Identification of an *Arabidopsis* mitochondrial succinate–fumarate translocator

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Abstract Complementation of a yeast *acr1* mutant carrying a deletion of the succinate/fumarate carrier gene enabled functional identification of a mitochondrial succinate translocator from *Arabidopsis thaliana* (AtmSFC1). Thus complementation of yeast mutants is applicable also for identification and characterization of organellar transporters. Reverse transcription polymerase chain reaction and promoter-GUS fusion showed expression of AtmSFC1 in 2 day old dark grown seedlings, which declined in cotyledons during further development, consistent with a role in export of fumarate for gluconeogenesis during lipid mobilization at early germination of *Arabidopsis* seeds. In mature plants, expression was found in developing and germinating pollen, suggesting a role in ethanolic fermentation.

Key words: Mitochondrial carrier family; Succinate transporter; Anaplerotic reaction; Gluconeogenesis; *Arabidopsis thaliana*

1. Introduction

The exchange of metabolites between different organelles is of central importance, since individual steps of a given metabolic pathway are carried out in different compartments. Intermediates of the mitochondrial tricarboxylic acid (TCA) cycle are used as precursors for various compounds including amino acids, nucleobases and porphyrins. The constant biosynthetic flow of carbon out of the TCA cycle has to be countered via anaplerotic reactions. Both in yeast and in plants the glyoxylate cycle serves as a major anaplerotic pathway leading to succinate, which enters the mitochondria to replenish the TCA cycle. In yeast, the glyoxylate cycle is essential for the growth on acetate or ethanol as sole carbon sources. Acetate and ethanol are converted into acetyl-CoA, and subsequently into succinate in the cytosol. In plants, the glyoxylate cycle converts acetyl-CoA derived from β-oxidation of fatty acids into succinate, which is exported from glyoxysomes and imported into mitochondria. This pathway is important especially for oilseed plants, which extensively convert storage lipids to soluble carbohydrates during germination and the following period of heterotrophic growth. In addition, the glyoxylate cycle seems also to be operative to mobilize thylakoid membrane lipids during senescence and during pollen development [9].

Specific carrier proteins in the inner mitochondrial membrane catalyze solute exchange with the cytosol. In yeast most of these carriers share structural features: a molecular mass of ~30 kDa, a tripartite structure (three repeats of ~100 amino acids) and the presence of two transmembrane α-helices separated by hydrophilic loops in each repeat. A consensus [P-X-(DE)-X-(LIVAT)-(RK)-X-(LRH)-(LIVMFY)], named 'mitochondrial energy transfer signature', is present in up to three copies at the C-terminus of the first helix in each repeat [1,2]. These transporters fall into the mitochondrial carrier family (MCF) with 35 members *Saccharomyces cerevisiae* [3]. Genetic studies identified the function of three MCF members: ARG11, an ornithine/arginine exchanger, DIC1, a dicarboxylate transporter and ACR1, a succinate/fumarate exchanger.

The *Arabidopsis* genome contains at least 48 MCF members. Until now, only few of these MCF members have been characterized: two adenine nucleotide translocators, two uncoupling proteins [4–8] and dicarboxylate carrier AtDTC [4–8]. Because of the particular importance of the mitochondrial succinate carrier [11], the aim of this work was to identify the AtmSFC1 succinate–fumarate translocator gene from *Arabidopsis* by suppression cloning in the *acr1* yeast mutant. Expression studies were used to provide first insights into the physiological role of AtmSFC1.

2. Materials and methods

2.1. Yeast mutants

Yeast strain Σ23344c (Mata, *ura3*) [12] was used to generate a *knock-out* mutant in *ACR1*. *ACR1* was deleted by a disruption cassette containing *loxP* sites flanking *kan* [13]. The *acr1* mutant GEM60 was selected on YPD containing 200 mg/l G418; deletion was confirmed by polymerase chain reaction (PCR). Selective conditions for identification of plant succinate transporters were 1.7 g/l yeast nitrogen base without amino acids/ammonium sulfate, 5 g/l $(NH_4)_2SO_4$, 2% ethanol as sole carbon source.

2.2. Cloning of AtmSFC1

AtmSFC1 was amplified by PCR on an *Arabidopsis* cDNA library [14] using the primers: 5′-ACGGCGGCCGCATGGCGACGAGAACGGAATC-3′, containing a *Not*I site and 5′-ACGGGTCCCTATAAAGGAGCATTCCGAAG-3′, containing a *Bam*HI site. The PCR product was restricted with *Not*I and *Bam*HI and ligated into pDR195 [15].

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Abbreviations: MCF, mitochondrial carrier family

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Fig. 1. Functional complementation of the yeast strain GEM60. A: The yeast strain Σ23344c was transformed with pDR195. B: GEM60 strain (*acr1*) was transformed with the empty vector pDR195 or (C) with AtmSFC1 cloned in pDR195.

2.3. Expression analysis

Arabidopsis thaliana L. Heynh. ecotype Col-0 was used for plant studies. With the exception of roots, which were obtained from plants cultured on MS agar plates, all plant organs were harvested from greenhouse-grown plants. For RT-PCR (reverse transcription PCR), RNA was converted to cDNA using Retroscript (Ambion). A 340 bp cDNA fragment was amplified by 25 PCR cycles using specific primers for *AtmSFC1* (5′-GCTCTCGCCATTGTTACACCCTT-3′; 5′-TCACCACATCAAACGGTCCTGT-3′). A 377 bp cDNA fragment of the constitutively expressed actin 2 gene (*AtACT2*) was used as control [16]. Identity of the amplicons was confirmed by sequencing.

2.4. Promoter-GUS fusions

Two different fragments (1240 and 800 bp) of the promoter region (up to the next open reading frame (ORF)) including the *AtmSFC1* start codon were amplified on *Arabidopsis* genomic DNA using primers 5′-CTGACCACTAGTCTGTTTATCTTTGTATAAAG-3′; 5′-CTGACCACTAGTAAAGCTTAATCACTCTTTCC-3′ and 5′-CTGACCACCCGGGACCATATCTGATTCCTCGGCCGG-3′. Forward primers contain *Spe*I restriction sites, the reverse primer a *Sma*I site. The PCR product was cleaved with *Spe*I and *Sma*I and ligated in frame to the GUS gene in pCB308 [17]. *Agrobacterium tumefaciens* GV3101 was transformed with the resulting plasmids. *Arabidopsis thaliana* L. Heynh. ecotype Col-0 grown in soil in the greenhouse, was transformed by floral dipping [18]. Transgenic *Arabidopsis* plants were selected for BASTA resistance.

2.5. Histochemical analysis of GUS activity

T2 transformants were grown in soil. At different growth times tissues were harvested. For seedling staining, plants were grown on solid MS. For histochemical assays of β-glucuronidase activity, tissues were incubated in GUS-staining solution containing 100 mM sodium phosphate (pH 7), 10 mM EDTA, 3 mM $K_4[Fe(CN)_6]$, 0.5 mM $K_3[Fe(CN)_6]$, 0.2% (v/v) Triton X-100, 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) for ~18 h at 37°C [19].

2.6. Pollen germination and staining

Pollen was collected from dehiscent anthers of plants grown in greenhouse and dabbed on agar plates with a thin layer of germination medium consisting in 17% sucrose, 2 mM KCl, 1.6 mM boric acid pH 7.0. Plates were incubated at 27°C for 3 h. For GUS activity determination, pollen tubes were stained as described above.

3. Results

The mitochondrial succinate/fumarate carrier provides a link between the glyoxylate cycle in glyoxysomes, the TCA cycle in mitochondria and gluconeogenesis in the cytosol. In *Saccharomyces cerevisiae*, this transporter is encoded by *ACR1* [10]. An *acr1* yeast mutant GEM60 was generated in the yeast background 23344c, which is unable to grow on ethanol as sole carbon source (Fig. 1).

Database searches identified a gene (At5g01340) sharing 35% identity to yeast *ACR1*. The corresponding cDNA *AtmSFC1* was cloned via RT-PCR into the yeast expression vector pDR195. Expression of *AtmSFC1* restored the capability of GEM60 to grow on ethanol (Fig. 1), indicating that AtmSFC1 imports succinate into mitochondria in exchange for fumarate. *AtmSFC1* encodes a protein of 310 amino acids with a calculated molecular mass of 34 kDa. PROSITE identified three repeats of the mitochondrial energy transfer signature behind the first, third and fifth membrane-spanning domains. As in the case of many other mitochondrial carriers, no mitochondrial signal sequence was found. Various mem-

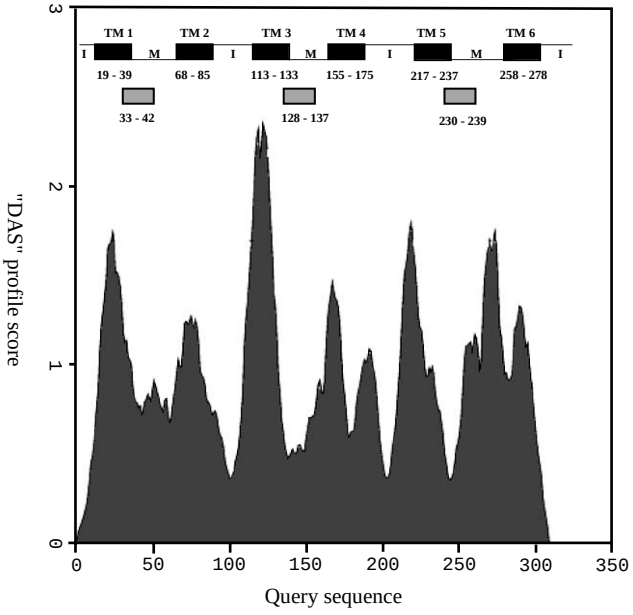

Fig. 2. Hydrophobicity plot of AtmSFC1 obtained with DAS. Six hydrophobic regions are depicted and the three mitochondrial energy transfer signatures are in gray boxes (amino acid positions are given).

brane protein prediction programs (http://www.aramemnon.de) did not predict the presence of six transmembrane domains typical for MCF carriers [2]. Instead, the programs predicted between zero and three transmembrane spans. Nevertheless, hydrophobicity plots generated with the dense alignment surface method (DAS; http://www.sbc.su.se/~miklos/DAS/maindas.html) show six hydrophobic regions in accordance with the typical structure of the members of the MCF (Fig. 2).

By querying of transporter databases, e.g. ARAMEMNON [20], at least 51 MCF members were identified. The family is highly divergent, full alignment showed only eight perfectly conserved residues. Since a similarity tree fell into two major groups plus a few very distantly related members, the phylogenetic analysis was carried out with the subgroup containing the mitochondrial amino acid transporters, several other *Arabidopsis* MCF members and selected fungal and mammalian members. The members were classified into six clades: (i) two clades of di- and tricarboxylate carriers, (ii) amino acid carriers, (iii) uncoupling proteins (UCPs), and (iv) at least two groups for which no function has been assigned yet (Fig. 3). Within the di- and tricarboxylate carrier group II, ACR1 is most closely related to AtmSFC1. The clade also includes the yeast and mammalian citrate carriers, whereas the *Arabidopsis* di- and tricarboxylate carrier AtDTC [8] falls into a different clade. No other *Arabidopsis* protein fell into the di- and tricarboxylate carrier group II clade.

As the cellular expression pattern might provide hints for physiological function, total RNA was extracted from different tissues of *Arabidopsis* plants. Since the expression level was too low to be detected by RNA gel blot analysis, RT-PCR was carried out. *AtmSFC1* expression was found in all

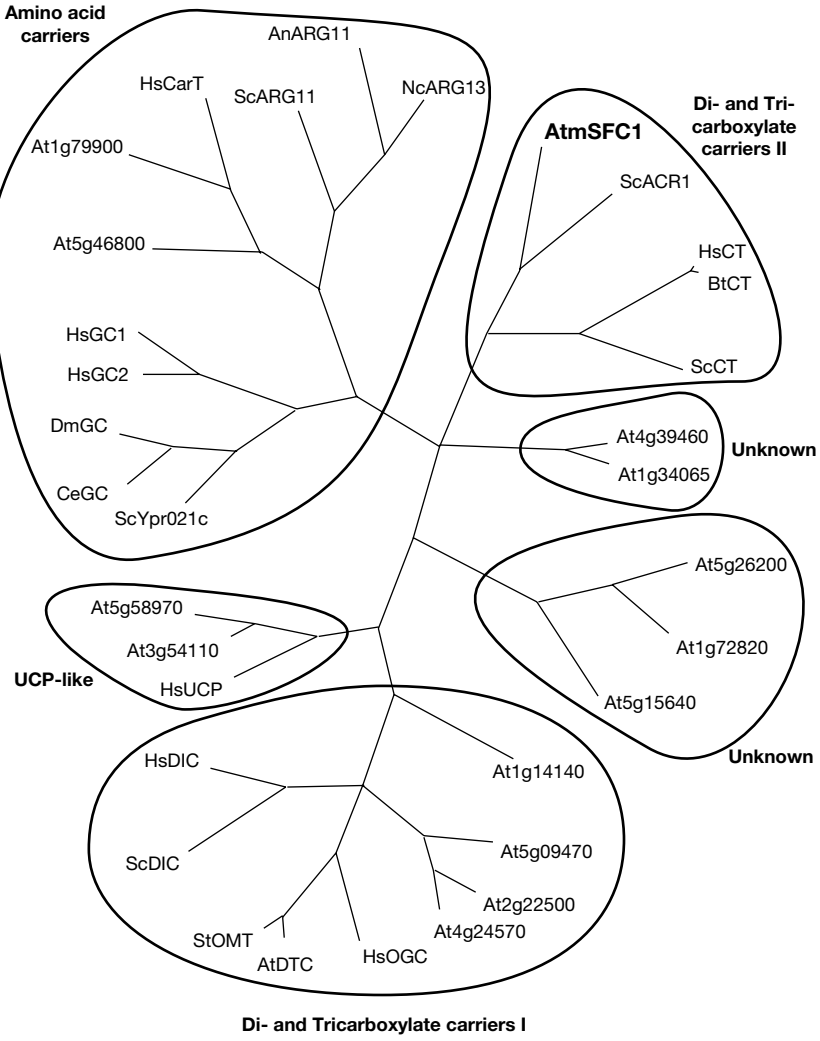

Fig. 3. Phylogenetic analyses of a multiple alignment of the deduced protein sequence of AtmSFC1 and other mitochondrial carriers. The alignment was restricted to the conserved domains (between positions 20 and 296 in the AtmSFC1 amino acid sequence). Maximum parsimony analyses were performed using PAUP 4b10 with all amino acid characters unweighted and gaps scored as missing characters. Heuristic tree searches were executed using 1000 random sequence additions and the tree bisection–reconnection branch-swapping algorithm with random sequence analysis. The complete alignment was based on 342 sites; 306 were phylogenetically informative. The sequences were retrieved from the GenBank data base (http://www.ncbi.nlm.nih.gov/) and have the following accession numbers: AtmSFC1, NP195754; ScACR1, CAA80973; HsCT, NP005975; BtCT, P79110; ScCT, NP009850; At4g39460, NP568060; At1g34065, NP564436; At5g26200, NP197992; At1g72820, NP565048; At5g15640, NP568317; At1g14140, NP172866; At5g09470, NP196509; At2g22500, NP179836; At4g24570, NP194188; HsOGC, NP003553; AtDTC, CAC84549; StOMT, X99853; ScDIC, AAB71336; HsDIC, NP036272; HsUCP, NP003346; At3g54110, NP190979; At5g58970, NP568894; ScYpr021c, NP015346; CeGC, NP497274; DmGC, AAF57048; HsGC1, CAD21007; HsGC2, CAD21008; At5g46800, NP568670; At1g79900, NP178108; HsCarT, O43772; ScARG11, CAA60862; AnARG11, AAD44763; NcARG13, AAF87777.

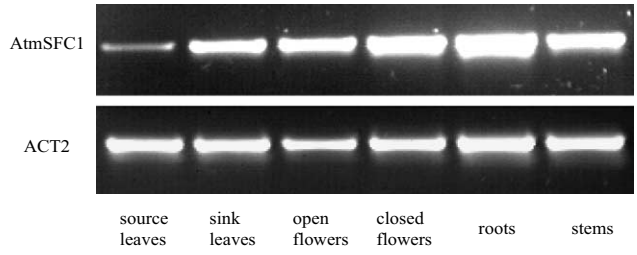

Fig. 4. Expression of AtmSFC1 mRNA in different *Arabidopsis* organs. RNAs were extracted and converted to cDNA by reverse transcriptase, and a 340 bp AtmSFC1 fragment was amplified by 25 PCR cycles. As control, a 377 bp actin2 fragment was amplified by 20 PCR cycles (ACT2).

organs analyzed with a slightly elevated level in roots (Fig. 4), a pattern consistent with housekeeping functions.

To investigate expression in more detail, two promoter fragments (1240 and 800 bp) were fused transcriptionally to GUS. Expression patterns were comparable in both constructs, thus the region between −800 and −1240 bp did not seem to contain additional regulatory elements. As AtmSFC1 is expected to play a role in mobilization of storage lipids in cotyledons, seedlings were grown in darkness and analyzed 2 days after imbibition. GUS activity was found in cotyledons, hypocotyl and to a minor extent in root tips (Fig. 5A), whereas 5 day old seedlings showed no or only marginal GUS activity (Fig. 5B), indicating that AtmSFC1 expression correlates with that of glyoxylate cycle genes during post-germination. At later stages, the promoter was active in most leaves; however, expression was preferentially found towards leaf margins, sometimes in cloudy patterns (Fig. 5C). In mature leaves, staining was localized in patches of veins and in trichomes (Fig. 5D,E). Analysis of 3 month old plants showed expression in flowers especially in stigmatic papillae of the carpel and anthers (Fig. 5F–H). As glyoxylate cycle gene expression increases during pollen development [21], pollen expression of *AtmSFC1* was investigated. Pollen grains as well as in vitro germinated pollen tubes showed GUS-staining (Fig. 5I,J). Developing siliques showed expression in abscission zones (Fig. 5K).

4. Discussion

Despite the importance of lipid catabolism in early plant development and of intracellular compartmentation in this process, little was known about the carriers involved. So far, the most efficient methodology to characterize mitochondrial transporters was a candidate gene approach. Candidates were expressed in *Escherichia coli*, purified proteins were reconstituted in lipid vesicles and transport properties were determined [22]. The approach is difficult and laborious and no standard protocols applicable for all candidates are available [23]. Nevertheless, this approach enabled the identification of the first di- and tricarboxylate carrier AtDTC from *Arabidopsis* [8]. Here, an alternative approach was used, allowing identification of mitochondrial carriers via suppression of auxo- or bradytrophic phenotypes of yeast mutants. To demonstrate the feasibility, the yeast *acr1* mutant was complemented with a plant succinate transporter candidate gene. An *Arabidopsis* gene *AtmSFC1* was identified, showing the validity of yeast complementation also for the functional assignment of candidate genes which products localize in intracellular membranes.

AtmSFC1 shows the typical features of mitochondrial carriers, namely a relatively low molecular mass of around 30 kDa, a tripartite structure and three signature motifs. However, only a hydrophobicity plot generated by the DAS prediction server for prokaryotic transmembrane proteins shows a profile consistent with the existence of six transmembrane domains (Fig. 2).

Phylogenetic analysis of the MCF family shows that AtmSFC1 falls into a clade together with human and yeast citrate transporters (Fig. 3). A second clade contains di- and tricarboxylate carriers, including AtDTC, which transports various di- and tricarboxylates [8]. Broad substrate spectra seem general characteristics for all transporters of the MCF family, e.g. ScACR1 has highest affinities for succinate and fumarate, but also recognizes oxoglutarate, oxalacetate, malate, phosphoenolpyruvate, citrate or isocitrate [10]. This reflects that transporters do not have a simple binding pocket, but recognition and exclusion take place along the translocation pathway in the pore, thus requiring release of the substrate on the other side of the protein. From an evolutionary perspective promiscuity may be advantageous since mutations thus allow rapid adaptation to new tasks. This may also explain why so far only few mutants in transport processes across the inner mitochondrial membrane have been identified: carriers have overlapping substrate specificities, and in most cases are thus able to compensate deficiencies. Therefore, it is likely that AtmSFC1 transports other substrates besides succinate and fumarate.

Also in plants, transport of succinate across the mitochondrial membrane is of central importance for utilization of storage lipids during germination. Therefore, the identification of the mitochondrial succinate/fumarate carrier could be relevant for engineering efficiency of seed germination. Yeast ACR1 is co-regulated with genes coding for glyoxylate cycle and gluconeogenesis enzymes, like isocitrate lyase 1 (ICL1), malate synthase 1 (MLS1), phosphoenolpyruvate carboxykinase 1 (PCK1) and fructose 1,6-bisphosphatase 1 (FBP1). Furthermore, *ACR1* is subject to glucose repression [24]. Similarly, glyoxylate cycle genes are turned on early and transiently during germination of oil seeds and are subject to carbon catabolite repression [25–27]. Consistent with a role in fatty acid metabolism, *AtmSFC1* expression is also transiently induced in early phases of seedling development and declines rapidly (Fig. 5).

In pollen from *Brassica napus*, mRNAs for key enzymes of the glyoxylate cycle have been found [21]. Besides entrance of pyruvate into mitochondria for the TCA cycle, pollen may use ethanolic fermentation via pyruvate decarboxylase as a pyruvate dehydrogenase (PDH) bypass serving energy and lipid biosynthesis [28]. Thus in the PDH bypass, cytosolic acetyl-CoA may be converted into succinate, which could then enter mitochondria via a succinate transporter. The preferential expression of AtmSFC1 in developing and germinating *Arabidopsis* pollen suggests a role in ethanolic fermentation.

In summary, a novel mitochondrial carrier for succinate/fumarate from *Arabidopsis* has been identified. This transporter potentially links the glyoxylate cycle, TCA cycle and gluconeogenesis enabling the mobilization of storage lipids during germination and the supply of energy for pollen. To further explore the role of AtmSFC1 in *Arabidopsis* the anal-

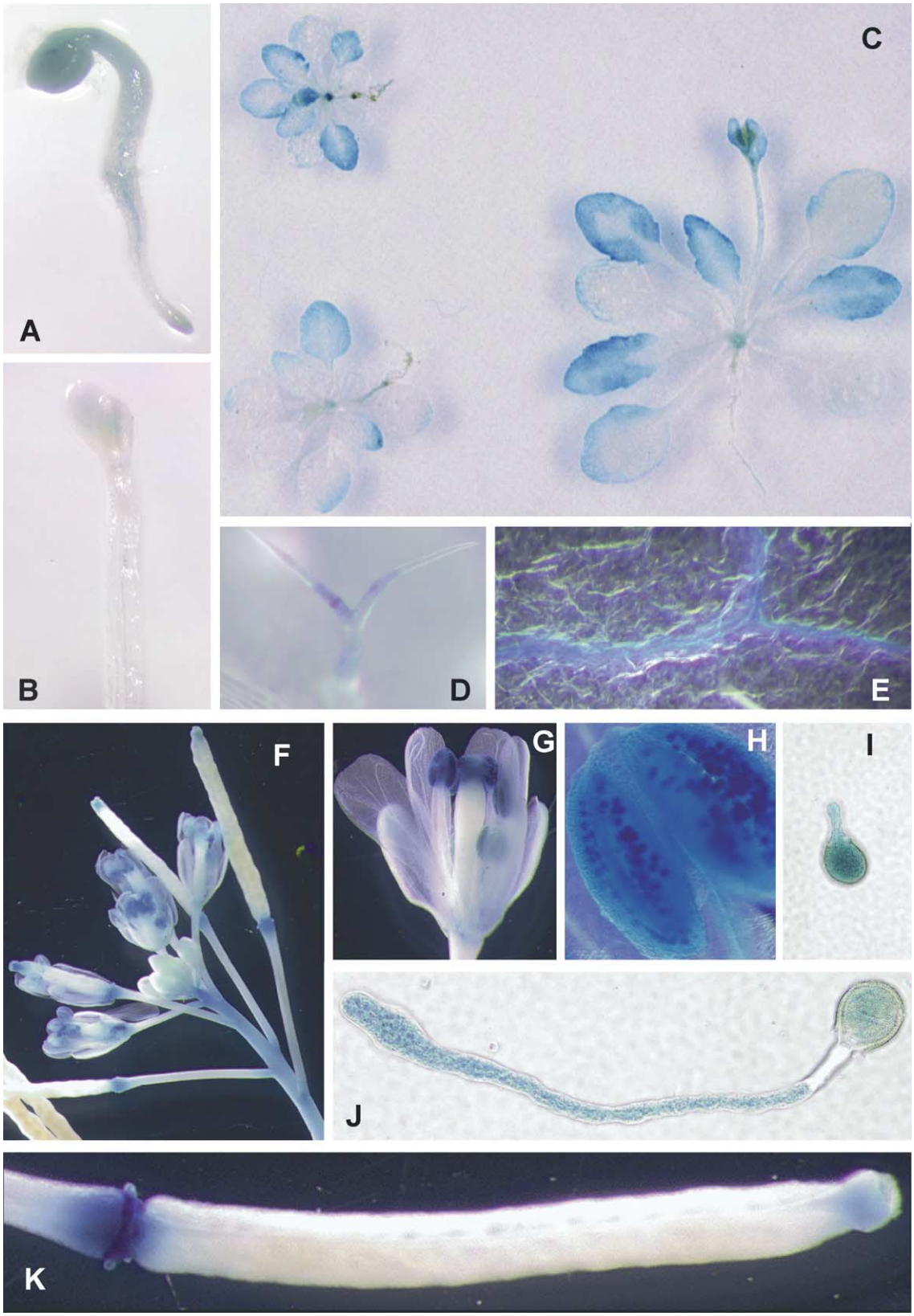

Fig. 5. GUS-staining of *Arabidopsis* plants transformed with the AtmSFC1 promoter–GUS fusion. Histochemical analysis of promoter activity in 2 and 5 day old seedlings (panels A and B) and 3 week old plants (panel C). Details of expression in trichomes (panel D), leaf veins (panel E), inflorescence and flower (panels F and G), anthers (panel H), germinating pollen (panels I and J) and siliques (panel K).

ysis of mutants may be of invaluable importance. Probably due to the small size, no insertion mutants have been identified in the various mutant collections using reverse genetics. Since one may expect that single mutants will not show phenotypes due to promiscuity, RNAi approaches aiming at inhibition of several MCF members with overlapping substrate specificity may be a means to test the role of AtmSFC1.

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