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# Fibrillin influence on plastid ultrastructure and pigment content in tomato fruit

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**Abstract**

The protein termed fibrillin is involved in the formation of lipoprotein structures, such as plastoglobules and fibrils in certain chromoplast types, which have been implicated in the over-production of pigments due to a sink effect. In order to examine its effect in differentiating chromoplasts of a non-fibrillar type, the pepper fibrillin gene was expressed in tomato fruit. Both the transcript and protein were found to accumulate during tomato fruit ripening from an early mature green stage. However, formation of carotenoid deposition structures in tomato chromoplasts, such as fibrils, was not observed. Nevertheless, a two-fold increase in carotenoid content and associated carotenoid derived flavour volatiles (6-methyl-5-hepten-2-one, geranylacetone, β-ionone and β-cyclocitral) was observed. An unexpected phenotypic observation in the transgenic fruit was the delayed loss of thylakoids in differentiating chromoplasts, leading to the transient formation of plastids exhibiting a typical chromoplastic zone adjacent to a protected chloroplastic zone with preserved thylakoids. An *in vitro* assay has been developed to monitor fibrillin activity on thylakoids: data were obtained suggesting a membrane protection role for fibrillin, more specifically against moderate uncoupling effects.


*Keywords:* Chloroplast; Chromoplast; Fruit ripening; *Lycopersicon esculentum*; Membrane pore; Thylakoid integrity

## 1. Introduction

Proteins referred to as plant fibrillins, or plastid lipid associated proteins, are widespread from cyanobacteria to higher plants, found in association with a variety of different lipidic structures (for review see Simkin et al., 2004a). A fibrillin gene was cloned from pepper where the protein was found to be the major protein in carotenoid-storing fibrillar structures within the chromoplasts of the red fruit (Deruère et al., 1994a,b). The corresponding protein in cucumber flower chromoplasts is usually depicted as a "carotenoid-associated" protein (see Vishnevetsky et al., 1999). The fibrillin protein was viewed as mediating storage of excess carotenoid in a context of high levels of carotenoid biosynthesis concomitant to loss of photosynthetic membranes. Further studies have clouded this view: fibrillin, and its homologues are also thylakoid-associated and they accumulate under stress conditions (Chen et al., 1998; Gillet et al., 1998; Leitner-Dagan et al., 2006). It should also be mentioned that tomato and rice contain 10 distantly related genes and *Arabidopsis thaliana*, surprisingly, 13 (Laizet et al., 2004). In *Arabidopsis*, most of the encoded proteins are thylakoid-located and some are plastoglobule-associated (Friso et al., 2004; Ytterberg et al., 2006; Vidi et al., 2006). Evidence is also available in other species for the association of these proteins with various lipid structures,

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for example lipid globules under non-stress conditions (Ting et al., 1998; Hernández-Pinzón et al., 1999; Kessler et al., 1999; Smith et al., 2000). In this article, we use the term fibrillin to refer to the originally identified fibrillin from pepper or to its closest homologues from other species.

Constitutive over-expression of the pepper fibrillin protein in tobacco leaves leads to a slightly faster development of plants (up to the flowering stage; Rey et al., 2000) and potato expressing an anti-sense construction of the endogenous fibrillin-like transcript exhibits a stunted growth and reduced values of non-photochemical quenching of chlorophyll fluorescence (Monte et al., 1999). *Arabidopsis* mutants with altered levels of fibrillin show that the protein enhances the tolerance of photosystem II in leaves (Yang et al., 2006).

In the present article, the pepper fibrillin gene was over-expressed in tomato fruit, which was chosen for two reasons. First, although tomato fruit accumulate carotenoids during ripening, they do not form fibrils in their chromoplasts. Secondly, at the onset of ripening, green tomato fruit also offers the opportunity to examine the influence of fibrillin during plastid differentiation, a process characterised by the extensive re-organisation of internal lipidic structures. The pepper gene was chosen since the encoded protein has a demonstrated function of fibril assembly in pepper. Our *in planta* phenotypic observations allowed us to design *in vitro* experiments to monitor, for the first time, the activity of fibrillin on plastid membranes.

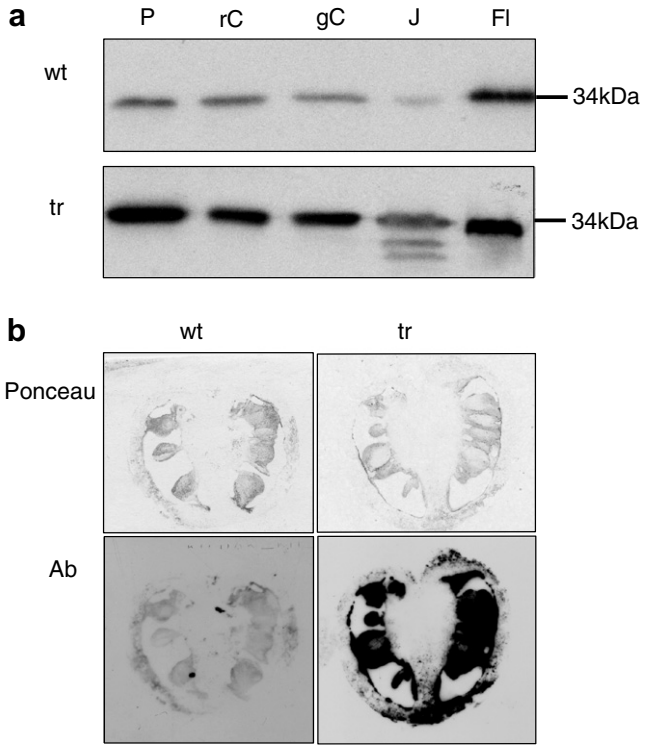


Fig. 1. Detection of fibrillin in wild type (wt) and a transgenic (tr) tomato line (*fib*FIB1). (a) Immunodetection of fibrillin in protein extracts from various ripe tomato fruit tissues and flowers. Extracts were fractionated by gel electrophoresis and blotted onto a membrane. P: pericarp, rC: red columella, gC: green columella, J: jelly, Fl: flowers. (b) Nitrocellulose tissue blots of ripe fruit pericarp. The upper panels show the efficiency of transfer determined by red Ponceau staining. The lower panels (Ab) show the immunodetection with fibrillin antibodies.

## 2. Results and discussion

### 2.1. Generation of fibrillin over-expressing tomato plants and immunolocalisation of the fibrillin protein

The genomic sequence of the pepper fibrillin gene (both introns and its own promoter; Deruère et al., 1994b) was inserted in a plant transformation vector. *Agrobacterium* mediated transformation was used to generate transgenic tomato plants. Several primary transgenic lines were selected and maintained up to the T2 generation. Using antibodies directed against the N-terminal sequence of the pepper fibrillin mature polypeptide, the presence of the endogenous protein was detected in the pericarp, the red portion of the columella, the green portion of columella and the jelly of wild type (wt) ripe fruit. Two transgenic lines (termed *fib*FIB1 and *fib*FIB2) showing a more than 10-fold increase in fibrillin protein in all these fruit tissues were selected for further study. As an illustration, line *fib*-FIB1 is shown in Fig. 1a. A second complementary approach, tissue blots, confirmed the accumulation of the fibrillin protein in the transgenic fruit (Fig. 1b). In this case, fibrillin accumulation is visible in the inner and outer pericarp and prominently in the jelly (which is not the case in the protein extracts shown in Fig. 1a). This is likely due to a greater efficiency of protein transfer to the nitrocellulose membrane from the jelly than from the pericarp. In flowers from transgenic lines, the increase in fibrillin levels appears lower since greater levels of endogenous protein were already observed in the wt flowers (compare, in Fig. 1a, levels in flowers in both wt and transgenic lines to levels in columella, for example).

It can be mentioned that the first intron of the pepper fibrillin gene is necessary for transcript accumulation in tobacco leaves and light is necessary for its splicing (Rey et al., 2000). This is the reason for using a construct containing the two original introns. In addition all experiments were performed with light-exposed fruits. Despite this experimental design, optimal for fibrillin protein accumulation, the observed level of this protein in the best over-expressing tomato lines was less than that detected in pepper fruit (not shown). This could be due to a counter-selection against high expressers during production of the transgenic plants.

### 2.2. Fibrillin mRNA and protein accumulation during fruit ripening

Since our primary interest was fruit development, eight fruit stages from ovary to ripe fruit were chosen (Fig. 2a). Using primers specific for the transgene, RT-PCR showed an increase in transcript level at the early

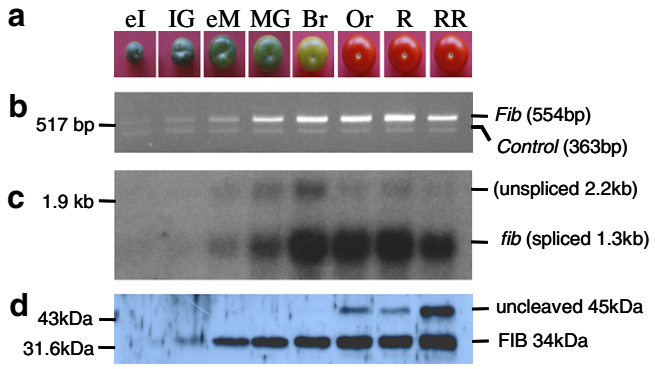


Fig. 2. Fibrillin mRNA and protein accumulation pattern during fruit development in transgenic tomato. (a) Fruit stages. eI: early immature green, IG: immature green, eM: early mature green, MG: mature green, Br: Breaker, Or: orange, R: red, RR: fully ripe. (b) Transcript levels in the fruit stages shown in (a) determined by RT-PCR followed by electrophoretic separation of the products. The expected fibrillin RT-PCR product is 554 bp long. Prior to these reactions, standardisation of the amount of total RNA in the various samples was ensured by visual inspection of the major RNA bands after electrophoretic separation. To exclude differences in the efficiency of the RT and PCR reactions from sample to sample, a reference RNA (globin mRNA, yielding a 363 bp RT-PCR product) was added prior to the reaction and subjected to the same reactions, as described (Simkin et al., 2000). See Section 4 for details. (c) Transcript levels in the fruit stages shown in (a) determined by RNA gel blot analysis using a radio-labelled fibrillin cDNA as a probe. The occurrence of a spliced form (about 1.3 kb) and of an unspliced form (about 2.2 kb) has been observed before in pepper fruit (Deruère et al., 1994b). (d) Fibrillin protein levels in the fruit stages shown in (a) determined by immunodetection using anti-fibrillin antibodies, after electrophoretic separation and membrane blotting of protein samples. A form, which probably corresponds to an uncleaved precursor, is also visible. As shown in Fig. 1, the transgenic form is the dominant one in the detected signal. Standard size markers were used to estimate the apparent MW of the fibrillin polypeptide and confirm the expected sizes (based on published sequences) of its transcripts and RT-PCR product. Positions of relevant markers are shown on the left side of the gels.

mature green up to the breaker stage (Fig. 2b). Then transcript levels remain constant throughout ripening, with a slight decrease in fully ripe fruit (15 days post-breaker). This result was confirmed by northern blot (Fig. 2c) using total RNA. This is in agreement with previous data on the regulation of this fibrillin gene promoter in pepper (Deruère et al., 1994a,b) and when fused to a reporter gene and introduced into transgenic tomato (Kuntz et al., 1998). Total protein was extracted from the same fruit used for RT-PCR. Protein gel blot revealed a continuous accumulation of the mature protein (34 kDa) from the immature green stage up to the fully ripe stage (Fig. 2d). As ripening proceeded, a 45 kDa polypeptide was also observed, which probably represents an unprocessed form. This form is absent in green tissues and has not been observed in pepper fruit (Deruère et al., 1994a).

### 2.3. The effects of the presence of fibrillin on carotenoid accumulation and carotenoid derived volatile in ripe fruit

Carotenoid content was accurately measured in total extracts prepared from 4 days post-breaker (red) fruit. The analysis revealed a modification of the carotenoid content in the transgenic fruit of both *fib*FIB1 and *fib*FIB2 plants. A typical experiment is shown in Fig. 3a. In total extracts, a 95% increase in total carotenoid content was observed when compared to extracts from wt fruits. This increase was observed at the levels of lycopene (118%) and β-carotene (64%). Smaller increases, which may not be significant, were observed for phytoene and lutein. No significant difference was observed for α-tocopherol.

Diced fruit at the same stage of development were taken for quantification of the carotenoid derived volatiles β-ionone and β-cyclocitral (derived from β-carotene), citral and 6-methyl-5-hepten-2-one (MHO; both derived from lycopene) and geranylacetone (derived from phytoene). The analysis revealed a modification of the carotenoid derivative content in the transgenic fruit of both *fib*FIB1 and *fib*FIB2 plants. A typical experiment is shown in Fig. 3b. A 36% and 74% increase in β-ionone and β-cyclocitral were observed respectively in transgenic fruit. Furthermore, we observed a 50%, 122% and a 223% increase in citral, MHO and geranylacetone, respectively. It seems likely that the elevated levels of carotenoid derivatives is the direct consequence of the elevated levels of carotenoids (Lewinsohn et al., 2005) and that the carotenoids

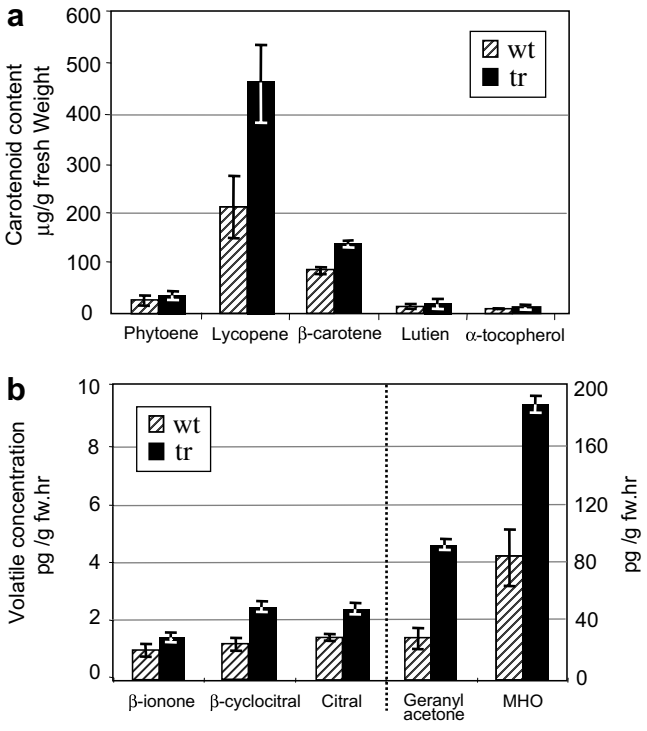


Fig. 3. Comparison of carotenoids, carotenoid derived volatiles and α-tocopherol content in fruit of wild type (wt) and transgenic (tr) plants. (a) Carotenoid and β-tocopherol content measured after HPLC fractionation. (b) Quantification of carotenoid-derived volatiles by gas chromatography. Error bars were calculated from 4 (panel a) or 4–6 (panel b) separate measurements using separately collected fruit samples. In addition, a given sample (for a single measurement) consisted of extracts from several fruits that had been pooled.

accumulating as a consequence of fibrillin over-expression are accessible for cleavage activity.

Carotenoids and tocopherols are hydrophobic antioxidants and high dietary intake of fruit and vegetables rich in these compounds can prevent the onset of chronic disease states. Tomato fruit and derived products are the principal source of lycopene (a potent antioxidant) in the diet, which has been associated with the prevention of certain cancers. Besides lycopene, tomato is an important source of β-carotene and contains other health promoting phytochemicals such as polyphenols, flavonoids, α-tocopherol and vitamin C. Therefore, tomato fruits have been an area of intense effort to elevate levels of these health-promoting phytochemicals (Fraser and Bramley, 2004). Typically, amplification of specific steps in a pathway has been the strategy adopted to elevate carotenoid content. Recently, carotenoid elevation in tomato fruit has been associated with increased plastid number/area (Cookson et al., 2003; Liu et al., 2004). Our data show that fibrillin over-expression provides an additional potential approach to elevate carotenoid and carotenoid derived volatiles derivatives in fruit.

### 2.4. Influence of fibrillin over-expression on plastoglobule arrangement

In order to visualise possible effects of fibrillin over-expression on lipid structures, transmission electron microscopy was used. Wt and transgenic fruit were examined sequentially through mature green to ripe stages, where plastid transition occurs.

In wt plants, plastoglobules are randomly dispersed throughout the chloroplasts or chromoplasts, and interpolated between membrane systems (Fig. 4A–D). In plastids of transgenic fruit (Fig. 4E–I), plastoglobules are found as grape like clusters: this is particularly visible (Fig. 4I) at the red stages (when the accumulation of the fibrillin protein is highest, as shown in Fig. 2) but also in some green plastids (not shown).

A similar effect on plastoglobules was also observed by Rey et al. (2000) who showed that the over-expression of the fibrillin protein, in the leaf chloroplasts of transgenic tobacco, led to grouped plastoglobule clusters, the collective number of clusters being close to the number of individual globules in wt leaves. Fibrillin may play a role in maintaining the structural integrity of plastoglobules in a similar fashion to the carotene globule protein (Cpg) localised at the periphery of the carotenoid globules of *Dunaliella bardawil* chloroplasts (Katz et al., 1995). Recently, seven proteins of the fibrillin family were found associated with plastoglobules in the chloroplasts of *A. thaliana* (Ytterberg et al., 2006; Vidi et al., 2006). It is, however, clear that the over-expression of a single member of the family (namely the one used here) can affect plastoglobules distribution. The role of fibrillin has, however, to be viewed in a dynamic perspective, since data indicate that plastoglobules can be coupled to thylakoids and exchange materials with them, and that they are connected to each other (Austin et al., 2006). Fibrillin could either reduce plastoglobule coalescence, or trigger the opposite process (increased rearrangement of larger globules into smaller ones) or increase the formation of plastoglobules from thylakoids.

A direct link between plastoglobule rearrangement and increased carotenoid content in transgenic tomato fruit could not be established using the present lines (lines with a higher increase in total carotenoids would probably be required to demonstrate a correlative link to plastoglobule total volume). What is clear, however, is that no new deposition structure of carotenoids such as fibrils (which are easily distinguished in pepper chromoplast electron micrographs), were observed in chromoplasts of transgenic tomato plants. Furthermore, fibrils can be concentrated and purified by fractionation of lysed pepper chromoplasts on sucrose gradients but, in the case of the present tomato transgenic lines, examination of such purified chromoplastic fractions by electron microscopy did not reveal the presence of new structures either (not shown). It should be pointed out that pepper chromoplasts predominantly contain carotenoids esterified by fatty acids, which may be necessary for fibril formation. Such esterified carotenoids are largely absent from tomato chromoplasts.

### 2.5. Influence of fibrillin over-expression on chloroplast/chromoplast transition

Harris and Spur (1969a,b) viewed lycopene crystal formation in tomato fruit as associated with existing thylakoids, sometimes even with grana at an early stage. An alternative view proposes that, during ripening, the thylakoids progressively disorganise while specific membrane vesicles and carotenoid-storage structure arise (Cheung et al., 1993; Deruère et al., 1994a). This is confirmed by our observations which indicate that, at the transition stage, two distinct phenomena can be observed in wt plastids, namely thylakoid disassembly (Fig. 4B) and synthesis of new membranes (Fig. 4C) where carotenoid crystals are growing. The latter phenomenon involves typical vesicle formation from the inner envelope of the plastid (Fig. 4B–D), indicating that the newly synthesised membranes are not directly formed inside the stroma from pre-existing thylakoids loosing their set of photosynthetic proteins. Although mechanistically different, both thylakoid disappearance and new membrane formation are progressive and concomitant phenomena, often occurring in spatial proximity (see insert in Fig. 4).

Surprisingly, chromoplasts from the transgenic lines often show, at an early ripening stage, a zone with a newly synthesised membrane network including carotenoid crystals typical of chromoplasts, while a separate stroma area appears to contain rather intact thylakoid membranes with grana stackings (Fig. 4H). This chloroplast-chromoplast co-existence within a single plastid is transient and, therefore, is not simultaneously observed in all plastids. It is specific to the fibrillin-overexpressing lines since such

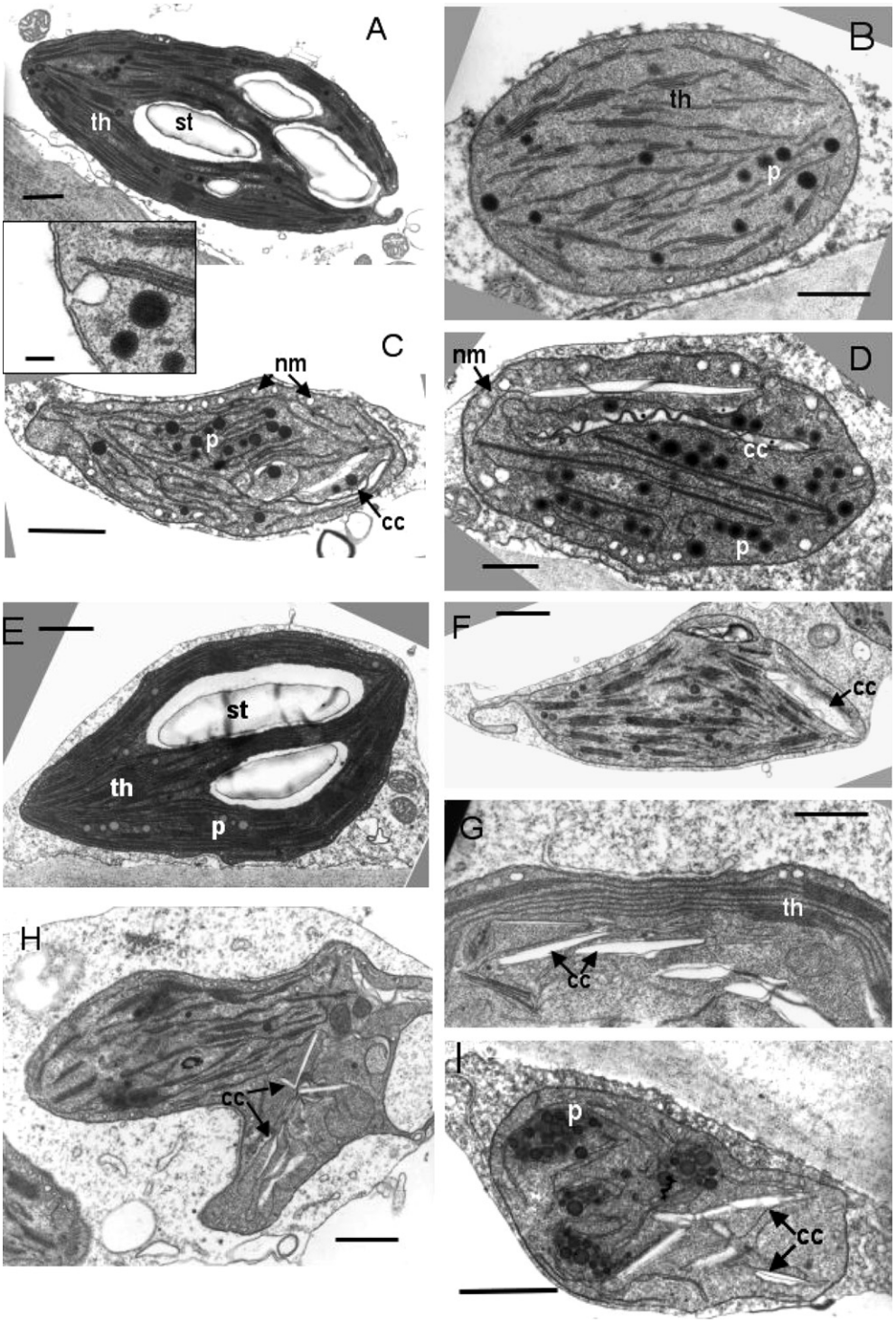


Fig. 4. Transmission electron micrographs of green and ripening fruit plastids from wt (A–D) and fibrillin over-expressing tomato plants (E–I). The insert illustrates the spatial proximity between thylakoids and membranes newly synthesised from the inner envelope. Panels f and h represent two different aspects of chloroplast to chromoplast transition. Lettering: p, plastoglobules; nm, newly synthesised membranes; th, thylakoids; cc, membranes enclosing carotenoid crystals (in white). Starch granules (st) are visible in chloroplasts. Scale bars: 50 nm for insert, 500 nm for b, d, g, 1 μm for a, c, e, f, h, i.

structures were never observed in wt plants. Furthermore, in the transgenic lines, carotenoid crystals grow from stellate nucleation centres (Fig. 4H) instead of developing in the long axis as in the wt chromoplast. The co-existence of two such zones within a single plastid leads to an abnormal shape resulting from the juxtaposition of an ovoid chloroplast-like shape and a more distorted chromoplast-like shape. At a later stage of plastid evolution in transgenic fruit, the thylakoid network disintegrates and is replaced by new membranes and clustered plastoglobules (Fig. 4I).

In its homologous system (namely pepper chromoplasts), fibrillin protein accumulation accompanies thylakoid disappearance. Therefore, it was unexpected that, in tomato fruit, the accumulation of the same fibrillin protein can lead to the prolonged conservation of thylakoids. It should also be mentioned that no formation of lycopene crystals is visible in zones of prolonged thylakoid conservation, which again points to the fact that thylakoid fate and appearance of new chromoplastic structures are independent phenomena.

In pepper fruit, an intermediate stage, retaining chlorophyll while accumulating carotenoid, can be observed under certain growth conditions, and leads to a transient brownish colour at the edge of green and reddening sectors (see Deruère et al., 1994a). This colour is retained throughout ripening in mutants such as Sweet Chocolate or Negral (Hornero-Mendez et al., 2000). In tomato, the green flesh mutant shows slowed chlorophyll catabolism and consequently a rusty-red colour (Cheung et al., 1993; Akhtar et al., 1999). Such a colour is not observed here because thylakoid conservation is a transient phenomenon and is not observed in all differentiating chromoplasts. In addition, to our knowledge, the abnormal shape of plastid described here is found neither in normal reddening pepper fruit, nor in any of the chlorophyll-retaining mutants mentioned above (Cheung et al., 1993; Deruère et al., 1994a).

### 2.6. *In vitro* influence of fibrillin on isolated thylakoids

Since the most striking effect of over-expressed fibrillin was on thylakoid persistence, experiments were designed to monitor *in vitro* effects of fibrillin on these membranes. Fibrillin was produced in *Escherichia coli* with an N-terminal His-tag and was purified by affinity chromatography on a Ni-NTA IMAC column from which it was eluted using an imidazole-containing buffer. Thylakoid electron transport or ATP synthase activity was measured, with or without recombinant fibrillin added as aliquots from its stock suspension.

ATP synthase activity was measured in a Tris buffer alone, in the buffer containing 3.8 mM imidazole and in the buffer containing an aliquot of fibrillin (3.8 mM final concentration of imidazole) (Fig. 5a). Strikingly, imidazole drastically reduced the ATP synthase activity to 30% of the control activity with the Tris buffer alone (suggesting an uncoupling effect of imidazole). However, this inhibitory effect was abolished in the presence of the recombinant fibrillin (final concentration 12.5 μg/ml). Measuring the electron flow across the electron transport chain in thylakoid membranes, an uncoupling-like effect of imidazole (3.8 mM) was also observed with the electron flow being drastically enhanced to about 175% of the control (assay in buffer without imidazole). Addition of recombinant EYFP (14 μg/ml) had no effect on the uncoupling effect of imidazole, unlike fibrillin which, when included in the assay (at 14 μg/ml), limited the increase in the electron flow to 130% of the control. EYFP and catalase were chosen

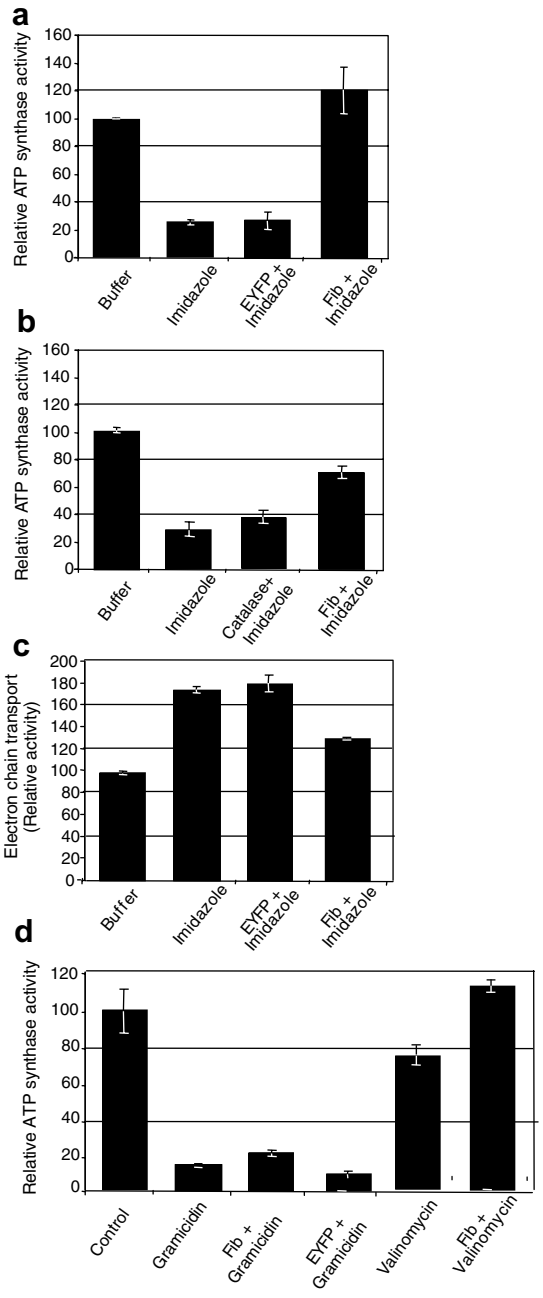


Fig. 5. *In vitro* effect of recombinant fibrillin on enzyme activities associated with wt thylakoids. (a) ATP synthase activity of isolated thylakoids was measured in buffer alone (final concentration: 0.25 mM Tris pH 7.6, 6.3 mM NaCl), or imidazole (final concentration 3.8 mM) in the same buffer, or in the presence of EYFP (12.5 μg/ml) in the same imidazole-containing buffer, or in the presence of fibrillin (12.5 μg/ml) in the same imidazole-containing buffer. (b) As in (a) but EYFP was replaced by recombinant catalase. Note that all 3 recombinant His-tagged proteins (fibrillin, EYFP and catalase) were purified using the same protocol. (c) Electron transport chain activity in isolated thylakoids was measured under the same conditions as described in (a). DCIP was used as a final electron acceptor. (d) ATP synthase activities measured as in (a), in the presence of gramicidin (0.3 μM) or gramicidin (0.3 μM) plus recombinant EYFP (12.5 μg/ml) or gramicidin (0.3 μM) plus recombinant fibrillin (12.5 μg/ml) or in the presence of valinomycin (11 μM) plus recombinant fibrillin (12.5 μg/ml). Since gramicidin and valinomycin were dissolved in ethanol, the same volume of ethanol was added to the control. Other details are given in Section 4. Error bars are the standard deviation for three measurements.

since they could be purified (taking advantage of their His-tag) using the same method described for fibrillin. In addition, EYFP has a MW similar to that of fibrillin. BSA was chosen as this protein is often used to bind to non-specific sites, such as on membranes.

Solely to further investigate the mode of action of imidazole on membranes, liposomes entrapping the fluorescent dye carboxy-fluorescein (CF) were used. Such a system allows monitoring of leakage through a model lipidic bilayer structure, which is believed to occur due to the formation of defects and/or transient pores (Silvander et al., 2003). As shown in Fig. 6, liposome permeability to CF is increased upon imidazole addition. We therefore conclude that imidazole mimics a (mild) uncoupling effect.

The above experiments using thylakoids were repeated using a fibrillin stock from which imidazole was largely removed by filtration (see Section 4). Other uncouplers were then used. As shown in Fig. 5d, ATPase activity is reduced to less than 20% of its original activity in the presence of a low concentration of gramicidin (0.3 μM) and this uncoupling effect is partially counteracted in the presence of fibrillin. This effect of fibrillin counteracting the gramicidin is slight (but was observed in several independent experiments) which is not surprising considering firstly the powerful activity of gramicidin, and secondly the reduced stability of fibrillin activity after filtration (not shown). Regarding the latter point, it should be mentioned that these experiments were performed each time with a freshly prepared suspension. The counteracting activity of fibrillin was also observed at 0.6 μM gramicidin (which lead to further reduction in ATPase activity in the absence of fibrillin; not shown). In contrast, EYFP at the same concentration did not show this effect of counteracting mild uncoupling effects. It should be mentioned that this fibrillin effect was only observed when added to thylakoids prior to gramicidin (not shown).

To confirm this effect of fibrillin, a third uncoupler was used, namely valinomycin, which is known to catalyse proton flux at certain concentrations (Brookes et al., 1997). As shown in Fig. 5d, at the valinomycin concentration used (11 μM), a mild uncoupling effect was obtained, which was also counteracted by fibrillin (when added to thylakoids prior to the treatment with valinomycin).

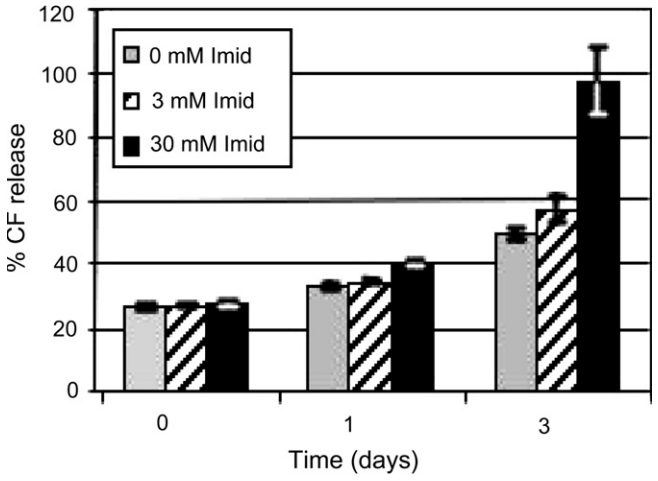


Fig. 6. Effect of imidazole on the leakage of liposomes. Phosphatidyl choline liposomes loaded with carboxyfluorescein (CF) were incubated with increasing imidazole concentrations. Stability of liposomes was estimated as the release of CF and related increase in fluorescence (self-quenching of CF is abolished by its dilution in the medium).

## 3. Concluding remarks

The fact that pepper fibrillin, and its homologue from potato, are synthesised and associate with thylakoids under (photo-oxidative) stress conditions in leaves has led to the hypothesis that they are involved in the protection of thylakoids under such adverse conditions (see Gillet et al., 1998; Rey et al., 2000). This is confirmed by observations of modified chlorophyll fluorescence in potato (Monte et al., 1999) or *Arabidopsis* (Yang et al., 2006) mutants with altered fibrillin levels. The latter report also indicated that fibrillin mediates ABA-induced photoprotection. We show here that fibrillin protects thylakoids both *in vivo* (during chloroplast to chromoplast transition) and *in vitro* (against moderate uncoupling effects).

Under stress conditions, thylakoid protein degradation may leave membranes partially unprotected and consequently sensitive to agents that may induce pore formation and most likely other severe alterations. The immunolocalisation of fibrillin to stromal lamellae of thylakoids (Rey et al., 2000) could be interpreted as a direct structuring role of the membrane bilayer. The role of fibrillin could be viewed as a replacement of the missing thylakoid proteins during a transient phase, before the latter proteins can be replaced post-stress.

Alternatively, stress may induce changes in the lipid composition of membranes, which may trigger formation of plastoglobules as a protective mechanism (to store neutral lipids which are incompatible with the maintenance of a lipid bilayer structure). Since fibrillin fulfils a structural role in plastoglobules, and if we assume that it favours their formation but also the reversibility of the process, then fibrillin may be viewed as fulfilling an indirect membrane protection role by structuring these globules, their interconnexion zones (Austin et al., 2006) and possibly, in addition, their formation zones in thylakoids.

During an early stage of fruit ripening, thylakoids are likely to be subject to increased catalytic activities, in this case leading to the breakdown of these membranes in an irreversible way. Over-expressing fibrillin in tomato fruit can be viewed as applying a protective mechanism to these membranes similar to that normally occurring in stressed leaf thylakoids. In other words, the steady membrane breakdown process could be slowed down (but not blocked as under stress), due to the excess amount of fibrillin, by the transient maintenance of lipid structures. Whether it

involves the direct or the indirect (or both) mechanisms hypothesised above in the case of stressed membranes is out of the scope of this paper but will be examined in the future.

It should also be kept in mind that fibrillin is present at a low level in the absence of stress, suggesting that it fulfils additional roles, which remain to be determined, during normal membrane maintenance.

It is conceivable that, *in vivo*, a number of proteins could contribute to structuring effects similar to the one shown here *in vitro* for fibrillin. We anticipate that some intrinsic plastid membrane proteins would show a comparable activity, a hypothesis that remains untested due to experimental difficulties. However, being able to monitor fibrillin activity *in vitro*, as described here, is an important breakthrough, since no direct enzyme assay is available for this protein. The development of this *in vitro* assay for fibrillin activity will now allow further examination of these possible modes of action for fibrillin and enable us to study the relation between fibrillin structure and function. The present data seem to favour a direct and broad structuring role for fibrillin on membranes. However, they do not exclude an indirect role, namely structuring of plastoglobules connected to thylakoids. Weak uncouplers or diluted strong uncouplers might preferentially act on particular membrane zones, such as those from which plastoglobules are formed by membrane blistering. The over-expressed fibrillin might reinforce these zones by stabilizing their associated globules (or pre-globules being formed), thus reducing leakage. The development of this *in vitro* assay for fibrillin activity will now allow further examination of these possible modes of action for fibrillin and enable us to study the relation between fibrillin structure and function.

Considering the sequence similarity between the corresponding fibrillin polypeptides from various plants (Laizet et al., 2004) and especially between pepper and tomato (91% identity and 96% similarity considering the mature polypeptides), we assume that the data obtained here in the case of accumulation of the pepper fibrillin can be generalised to the corresponding polypeptides from other plants.

## 4. Materials and methods

### 4.1. Plant material and treatment

Tomato (*Lycopersicon esculentum*, cv. Ailsa Craig) and tobacco (*Nicotiana tabacum* cv. Little Havana) plants were grown under controlled growth room conditions and were kept at culture room temperature (24–26 °C) with a 16-h photoperiod (Light: 280 $\mu E\, m^{-2}\, s^{-1}$; Dark: 0,06 $\mu E\, m^{-2}\, s^{-1}$). For fruit developmental studies, the fruits were divided into eight developmental stages from 1 cm to 7 cm in diameter. The fruit were of a green colour through stages 1–4, becoming yellow and orange at stages 5 and 6, respectively, and red at stages 7 and 8. Transgenic tomato plants were produced using *Agrobacterium*-mediated transformation with kanamycin resistance as a selectable marker. Full-length genomic sequence of the *Capsicum annuum* fibrillin gene (Deruère et al., 1994b) was introduced in the sense orientation under the control of its own promoter and followed by the *nos* 3′ terminator (Kuntz et al., 1998). Introduction and inheritance of the transgene were confirmed by PCR using specific primers.

### 4.2. Thylakoid extraction

Tobacco leaves were rinsed in deionised water prior to homogenisation in buffer (0.45 M Sorbitol, 20 mM Tricine–KOH, 10 mM EDTA, 10 mM $Na_2HCO_3$, 0.1% BSA, pH 8.4) in a waring blender for $3 \times 4$ seconds. The homogenate was filtered on muslin and cheese cloth then centrifuged at 3000*g* for 10 min at 4 °C. The chloroplast pellet was resuspended in RB buffer (0.3 M Sorbitol, 20 mM Tricine–KOH, 5 mM $MgCl_2$, 25 mM EDTA, pH 7.6), loaded on a 40% Percoll cushion in RB buffer and centrifuged 3 min at 5100*g*. The pelleted chloroplasts were lysed in 10 mM $MgCl_2$. After recentrifugation, membranes were collected and rinsed three times with RB buffer. Chlorophyll content was estimated at 652 nm after solubilisation in 80% acetone. These extracts were used for ATP synthase and electron chain activities and for Blue native gel electrophoresis.

### 4.3. Extraction and immunodetection of total proteins

Total protein was extracted from frozen ground material using the Hurkman and Tanaka method (1986). Three hundred milligrams of frozen material was added to 600 µl extraction buffer (0.7 M sucrose, 0.5 M Tris pH 8.5, EDTA, 0.1 M NaCl, 2 mM PMSF) containing 2% β-mercaptoethanol. An equal volume of phenol is added and the samples agitated for 20 min. After centrifugation, the phenol phase is recovered and the proteins precipitated in 5 volumes of methanol 100%/NH4 acetate (0.1 M). The samples were rinsed and resuspended in SDS/PAGE buffer. Protein concentration was determined by the Lowry method (DC Protein Assay BioRad). Protein samples were fractionated by SDS/PAGE and electroblotted onto Immobilon-P nitrocellulose. Membranes were blocked in 10% milk and immunodetected with antibodies against the N-terminal region of the fibrillin protein. Immunodetection was performed using the HRP conjugate substrate kit (Biorad) and the ECL Western blotting kit (Amersham) as recommended by the suppliers.

### 4.4. Tissue printing on nitrocellulose

Nitrocellulose membrane was pre-wetted in sterile distilled water and blotted damp dry on 3 MM Whatman

paper. The surface of a freshly cut fruit was blotted onto the membrane (Spruce et al., 1987; Wingate et al., 1991). Fibrillin was immunodetected as described above and the membranes were then coloured with Ponceau Red to show the protein transfer efficiency.

### 4.5. Extraction of total RNA and measurement of mRNA by RT-PCR

Frozen ground material was added to extraction buffer (0.1 M Tris pH 8.0, 10 mM EDTA, 0.1 M LiCl, 1% SDS) mixed with 1 volume water-saturated phenol preheated to 65 °C (leaf), 80 °C (fruit) and vortexed. The samples were centrifuged and the aqueous phase was recovered and re-extracted with 1 ml of chloroform. The aqueous phase was collected upon centrifugation and precipitated overnight with 0.5 volumes 6 M LiCl. Following centrifugation, the pellet was washed with 70% ethanol and then 100% ethanol, dried and resuspended in RNA resuspension buffer (10 mM Tris pH 7.5, 1 mM EDTA, 1% SDS) and precipitated in 2 volumes absolute ethanol and 0.1 volumes Na acetate. RNA samples were treated with 20 μg/ml Proteinase K in buffer (10 mM Tris pH 7.0, 0.4% SDS) at 50 °C and re-purified by phenol/chloroform extraction. Samples were checked for DNA contamination by PCR. Concentration and purity of total plant RNA was determined by spectrophotometric analysis. All RNA samples in each experiment were analysed by formaldehyde agarose gel electrophoresis and visual inspection of rRNA bands upon ethidium bromide staining. Samples were treated with DNase in 25 μl buffer (20 mM Tris pH 7.0, 6 mM MgCl, 40 u RNase inhibitor (RNaseOUT, BRL), 0.1 u DNaseI) to remove DNA contamination.

Reverse transcription was carried out using 500 ng of total RNA and oligo-dT as a primer. The reaction mixture included 1 mM dNTPs, 0.5 μM oligo-dT, 20 u RNase inhibitor, 10 mM DTT, 1× RT buffer and 150 u M-MLV reverse transcriptase (BRL) in total volume of 20 μl. To exclude differences in the efficiency of the RT and PCR reactions from sample to sample, a reference RNA, 10 pg of control RNA (rabbit globin mRNA from reticulocyte polyribosomes, BRL), was added prior to the reaction and used as a control (subjected to the same reactions) as already described (Simkin et al., 2000, 2003). Each reaction was carried out in duplicate. The reaction mixture was incubated for 10 min at 20 °C, 35 min at 37 °C and then 15 min at 42 °C. Duplicate samples were pooled to give final volume of 40 μl for PCR. The PCR reaction contained 100–250 ng of each primer, 1× *Taq* polymerase buffer, 5 mM MgCl, 0.30 mM dNTPs, 1.5 u *Taq* polymerase (BRL) and 2 μl RT reaction mixture (12.5 ng RNA/μl) in a total volume of 50 μl. Final concentration was 0.5 ng/μl reaction. The primers used for the control reactions were CTGGGCAGGCTGCTGGT and GATCTCAGTGGTATTTGTGAG. The fibrillin specific primers were: GATGCTGAGACTTTAACCGTC and CACGACTCTGAG AACATGTCT. The amplification reactions included 18 cycles of 20 s at 94 °C, 20 s at 50 °C and 20 s at 72 °C.

### 4.6. Overproduction of pepper fibrillin in E. coli

A cDNA clone encoding a 28.7-kDa mature protein, corresponding to amino acids 59–322, was amplified by RT-PCR with ampliTaq Gold (Applied Biosystems) to generate SphI and PstI sites at the 5′ and 3′ ends, respectively, by using the following primers: pepmat5: (GCATGCGCTACCAATTACGACAAGGAAGAT) and pepmat3: (CTGCAGTTAAGGCTTCAAGAGGGGACT). The PCR fragment has been cloned in pQE30 vector (Qiagen) and transformed in *E. coli* M15 bacterial strain (Qiagen), Cells were grown in LB medium with 100 μg/ml amplicillin and 50 μg/ml kanamycin at 37 °C under vigourous shaking until $OD_{600} = 0.6$. N-terminal His-tagged fibrillin (approx. 30.3 kDa) was induced with 0.5 mM IPTG for 3 h at 30 °C.

### 4.7. Purification of recombinant protein

Cells were disrupted in a French pressure cell, and the resulting crude extract was clarified by centrifugation at 14,000 rpm for 10 min. The recombinant fibrillin was purified by immobilised metal ion affinity chromatography (IMAC) (Chelating Sepharose Fast Flow, Amersham Biosciences) as instructed by the manufacturer. Buffer used for purification was 20 mM Tris pH 8, 500 mM NaCl with 10, 50, 300 mM Imidazole respectively for binding, washing and elution of protein from the column. Fibrillin was either used directly from a stock suspension in this elution buffer or first subjected to PD-10 spin column (Amersham) or Biogel P6 (Biorad) centrifugation to remove most of the imidazole. This filtration step reduced the residual imidazole concentration in the stock suspension from 300 mM to ca. 2.5 mM. Purified protein was obtained at a yield of 2.5 mg/g of induced culture pellet and aliquoted at −80 °C for experiments. The amount of proteins was analysed by the method of Bradford using bovine serum albumin as the standard. The quality of the purification was checked by SDS/PAGE: only a single polypeptide was detected.

### 4.8. ATP synthase activity

Thylakoids ATP synthase activity was assayed as the amount of free phosphate (Tausky and Shorr, 1953). The reaction mixture contained 31.3 mM Tris pH 8.0, 6.3 mM $MgCl_2$, 1.3 mM phosphate, 1.3 mM ADP, 62.5 μM phenazine methosulfate and thylakoids (0.125 mg chlorophyll/ml). Recombinant fibrillin was added from its stock suspension and thus diluted to 12.5 μg/ml final concentration. Controls were performed using recombinant EYFP or catalase (12.5 μg/ml) or BSA (25 μg/ml). When imidazole was used as an uncoupler, its final concentration was 3.8 mM. When gramicidin or valinomycin were used (and fibrillin

chromatographed in order to remove imidazole; see above) the final concentration of residual imidazole was ca. 3.2 μM (its effect on ATP synthase activity was negligible). After these additions, thylakoids were kept on ice for 5 min before the ATP synthase assay, which was run 3 min at room temperature under 90 klux illumination.

### 4.9. Electron transport chain assay

DCIP (0.2 mM final concentration) was used as final electron acceptor during measurement of electron chain transport in thylakoid suspensions (final concentration 0.15 mg chlorophyll/ml) in RB buffer. Reduction of DCIP was followed at 600 nm using an UVIKON spectrophotometer under 90 klux illumination. Values were calculated as the slope of DCIP reduction during the time in three independent experiments. When present, imidazole final concentration was 3.8 mM. Protein concentrations were as in ATP synthase activity assays.

### 4.10. Liposomes preparation and leakage assays

A chloroform solution of 25 mg of L-phosphatidylcholine type X–E (Sigma–Aldrich) was dried under a nitrogen flow and then under vacuum for at least 2 h. The lipid film was then resuspended in 2.5 ml of a Tris 20 mM pH 7.5, NaCl 150 mM buffer containing 50 mM 5-6-carboxyfluorescein (CF) (Fluka) and sonicated for 2 h at 4 °C (60 V). CF is an hydrophilic fluorescent reagent self-quenched at high concentration (50 mM). Release of the dye increases the fluorescence due to dequenching resulting from dilution. Untrapped CF was separated from liposomes by gel filtration on Sephadex G50 or PD-10 column (Amersham) equilibrated in the same Tris–NaCl buffer. Liposome integrity was checked by adding Triton X-100 (0.2%) to an aliquot (resulting in complete lysis). The average liposome radius was estimated by laser light scattering to be 53 nm.

For leakage assay, 60 μl of CF entrapped liposomes were added to 140 μl of Tris–NaCl buffer containing (final concentration in 200 μl) 0, 3 or 30 mM imidazole. pH was equilibrated at 7.5 for each buffer. Fluorescence measurements were determined with an Hitachi 2500-F spectrofluorometer equipped with a continuous stirring device. Release of CF was recorded as an increase in CF fluorescence with an excitation wavelength of 490 nm and an emission wavelength of 520 nm. The excitation and emission slit width was 5 nm. Three consecutive measurements were taken per sample: a blank with 2.95 ml of Tris–NaCl buffer, after addition of 50 μl of the incubated liposome sample and finally after addition of 0.2% (vol/vol) Triton X-100 to calibrate the 100% release.

### 4.11. Carotenoid analysis

The method used to analyse and quantify carotenoids is detailed in Fraser et al. (2000). Typically tomato pericarp tissues were freeze-dried and ground into a powder, 10–50 mg aliquots were extracted using methanol:chloroform (1:3 v/v) and partitioned against 2 volumes of 50 mM Tris–HCl pH 7.0. The aqueous phase was re-extracted twice. HPLC separations were performed on a C30 reverse-phase column (250 × 4.6 mm) manufactured by YMC Co. Ltd., and purchased from Interchim (France). The mobile phases used were: methanol (A), water/methanol (20/80 by vol) containing 0.2% ammonium acetate (B) and *tert*-methyl butyl ether (C). The gradient used was 95% A/5% B isocratically for 12 min, a step to 80% A/5% B/15% C at 12 min, followed by a linear gradient to 30% A/5% B/65% C by 30 min (Fraser et al., 2000). Quantification was achieved from dose-response curves and identification of carotenoids was achieved by co-chromatography and comparative spectral properties acquired on-line.

### 4.12. Volatile analysis

Diced fruit were placed into glass vessels and assayed under normal light conditions. Experiments utilised glass cylinders (17 mm i.d. × 61 cm long, 127 ml volume) and collection of volatiles followed Turlings et al. (1991). Volatile emissions were collected for a period of 1 h in all experiments (Simkin et al., 2004b; Underwood et al., 2005). Briefly, clean humidified air was passed through the vessels (550 ml $min_{-1}$) and volatiles were trapped on 30 mg Super Q (80/100 mesh; Alltech). Five micrograms nonyl acetate (in 5 μl dichloromethane) was added as an internal standard and the super Q traps were eluted with 150 μl dichloromethane. Quantification of volatiles was performed on an HP 6890N GC according to Schmelz et al. (2001).

### 4.13. Electron microscopy

Fruit tissues were sequentially fixed with glutaraldehyde, osmium tetroxide and tannic acid, according to Carde (1987) and embedded in Epon. Plastid subfractions were fixed according to the same procedure but embedded in drops of low melting point agarose (2% in water) before further processing. Ultrathin sections (50 nm thick) were collected on bare 600 mesh copper grids and stained with uranyl and lead. Observations were made with a FEI CM10 electron microscope fitted with an AMT 2K digital camera.

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