entire protein (Figure 4a). Protein sequence analysis has revealed a highly conserved DRY (aspartic acid, arginine, tyrosine) motif [47] that is located close to the C terminus (Figure 4a).

The phylogenetic relationship using the neighbour-joining distance method has shown that the seven known members of the forisome protein family can be divided into three clades. A single clade comprises a forisome protein from C. gladiata. The second clade comprises the SEO2 and SEO3 proteins from M. truncatula. The third clade contains forisome proteins from V. faba and P. sativum, and the FOR1 and SEO1 proteins from M. truncatula (Figure 4b).

A promoter–reporter assay localized the temporal expression of forisome genes primarily to the young metaphloem sieve tubes of Fabaceae [45]. Detection of an antigen on the forisome body after application of an anti-VfFor1 anti-serum has indicated that For1 is a structural component of forisomes [45]. Moreover, using a green fluorescent protein (GFP)-tagged for1 promoter, it has been shown that for1 is expressed exclusively in immature SEs, which suggests that forisomes are formed during SE development, and that protein turnover at the mature state is low [45]. Other studies have shown that all three SEO proteins are building blocks of forisomes [42].

Structural organization of forisome proteins
The spatial structure of forisome proteins is unknown, therefore, we used existing sequence data and search tools.
to draw an initial physiochemical sketch of the forisome proteins identified thus far. Parameters, including isoelectric point (pI), molecular weight, and the number of negatively and positively charged amino acids, were scored using the Expert Protein Analysis System (ExPASY) Proteomics Server tool (http://www.expasy.org/tools/protparam.html) (Table 1). Secondary structural content from the amino acid composition of For1 and SEO1, 2 and 3 proteins was predicted, based on the Analytical Vector Decomposition method using the Comparative Sequence Analysis – Secondary Structural Content Prediction (SSCP) tool (http://coot.embl.de/SSCP). SSCP calculates predictions for the composition of secondary structural segments (helices, sheets and coils) using the amino acid composition as input information. Conserved domains of forisome protein family members are tracked using the NCBI Conserved Domain Search tool (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

The prediction of secondary structural content derived from amino acid composition of FOR and SEO1, 2 and 3 proteins has indicated that alpha helices, beta sheets and coiled-coil configurations are present at approximately equal levels (Table 1). Forisome proteins contain 650–700 amino acids, are 75–80 kDa in size, and have a pI of 6–7. pI and molecular weight of each forisome protein have been calculated using ExPASY Proteomics Server tool (Table 1). Leucine content is abundant (9–11%) in all forisome proteins. The DRY motif, a conserved domain of rhodopsin type G-protein-coupled receptors [43] is found in all forisome proteins with the exception of SEO3 of *M. truncatula* (Figure 4a). Conserved domain search identified protein domains that are reminiscent of thioredoxin folds in SEO2 (amino acids 307–450), SEO3 (amino acids 323–459) of *M. truncatula*, and in For1 (353–440) of *C. gladiata* (Table 1) [48].
Conventional Ca\(^{2+}\)-binding motifs have not been identified, which renders the Ca\(^{2+}\) sensing to be unique [49]. There are a number of speculations as to the apparent absence of Ca\(^{2+}\) binding sites: (i) essential forisome proteins including one containing a Ca\(^{2+}\)-binding site still await detection; (ii) the Ca\(^{2+}\)-binding site of forisome proteins is not yet recognized; (iii) forisome proteins provide the matrix for a Donnan phase in which Ca\(^{2+}\) is accumulated; or (iv) Ca\(^{2+}\) binding sites are formed by residues of more than one protein and thus remain invisible in searches for known Ca\(^{2+}\)-binding motifs [42].

Within forisomettes, several different proteins might be highly ordered spatially and associated intimately as suggested by striation [29,44]. The exact in vivo structure of individual forisome proteins might however prove difficult to elucidate, because proteins strongly interact in the anticipated tight lattice. A complex forisomette structure is expected to result from a multitude of electro- and hydrostatic interactions within and between the forisome proteins and their hydration shells. Ca\(^{2+}\)-induced changes in surface charge and electrical density might allow for massive and sudden binding of water molecules, which in turn could induce conformational shifts. It is also imaginable that Ca\(^{2+}\)-binding sites are present in niches that are formed by more than one protein and that the insertion of Ca\(^{2+}\) ions could trigger structural changes in the protein frame.

How forisomettes are kept together and communicate remains a mystery. Passage of weak electropotential waves (EPWs), during which minor amounts of Ca\(^{2+}\) are...
As soon as their Ca\(^{2+}\)-sensitivity emerged, forisomes have been readily used as biosensors to monitor Ca\(^{2+}\) changes in various systems. Pressure exerted by micropipettes on forisome-containing SE protoplasts causes forisome dispersion, as a result of Ca\(^{2+}\) influx, which shows the presence of mechano-sensitive Ca\(^{2+}\) channels in the SE plasma membrane [50].

*In vitro* dispersion of forisomes by Ca\(^{2+}\) application is reversed by extracts of aphid saliva that are secreted into sieve tubes during penetration by a feeding stylet [58]. The saliva-induced forisome contraction demonstrates that watery aphid saliva contains Ca\(^{2+}\)-binding proteins [58,59]. Thus, aphids prevent sieve-tube blockage and maintain food supply by binding Ca\(^{2+}\) set free by stylet damage [58].

Furthermore, forisomes also show that Ca\(^{2+}\) channels are involved in nitric oxide production [60]. Adding elicitors of NO production to phloem tissue triggers a massive NO production in CCs, which is accompanied by forisome dispersion in adjacent SEs. Finally, passage of EPWs along sieve tubes coincides with forisome dispersion. During EPW passage, Ca\(^{2+}\) channels are activated and give rise to Ca\(^{2+}\) influx and forisome dispersion. In this way, plants can exert remote control on sieve-tube occlusion [39].

### Table 1. Physicochemical parameters for the prediction of structure, function and potential biological role of forisome proteins.

<table>
<thead>
<tr>
<th>Protein</th>
<th>pl</th>
<th>Molecular weight (kDa)</th>
<th>Number of AA</th>
<th>Leu content (%)</th>
<th>Number of −ve charged AA (Asp+ Gln)</th>
<th>Number of +ve charged AA (Arg + Lys)</th>
<th>Alpha content (%)</th>
<th>Beta content (%)</th>
<th>Coil content (%)</th>
<th>Conserved domain search</th>
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<tbody>
<tr>
<td>Mt (SE01)</td>
<td>5.91</td>
<td>74,935</td>
<td>647</td>
<td>11.1</td>
<td>94</td>
<td>84</td>
<td>32.5</td>
<td>36.3</td>
<td>31.2</td>
<td>no hits found</td>
</tr>
<tr>
<td>Mt (SE02)</td>
<td>6.96</td>
<td>77,490</td>
<td>675</td>
<td>9.0</td>
<td>84</td>
<td>83</td>
<td>20.3</td>
<td>41.0</td>
<td>38.7</td>
<td>protein with thioredoxin fold</td>
</tr>
<tr>
<td>Mt (SE03)</td>
<td>6.49</td>
<td>80,456</td>
<td>701</td>
<td>10.0</td>
<td>92</td>
<td>86</td>
<td>41.6</td>
<td>29.7</td>
<td>28.7</td>
<td>protein with thioredoxin fold</td>
</tr>
<tr>
<td>Cg (For1)</td>
<td>6.17</td>
<td>76,727</td>
<td>668</td>
<td>10</td>
<td>90</td>
<td>84</td>
<td>33.7</td>
<td>34.2</td>
<td>32.1</td>
<td>protein with thioredoxin fold</td>
</tr>
<tr>
<td>Ps (SEO1)</td>
<td>6.41</td>
<td>78,548</td>
<td>685</td>
<td>9.2</td>
<td>83</td>
<td>77</td>
<td>26.7</td>
<td>37.9</td>
<td>35.5</td>
<td>no hits found</td>
</tr>
<tr>
<td>Vf (For1)</td>
<td>6.38</td>
<td>78,506</td>
<td>684</td>
<td>8.9</td>
<td>85</td>
<td>77</td>
<td>22.7</td>
<td>39.2</td>
<td>38.2</td>
<td>no hits found</td>
</tr>
</tbody>
</table>

Abbreviations AA amino acids; Mt Medicago truncatula; Cg Canavalia gladiata; Ps Pisum sativum; Vf Vicia faba.

Forisomes as candidate tools for nanotechnology

‘Smart’, or responsive, materials integrated in monitoring devices allow conversion of input signals into appropriate actions [25]. They encompass a number of different classes, such as piezoelectric materials that respond to applied electric fields, shape memory alloys that respond to heat or cold, and stimulus-sensitive materials (response to pH, temperature) and smart fluids that show excitation by electric or magnetic fields [12]. In the quest for novel materials that have the capacity to sense and process this information, which results in an appropriate response, biological compounds have received increased attention, and have given rise to the relatively new field of biometrics [61].

As mentioned before, forisome conformation is controlled by Ca\(^{2+}\) levels that occur in the low micromolar range. Since Ca\(^{2+}\) concentrations in this range are difficult to manipulate, control by electricity and pH could be more practicable under given conditions. Given the electrochemical excitability of pH-sensitive artificial contractile polymers [62,63], further studies on electrical control of forisome configuration [20] must be undertaken.

It has been found that the contracted state is not affected in the pH range from 5.0 to 9.5 [20], which confirms

Forisomes as biosensors in plants

As soon as their Ca\(^{2+}\)-sensitivity emerged, forisomes have been used readily as biosensors to monitor Ca\(^{2+}\) changes in various systems. Pressure exerted by micropipettes on forisome-containing SE protoplasts causes forisome dispersion, as a result of Ca\(^{2+}\) influx, which shows the presence of mechano-sensitive Ca\(^{2+}\) channels in the SE plasma membrane [50].
the observation that forisomes experience buffered pH conditions, varying only between pH 7.3 and 7.5, in SEs. However, forisomes respond in vitro to non-physiological pH values. Below pH 4.9 and above pH 9.0, forisomes expand. Up to pH 11.0, forisomes shorten by 30%, whereas the diameter increases by a factor 2.7; above pH 11.0, forisomes expand but their initial length cannot be restored. Above pH 12.0, the observed reduced stability of forisomes is ascribed to irreversible denaturation under these highly alkaline conditions [20,21].

Reversible and rapid (within 0.15–0.5 s) conformation shifts (threefold increase in volume) [40] of forisomes are accompanied by considerable mechanical forces [12,20]. The force exerted during contraction and expansion (about 0.1 μN [20]) is able to push and pull a flexible glass fiber (Figure 5). Upon Ca^{2+} supply, forisomes are able to pull a glass fiber towards a glass pipette, whereas addition of EDTA causes forisome expansion, which pushes the glass fiber away from the pipette. $F$, forisome; (i) volume-contracted form; (a) volume-dispersed form.

**Ongoing efforts and future perspectives**

Initial tests of forisomes as micro-valves, such as micro-stopcocks in microfluidity systems, nanomotors, or as smart sensors have been conducted [20,25,66,67]. Microfluidity systems based on the transport principles in sieve tubes have been designed recently [68]. Prototypes that incorporate forisomes have indicated that dispersed forisomes are able to occlude artificial microchannels. However, considerable particle passage at the forisome edges has demonstrated that there is insufficient adhesion between the forisomes and the wall material to achieve full occlusion by the forisome [67]. Thus, further searches for more adhesive wall material are necessary, or alternatively, means of constructing artificial sieve plates into microchannels to serve as holds for forisomes are required.

In addition, a self-powered forisome-based model systems that mimics the transmission of neuronal signals in humans for sensing and information transfer has been designed [3], which has provided information about fault location, severity, and spatial distribution of any disorder within the structure. Here, a nanoscale sensory layer of forisomes is patched together with piezoelectric elements [3]. The forisome-based nervous system, based on a common biomimetic control, actuator and sensor, can be converged from multiple tracks (multiple neuronal pads) into smaller and smaller output fiber nerves that eventually lead into one output fiber nerve going to the central data processing unit [3].

An attractive feature of forisomes is that their size can be tailored by genetic engineering of legumes. Therefore, thorough research efforts are required to identify and characterize the genes involved in forisome formation. Furthermore, developmental stages and location of forisome production need to be assessed to optimize yield and quality of forisomes. A detailed understanding of the relationship between dispersion/contraction and calcium binding also requires extensive studies of the structure of forisomettes and forisomes.

Forisomes have the additional advantage that they can be isolated easily en masse direct from living tissues [20]. Of particular importance for their nanotechnological potential is that forisome conformation can be controlled effectively in vitro, whereas their reactivity must persist over longer periods. In this respect, several technical problems have to be solved first. Like other phloem-specific proteins [38], forisomes oxidize rapidly, probably because the natural sieve-tube environment is semi-aerobic (<7% oxygen) [69]. This implies that dispersibility of forisomes is expected to decrease with time, which leads to unreliable dispersion rates unless antioxidants are added [21,70]. Controlled supply and withdrawal of calcium to the system is also a technical bottleneck, as well as the functionality of forisomes under room temperature conditions. A further limitation of forisomes is that they only operate under fluidic conditions. It is anticipated therefore that forisomes will be first applied as microactuators in microfluidics, before being utilized in other systems, although more wide-spread applications are certain within the realm of possibility [3,20].

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Review

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