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RESEARCH PAPER

The paramutated *SULFUREA* locus of tomato is involved in auxin biosynthesis

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Abstract

The tomato (*Solanum lycopersicum*) *sulfurea* mutation displays *trans*-inactivation of wild-type alleles in heterozygous plants, a phenomenon referred to as paramutation. Homozygous mutant plants and paramutated leaf tissue of heterozygous plants show a pigment-deficient phenotype. The molecular basis of this phenotype and the function of the *SULFUREA* gene (*SULF*) are unknown. Here, a comprehensive physiological analysis of the *sulfurea* mutant is reported which suggests a molecular function for the *SULFUREA* locus. It is found that the *sulf* mutant is auxin-deficient and that the pigment-deficient phenotype is likely to represent only a secondary consequence of the auxin deficiency. This is most strongly supported by the isolation of a suppressor mutant which shows an auxin overaccumulation phenotype and contains elevated levels of indole-3-acetic acid (IAA). Several lines of evidence point to a role of the *SULF* gene in tryptophan-independent auxin biosynthesis, a pathway whose biochemistry and enzymology is still completely unknown. Thus, the *sulfurea* mutant may provide a promising entry point into elucidating the tryptophan-independent pathway of IAA synthesis.

Key words: Auxin, auxin biosynthesis, paramutation, photosynthesis, *Solanum lycopersicum*, *sulfurea*.

Introduction

The tomato *sulfurea* mutant represents one of the first discovered examples of paramutation in plants (Hagemann, 1958). The mutant was isolated by Rudolf Hagemann in an X-ray mutagenesis experiment with tomato seeds (Hagemann, 1958, 1993; Hagemann and Berg, 1977). An additional independent allele was obtained by plant regeneration in tissue culture, presumably through somaclonal variation (Wisman *et al*., 1993). The *sulfurea* mutant displays a striking chlorophyll-deficient phenotype which gives the plants a nearly sulfurous colour and stimulated the naming of the mutant (Hagemann, 1958; Fig. 1A–C). Interestingly, although the *sulfurea* allele is recessive, the pigment deficiency appeared spontaneously in somatic tissues of heterozygous plants at high frequency (Fig. 1B, C). This phenomenon was initially referred to as 'somatic gene conversion' and later named paramutation (reviewed in Chandler *et al*., 2000; Chandler and Stam, 2004; Stam and Scheid, 2005; Bond and Finnegan, 2007; Chandler, 2007). Paramutation is an epigenetic mechanism that results in heritable changes in gene expression. It involves the *trans*-inactivation between a pair of homologous alleles: a paramutable allele and a paramutagenic allele. The paramutagenic allele is inactive (silenced) and capable of imposing its inactive expression state onto a susceptible (paramutable) allele. Remarkably, gene inactivation by paramutation is heritable in that an inactivated paramutable allele becomes itself paramutagenic and can infect new paramutable alleles brought in by crossing. The nature of this epigenetic inactivation mechanism is unknown, and two alternative models have been discussed (Chandler and Stam, 2004; Stam and Scheid, 2005; Bond and Finnegan, 2007; Chandler, 2007): a direct physical interaction between the paramutable and the paramutagenic alleles (pairing model) or an RNA-mediated gene inactivation mechanism (small RNA model). The recent successful identification of a first set of genes involved in paramutation in maize (Alleman *et al*., 2006; Woodhouse

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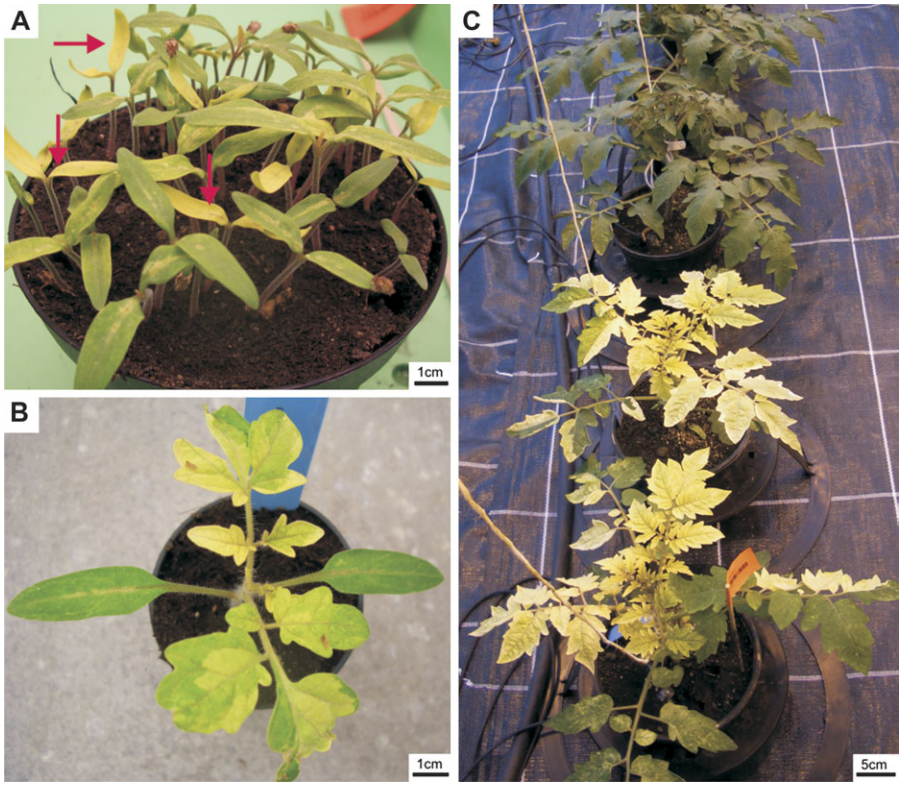

Fig. 1. Phenotype of the tomato *sulfurea* mutant. (A) Mendelian segregation in the progeny from a (branch of a) heterozygous plant that was not paramutated. Homozygous *sulf* seedlings are easily identifiable by their yellow cotyledons (red arrows). (B) Paramutation in primary leaves of a heterozygous *sulf* seedling. (C) Paramutated branches in heterozygous plants. These branches are genetically homozygous for *sulf*. Consequently, seeds obtained from fruits on these branches give rise to uniformly yellow seedlings.

et al., 2006; Hale *et al*., 2007) has begun to shed some light on the molecular mechanisms of paramutation. The involvement of an RNA-dependent RNA polymerase in the establishment of the paramutated state (Alleman *et al*., 2006) has provided circumstantial evidence in favour of a small RNA model, but the molecular details of the inactivation mechanism are still far from being understood.

Paramutation at the tomato *sulfurea* locus occurs in two different phenotypes: (i) homogeneously yellow leaf sectors or branches, described as $sulf^{pura}$ allele, or (ii) green-yellow variegated sectors or branches ($sulf^{vag}$ allele). Both the $sulf^{pura}$ and the $sulf^{vag}$ alleles were found to be paramutagenic in that they impose their presumed inactive status onto the wild-type allele in heterozygous plants (Hagemann and Berg, 1977; Hagemann, 1993). The *SULF* gene maps to the centromeric heterochromatin of chromosome 2 (Hagemann, 1993). This and the lack of any other genetic marker in close proximity (Tanksley *et al*., 1992) make the isolation of the gene very difficult.

The molecular basis of the *sulfurea* phenotype and the function of the *SULFUREA* gene (*SULF*) are still unknown. The pigment deficiency could be most readily explained by a primary defect in chlorophyll or carotenoid biosynthesis or, alternatively, by a defective photosynthetic apparatus. Here, evidence is provided that neither is the case. Instead, it is shown that the *sulfurea* mutant is deficient in auxin synthesis, most probably due to a block in tryptophan-independent indole-3-acetic acid (IAA) biosynthesis. While IAA biosynthesis via tryptophan has been studied for a long time and the gene products involved in the different steps of the tryptophan-dependent pathway have been mostly unravelled, the enzymology of the tryptophan-independent auxin biosynthetic pathway is still enigmatic (Östin *et al*., 1999; Cohen *et al*., 2003; Woodward and Bartel, 2005). Thus, the *sulfurea* mutant provides a promising genetic tool towards further elucidating the immense complexity of auxin metabolism in plants.

Materials and methods

Plant material and growth conditions

Seeds obtained from heterozygous *sulfurea* plants and wild-type seeds (*Solanum lycopersicum* cv. Lukullus) were germinated in soil. Homozygous *sulfurea* plants did not survive beyond the seedling stage. Heterozygous plants and wild-type control plants were grown in a growth chamber at 100 μmol quanta m^{-2} s^{-1} for 3 weeks, then transferred to the greenhouse and grown under standard conditions

(250 μmol quanta m^{-2} s^{-1}). To obtain material from paramutated tissue which is not photo-oxidatively damaged, heterozygous plants were grown under low light condition (50 μmol quanta m^{-2} s^{-1}). Completely paramutated leaf material was easily recognizable by its homogeneous yellow phenotype.

Tissue culture, regeneration, and in vitro *growth assays*

Surface-sterilized seeds from heterozygous plants were germinated on agar-solidified MS medium containing 20 g l^{-1} sucrose (2× MS; Murashige and Skoog, 1962). Cotyledons from wild-type and homozygous *sulf*^{pura} seedlings were regenerated on 2× MS medium containing 1 mg l^{-1} zeatin. To test for heterotrophic growth, regenerated wild-type and homozygous *sulf*^{pura} plantlets were grown under sterile conditions on 2× MS medium and cultivated under low light (15 μmol quanta m^{-2} s^{-1}). Etiolated growth was investigated by germinating surface-sterilized wild-type seeds and seeds harvested from completely paramutated branches on 2× MS medium followed by growth under sterile conditions in complete darkness for up to 2 months. To assess growth stimulation by auxins, regenerated wild-type and homozygous *sulf*^{pura} plantlets were grown on 2× MS medium containing 2.5 μM indole-3-acetic acid (IAA; Sigma) and/or 2.5 μM indole-3-butyric acid (IBA; Sigma) under sterile condition in low light (see above). Apical stem-feeding experiments were performed using sterile blocks of agar-solidified MS medium containing 2 mM IAA or 2 mM IBA. The agar-solidified media were cut into small cubes of approximately 2×2 mm. These agar blocks were placed on top of decapitated stems of *sulf*^{pura} and wild-type plantlets (grown on 2× MS under sterile conditions and low light). The auxin-containing agar blocks were exchanged every week.

Electron microscopy

Leaf samples were fixed for 2 h with glutaraldehyde (2.5%) in sodium-potassium phosphate buffer (0.1 M, pH 7.0) including paraformaldehyde (2%) and tannic acid (0.2%). After washing with phosphate buffer, the samples were incubated for 12 h in osmium tetroxide (1% in 50 mM sodium-potassium phosphate buffer, pH 7.0) and subsequently washed again with phosphate buffer. Samples were dehydrated in a graded series of ethanol followed by propylene oxide, then incubated in a 1:1 mixture (v/v) of propylene oxide and epoxide resin (ERL-4206; vinylcyclohexene dioxide) and finally transferred into pure ERL (Spurr, 1969) and polymerized overnight at 60 °C. Ultra-thin sections were contrasted with uranyl acetate and lead citrate. Electron micrographs were obtained with a Siemens EM 10 microscope using Scientia negative films. The negatives were scanned with an Epson 1680 Pro scanner at a resolution of 1200 dpi.

Photosynthesis measurements

Chlorophyll fluorescence was recorded with a pulse-amplitude modulated fluorimeter (Dual-PAM; Heinz Walz, Effeltrich, Germany). Plants were dark adapted for 1 h prior to determination of PSII quantum efficiency (F_v/F_m). The relative stoichiometries of plastocyanin (PC) per P_{700} were determined using the plastocyanin version of the Dual-PAM spectroscope (Dual-PAM-S; Heinz Walz GmbH, Effeltrich, Germany; Schöttler *et al*., 2007). Measurements were performed on intact leaves and transmission signals were normalized to leaf chlorophyll contents.

HPLC analyses of pigments

Chlorophylls and carotenoids were isolated from dried leaf tissue by extraction with 80% acetone followed by a second extraction with 100% acetone and combination of the two extracts (Wurbs *et al*., 2007). Separation, identification, and quantitation of the pigments were performed by HPLC using an Agilent 1100 Series HPLC system with a diode array detection unit (Agilent). All pigments were quantified by comparison to known amounts of standards. A YMC ODS-A 250×4.6 mm column+precolumn was used for chromatographic separation. Separation was performed as described by Thayer and Björkman (1990) with the following modifications: solvent A contained acetonitrile, methanol, and 100 mM TRIS/HCl, pH 8.0 (72:8:3 by vol.). Pigments were eluted with 100% solvent A for 5 min, followed by 100% solvent B for 20 min. The column was allowed to re-equilibrate in solvent A for 10 min prior to the next run.

Measurement of auxin contents

For the determination of auxin contents, seedlings or leaves were shock frozen in liquid nitrogen and homogenized. Aliquots of 100 mg fresh weight (FW) were lyophylized for each analysis. For the determination of free auxins, the plant material was extracted and purified on NH_2 columns as described previously (Chen *et al*., 1988; Ludwig-Müller and Cohen, 2002). To each sample, 100 ng $^{13}C_6$-IAA (Cambridge Isotope Laboratories, Andover, MA) were added as an internal standard. After elution from the column, the samples were dried, directly methylated with diazomethane (Cohen, 1984), and resuspended in ethyl acetate for gas chromatography-coupled mass spectrometry (GC-MS) analysis. Auxin conjugates were determined following strong alkaline hydrolysis of an extract obtained from 100 mg FW with 7 M NaOH for 3 h at 100 °C. After subsequent cooling, the extract was brought to pH 3.5 and the aqueous phase extracted twice with ethyl acetate. The organic phases were combined and, after evaporation under a stream of nitrogen, the sample was resuspended in 100 μl ethyl acetate for methylation. The amount of free IAA was subtracted from the total IAA amount after strong alkaline hydrolysis (total IAA) to obtain the amount of conjugated IAA. GC-MS analysis was performed as described previously (Jentschel *et al*., 2007) and values for IAA contents were calculated according to the principles of isotope dilution using the quinolinium ions of endogenous and $^{13}C_6$-IAA, respectively (Cohen *et al*., 1986).

Results

Photosynthetic electron transport and pigment biosynthesis in the sulfurea *mutant*

The pale green to yellow phenotype of homozygous *sulfurea* plants and paramutated tissues in heterozygous plants suggested a deficiency in photosynthesis and/or pigment biosynthesis (chlorophyll biosynthesis). To test these possibilities, heterozygous plants were raised under low-light conditions and chlorophyll contents and photosynthetic electron transport parameters were measured in fully paramutated *sulf*^{pura} leaves (Table 1). Surprisingly, although chlorophyll contents in the mutant only reached approximately 25% of those in wild-type plants and photosynthetic electron transport rates were very low, the measurements revealed the presence of a mainly functional photosynthetic electron transport chain in the *sulfurea* mutant (Table 1). The quantum yield of dark-adapted photosystem II (F_v/F_m), a measure of photosystem II (PSII) integrity, was

only moderately affected in the mutant and the contents of the soluble luminal electron carrier plastocyanin (PC) were almost unchanged. However, photosystem I (PSI) amounts were found to be strongly reduced in the *sulf* mutant (cf. values for P_{700} and PC/P_{700} in Table 1).

To test whether or not the *sulf* mutant carries a defect in pigment biosynthesis, chlorophyll and carotenoid accumulation were determined by HPLC analyses (Fig. 2A). As expected from the pale phenotype, pigment contents were significantly lower in the mutant than in the wild type. However, all major chlorophyll and carotenoid species were readily detectable in *sulf* leaf tissue (Fig. 2A) indicating that neither chlorophyll nor carotenoid biosyntheses are blocked in the mutant. In accordance with the assembly of a functional electron transport chain and intact pigment biosynthetic pathways in *sulfurea*, chloroplasts in the mutant were capable of forming thylakoid membranes and grana stacks (Fig. 2B). However, *sulf* chloroplasts exhibited altered thylakoid alignment, reduced formation of stromal lamellae (stroma thylakoids), increased accumulation of plastoglobules and lack of accumulation of starch granules (Fig. 2B). All these ultrastructural abnormalities are well in line with the severe pigment deficiency, the very low PSI contents and the resulting strongly reduced assimilation capacity (Table 1).

To explore the possibility that *sulf* functions outside of photosynthesis and pigment biosynthesis, the mutant was assayed for heterotrophic growth on tomato tissue culture media. All photosynthesis mutants are viable when grown on sucrose-containing media under low-light conditions, even if electron transport or carbon fixation are completely abolished (Kanevski and Maliga, 1994; Ruf *et al*., 1997; Hager *et al*., 1999). This is because provision of an external sugar source can complement all defects in photosynthetic carbon assimilation. By contrast, mutants whose pigment-deficient phenotype is only a secondary consequence of a mutation elsewhere, cannot necessarily be rescued by provision of sucrose. Survival and growth of seedlings or regenerated plantlets under heterotrophic conditions, therefore, represents a suitable test for a possible primary defect in photosynthesis. Interestingly, homozygous *sulf* seedlings were incapable of heterotrophic growth and died rapidly after germination, typically within 10–14 d (Fig. 3A, B). Similar observations were made when homozygous tissue was regenerated on sucrose and phytohormone-containing tissue culture medium. While shoot regeneration was readily achieved, the

Table 1. *Spectroscopic analysis of photosynthesis in leaves of wild-type tomato plants and the* sulfurea *mutant, both grown under low-light conditions*

F_v, variable fluorescence; F_m, maximum fluorescence; PC, plastocyanin; P_{700}, reaction centre chlorophyll of photosystem I. The low chlorophyll *a*:*b* value in the wild type and the low $PC:P_{700}$ ratio are due to plant growth under low-light conditions, which results in an adaptive increase in the antenna system (i.e. the light-harvesting complexes).

	Wild type	*sulfurea*
F_v/F_m	0.79±0.01	0.69±0.04
PC ($\Delta I/I \times 10^{-3}$ mg^{-1} Chl)	8.6±1.0	8.5±1.7
P_{700} ($\Delta I/I \times 10^{-3}$ mg^{-1} Chl)	46.6±4.0	9.2±2.8
PC/P_{700}	0.74±0.05	3.83±0.54
Chlorophyll (mg m^{-2})	518.1±49.0	132.4±17.1
Chlorophyll *a*/*b*	2.58±0.1	3.25±0.03

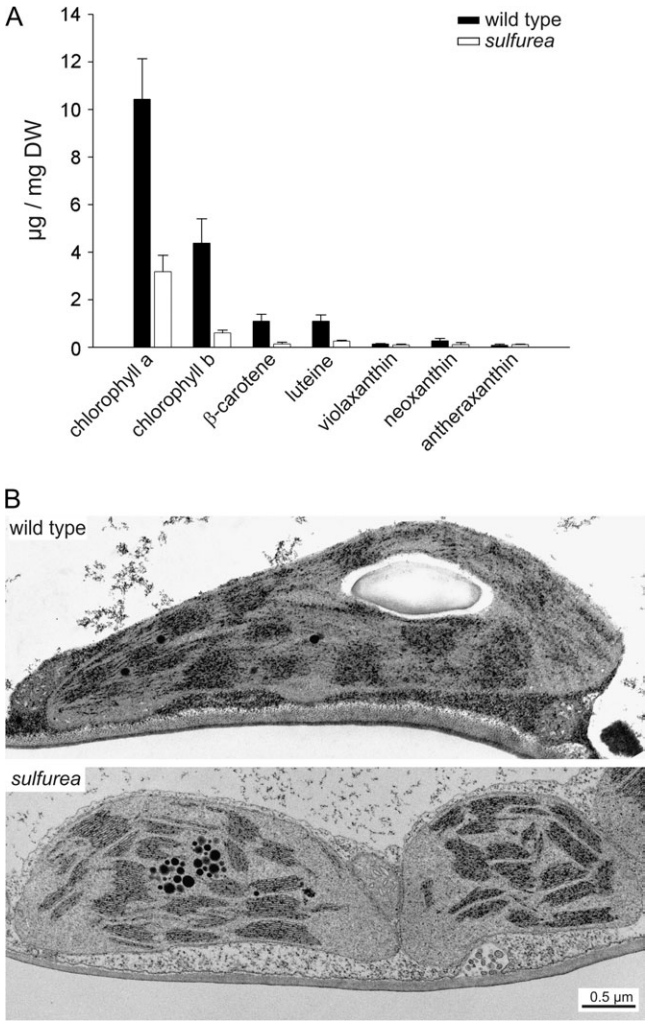

Fig. 2. Photosynthetic pigment contents and thylakoid formation in leaves of the *sulfurea* mutant and the wild type. Fully paramutated *sulf*^{pura} leaves from heterozygous plants were analysed. (A) Quantitation of chlorophyll and carotenoid accumulation by HPLC. Pigment contents are given in µg mg^{-1} dry weight (DW). (B) Electron micrograph showing formation of grana stacks in chloroplasts of the *sulfurea* mutant. While wild-type chloroplasts accumulate large starch granules (white body), mutant chloroplasts accumulate increased amounts of plastoglobules (dark spherical bodies). Also, formation of stroma thylakoids is strongly reduced in *sulfurea* chloroplasts.

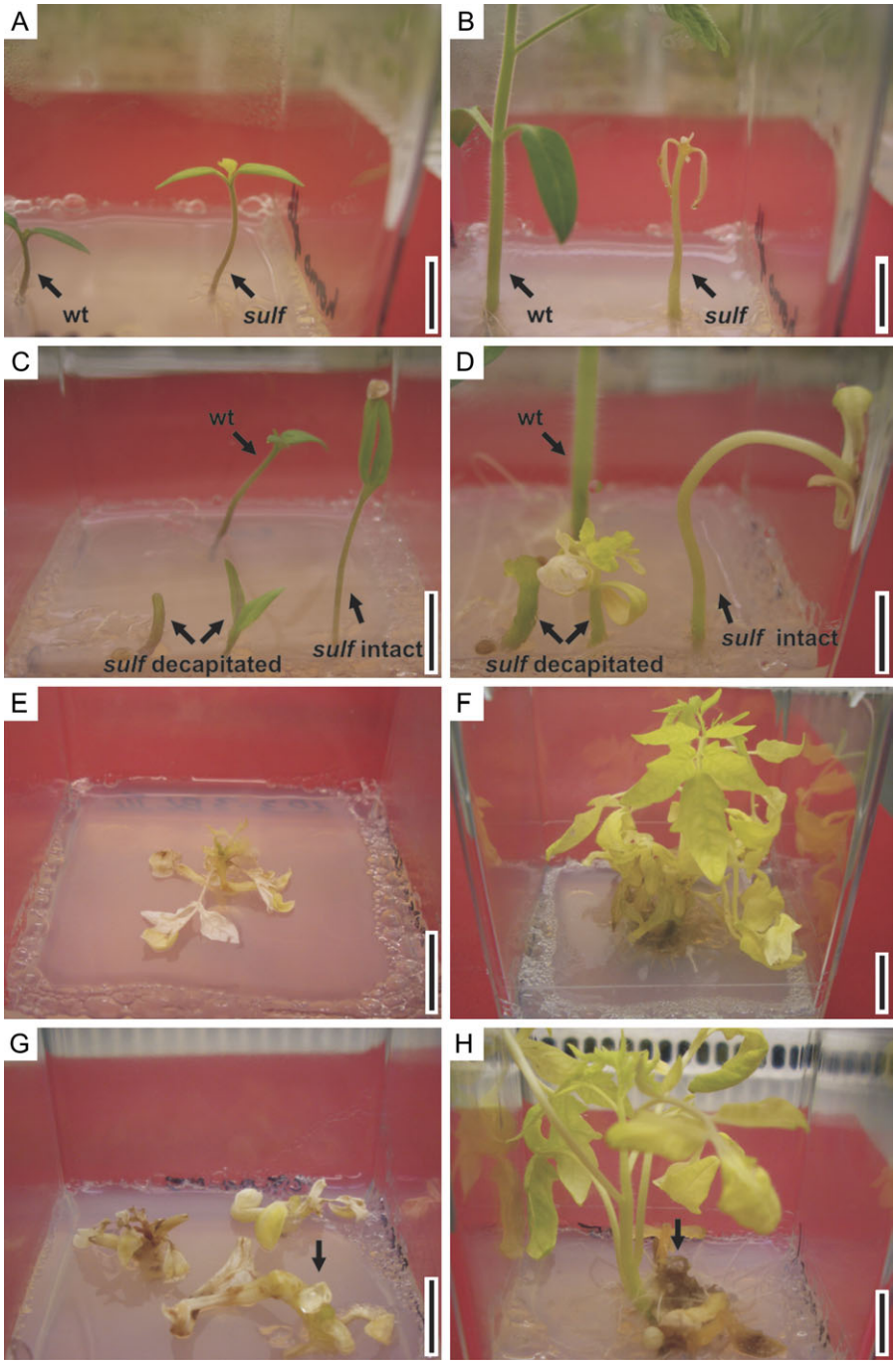

Fig. 3. Promotion of growth and development in the *sulfurea* mutant by auxin. (A) Normal germination of homozygous *sulfurea* seedlings. The pale green colour of cotyledons and initiating primary leaves is clearly visible. (B) Death of *sulfurea* seedlings after 10–14 d of growth on sucrose-containing medium. (C, D) Decapitation promotes survival of the *sulfurea* mutant. Shown is a comparison of intact and decapitated seedlings immediately after decapitation (C) and after 2 weeks (D). Growth and leaf development in the top half, as well as greening of the bottom part, are clearly visible. (E, F) Growth promotion of *sulfurea* shoots by IAA added to the culture medium. While control shoots without IAA in the medium rapidly die (E), the presence of 2.5 μM IAA induces normal shoot and leaf development (F). Pictures were taken after 4 weeks. (G, H) Induction of shoot and leaf development in *sulfurea* by application of auxin-containing agar blocks to the top of decapitated stems. (G) Control without IAA in the agar block. (H) Experiment with 2 mM IAA in the agar block. The arrows point to the agar blocks. Pictures were taken 4 weeks after decapitation and agar block application.

plantlets quickly ceased to grow and usually died after having developed two to four leaves (not shown). Taken together, the lack of heterotrophic growth as well as intact pigment biosynthesis and thylakoid biogenesis provide strong evidence against a primary defect in photosynthesis in the *sulfurea* mutant.

Wounding promotes survival of homozygous sulfurea *seedlings*

Lack of plant development in the presence of sugar as an externally supplied carbon source (Fig. 3A, B) suggested a growth defect in *sulfurea* that is not directly related to photosynthesis. To distinguish between a defective growth from the shoot apical meristem (SAM) and a general inability to grow and undergo cell division, etiolation experiments were carried out. Etiolation of seedlings strongly promotes hypocotyl growth (skotomorphogenesis) whereas growth from the SAM remains inhibited. After 2 months of growth on sucrose-containing medium in the dark, both wild-type seedlings and mutant seedlings reached hypocotyl lengths of over 30 cm, indicating that internodal growth and cell division are not generally impaired in *sulfurea* and, instead, suggesting that the growth defect may be limited to meristem outgrowth. This is consistent with wild type-like shoot regeneration, but lack of apical growth of the mutant in tissue culture (see above). Analysis of root development revealed that neither root growth nor gravitropism were significantly affected in homozygous *sulf* seedlings (data not shown).

Interestingly, when young *sulfurea* seedlings were decapitated and the two halves of the seedling cultured separately in synthetic medium (Fig. 3C), survival and growth of the mutant was significantly enhanced (Fig. 3D). While intact seedlings hardly developed primary leaves, the upper part of cut seedlings showed substantial leaf development (Fig. 3D). Moreover, the decapitated bottom part displayed strong greening (Fig. 3D) and the survival of both halves was extended for up to 4 weeks. A possible explanation for this surprising observation is that wounding induces a molecular response or a biochemical pathway that partially overcomes the defect present in the *sulfurea* mutant.

The sulfurea *mutant is auxin deficient*

Known wound-induced processes were considered next in order to identify possible causes of the enhanced growth and survival of *sulfurea* seedlings after decapitation. As synthesis of the phytohormone auxin is not only induced by wounding, but also involved in regulating apical growth (reviewed in Woodward and Bartel, 2005; Leyser, 2006; Teale *et al*., 2006; De Smet and Jürgens, 2007; Boutté *et al*., 2007), it was tested if the application of exogenous auxin would promote the survival and growth of *sulfurea* shoots. To this end, indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) was added to the growth medium (Fig. 3E, F). Alternatively, auxin-containing agar blocks were applied on top of decapitated wild-type and *sulfurea* stem sections (Fig. 3G, H). Interestingly, both sets of experiments revealed a strong growth-promoting effect of exogenously added auxins. While in the absence of auxin in the medium, *sulfurea* shoots died rapidly (Fig. 3E), the presence of either IAA or IBA (or a combination of both) resulted in normal leaf development and plant growth for up to 2 months (Fig. 3F). Similarly, the application of exogenous auxin via agar blocks placed on top of stem sections strongly promoted plant growth and development (Fig. 3G,H). These agar blocks mimic the presence of an auxin-producing shoot apical meristem. Their application to the *sulfurea* stem sections resulted in strong plant growth from axillary meristems with the resulting shoots reaching up to more than 10 cm height (Fig. 3H; data not shown). If auxin was omitted from the agar block, no growth and leaf development occurred beyond that induced by the wounding as resulting from the cutting process (Fig. 3G).

These data suggest that homozygous *sulfurea* plants suffer from auxin deficiency. Furthermore, the results indicate that *sulfurea* may not be completely auxin auxotrophic and that instead, the auxin deficiency is largely confined to synthesis in the shoot meristems. Induction of auxin synthesis by wounding or exogenous auxin feeding can partially overcome this defect.

The sulfurea *mutant may be defective in tryptophan-independent auxin biosynthesis*

In addition to shoot and leaf development, auxin is also involved in regulating floral development (Bernier *et al*., 1993; Christensen *et al*., 2000; Reinhardt *et al*., 2000; Cheng *et al*., 2006; Kim *et al*., 2007; Zhao, 2008). Therefore, flower development in heterozygous paramutated *sulfurea* plants was analysed. While the number of flowers developed per plant was comparable in the wild type and the mutant, paramutated branches of *sulfurea* plants displayed frequent abnormalities in floral morphology (affecting, on average, 10–20% of the flowers; Fig. 4A–F). These abnormalities were very diverse and included missing floral organs, fused organs, homeotically transformed organs or the presence of supernumerary organs, such as multiple carpels that were often fused (Fig. 4B–F). As similar phenotypes are common in auxin mutants (reviewed in Cheng and Zhao, 2007), this observation is consistent with a disturbed auxin homeostasis in *sulfurea* and a possible involvement of the *SULFUREA* locus in auxin metabolism.

Flowering plants, such as tomato, possess two distinct pathways of auxin biosynthesis: a tryptophan-dependent pathway (involving the intermediates tryptamine, indole-3-pyruvic acid, and indole-3-acetaldehyde) and at least one tryptophan-independent pathway (Östin *et al*., 1999), the biochemistry of which is not yet understood (but which is suspected to branch from either indole or indole-3-glycerol phosphate; reviewed in Cohen *et al*., 2003; Woodward and Bartel, 2005). The two pathways are non-redundant in that they are highly regulated in a tissue-specific and developmental stage-specific manner (Cohen *et al*., 2003; Woodward and Bartel, 2005; Zhao, 2008).

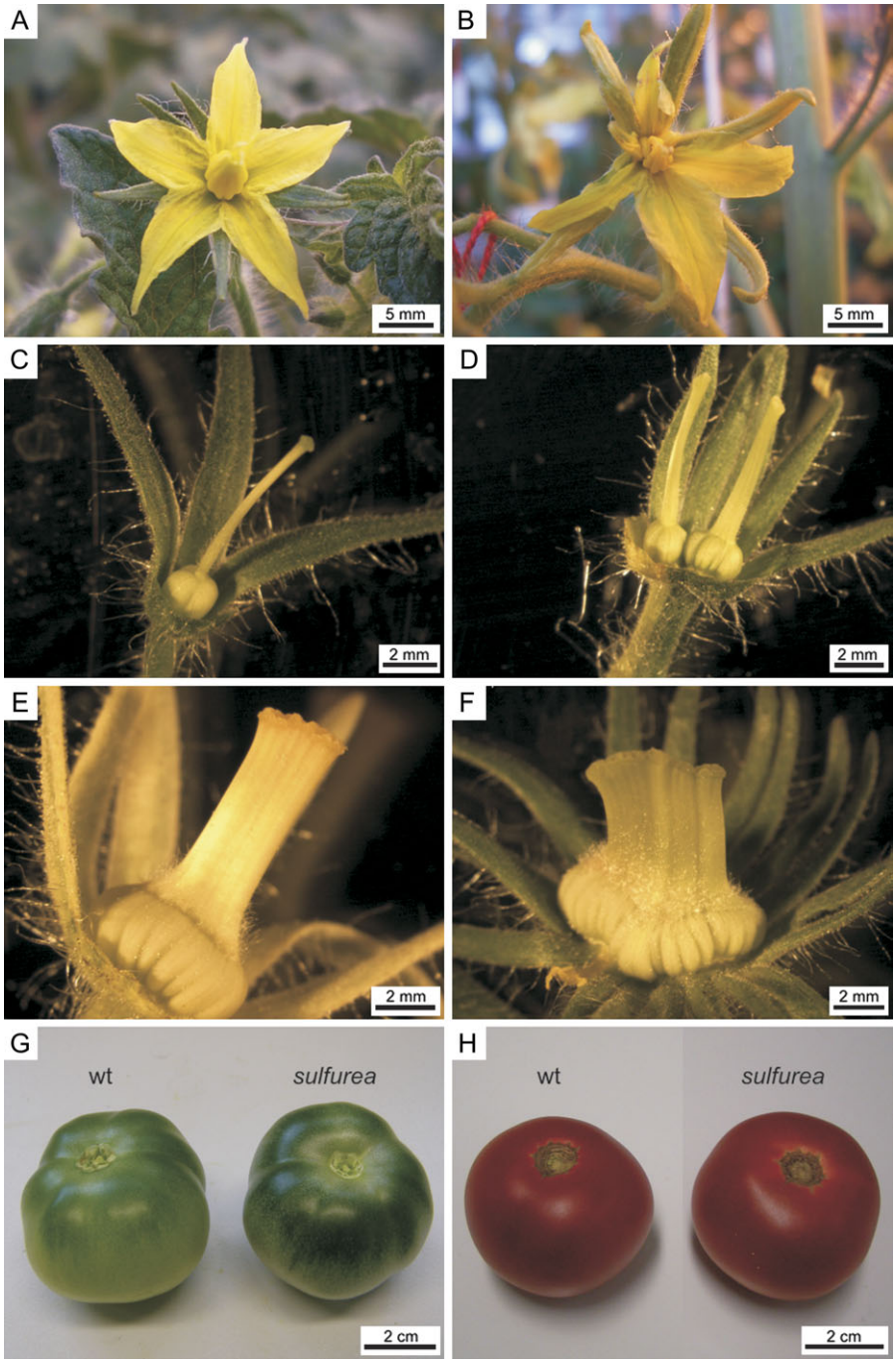

Fig. 4. Abnormal flower development, but normal fruit development in paramutated *sulfurea* plants. (A) Wild-type flower. (B) Flower of the *sulfurea* mutant displaying misshapen petals that are partially homeotically transformed into sepal-like organs. (C) Carpel in a wild-type flower. (D) Duplicated carpel in a flower of the *sulfurea* mutant. (E, F) Multiple fused carpels in flowers of the *sulfurea* mutant. (G, H) Normal fruit development in the *sulfurea* mutant. Mutant fruits were harvested from fully paramutated branches. Note the increased chlorophyll accumulation in the green *sulfurea* fruit.

Consequently, auxin synthesis in a given tissue or developmental condition is often largely dependent on only one of the two pathways. For example, rapidly growing tissues, like young seedlings and developing fruits are thought to rely on tryptophan-dependent auxin biosynthesis, whereas shoot meristems are believed to synthesize their auxin mainly via the tryptophan-independent pathway (Sitbon *et al*., 2000; Epstein *et al*., 2002). The first evidence for the occurrence of tryptophan-independent biosynthesis of IAA in tomato was provided by Epstein *et al*. (2002), who showed that, during fruit development, a switch between the two pathways occurs.

Several lines of evidence suggested tentatively that, in *sulfurea*, the tryptophan-dependent pathway is intact, while the tryptophan-independent pathway may be defective. First, inducibility of growth and development by auxin-containing agar blocks replacing the shoot apical meristem (SAM) indicated that *sulfurea* SAMs do not produce auxin (Fig. 3G, H). Second, wounding, which promoted growth and development of homozygous *sulfurea* seedlings (Fig. 3C, D), is known to induce tryptophan-dependent auxin biosynthesis (Sztein *et al*., 2002). Third, fruit development in the *sulfurea* mutant was unaffected (Fig. 4G, H) and strikingly, mutant fruits accumulated even more chlorophyll than wild-type fruits (Fig. 4G). This is consistent with auxin synthesis in developing fruits occurring via the tryptophan-dependent pathway (Srivastava and Handa, 2005; Woodward and Bartel, 2005).

To confirm that *sulfurea* is not defective in tryptophan-dependent auxin biosynthesis, intermediates of tryptophan-dependent IAA synthesis were applied in feeding experiments. The following substances were tested: tryptophan, tryptamine, indoleacetamide, and indole-3-pyruvate. In addition, indole, assumed to be a precursor in both pathways (Cohen *et al*., 2003) was tested. If the Trp-dependent pathway is not activated, then precursors of this pathway should not be able to restore the growth defect of *sulfurea*, while indole, which is also a precursor in the Trp-independent pathway cannot be converted to IAA if this pathway is defective in the mutant. In line with these considerations, none of these substances elicited a similar growth-promoting effect as the application of IAA or IBA (data not shown). However, some of the substances seemed to have slight positive effects, which were most pronounced for indole-3-pyruvate. This can most likely be explained with spontaneous decarboxylation of indole-3-pyruvate yielding indole-3-acetaldehyde which then can be oxidized to produce IAA.

Our feeding experiments with auxin-supplying agar blocks had tentatively indicated that auxin biosynthesis in the meristems rather than auxin transport is affected in the *sulfurea* mutant. To confirm this conclusion, a series of reciprocal grafting experiments was performed in which (i) wild-type scions were grafted onto *sulfurea* rootstocks, (ii) *sulfurea* scions were grafted onto wild-type rootstocks, (iii) *sulfurea* stem sections were inserted in between wild-type scions and rootstocks, and (iv) wild-type stem sections were inserted in between *sulfurea* scions and rootstocks. While wild-type scions grafted onto *sulfurea* rootstocks developed normally, the reciprocal grafting did not promote growth and development of *sulfurea* shoots, indicating that a wild-type rootstock cannot complement the suspected auxin deficiency in the *sulfurea* shoot meristems. Importantly, mutant stem sections inserted into wild-type plants survived, but did not affect plant growth or development, demonstrating that *sulfurea* stems support normal auxin transport. By contrast, wild-type stem sections inserted into mutant plants did not promote survival of the mutant, as expected.

To provide direct evidence for auxin deficiency in the *sulfurea* mutant, auxins were measured using gas chromatography-coupled mass spectrometry methods (GC-MS; Ludwig-Müller and Cohen, 2002; Jentschel *et al*., 2007). As a substantial fraction of auxin is present in conjugated form with either amino acids (amide-linked conjugates) or sugars (ester-linked conjugates; Woodward and Bartel, 2005; Seidel *et al*., 2006), the amounts of both free IAA and conjugated IAA were determined. To test for the suspected defect in tryptophan-independent auxin biosynthesis, both very young *sulfurea* seedlings (which predominantly synthesize their auxin via the tryptophan-dependent pathway) and older seedlings (in which a larger proportion of auxin comes from the tryptophan-independent pathway in the SAM) were included. While seedling age had no influence on the accumulation levels of either free or conjugated IAA in the wild type (data not shown), a strong effect was observed in the mutant. Very young seedlings (5–6 d after germination) showed a reduction in conjugated IAA, but not in free IAA (Fig. 5), indicating that they largely compensate the auxin deficiency by reducing the pool of conjugated (i.e. inactive) IAA, which may provide some buffer capacity. By contrast, older seedlings (10–11 d after germination) displayed a strong reduction in both free and conjugated IAA (Fig. 5), consistent with a lack of IAA synthesis in the SAM.

Isolation and phenotypic characterization of a suppressor mutant of sulfurea

Unlike most other epigenetic gene-silencing phenomena, gene inactivation by paramutation is not only somatically

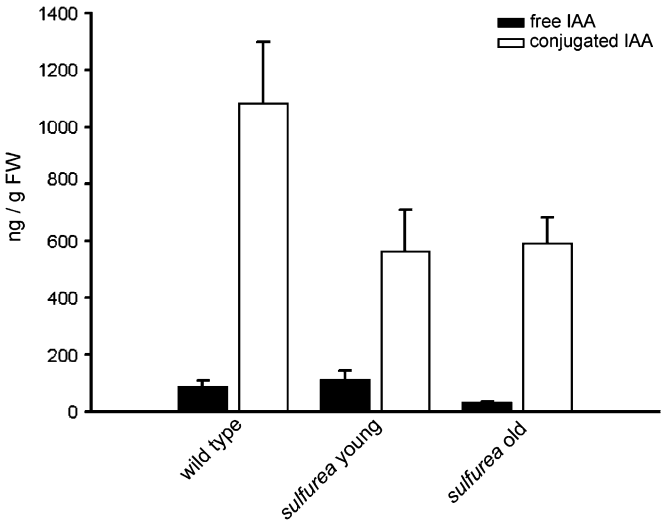

Fig. 5. Auxin deficiency in the *sulfurea* mutant. Accumulation of free and conjugated IAA was quantitated in wild-type seedlings, young mutant seedlings (5–6 d after germination) and older mutant seedlings (10–11 d after germination). IAA contents are given in ng g^{-1} fresh weight (FW). See text for details.

stable, but also heritable (Chandler *et al*., 2000; Chandler and Stam, 2004; Stam and Scheid, 2005; Bond and Finnegan, 2007; Chandler, 2007). To gain more insights into the mechanism of paramutation at the *sulfurea* locus and the involvement of *SULF* in auxin metabolism, a genetic screen for suppressors of the pale *sulfurea* phenotype was initiated. The establishment of an efficient shoot regeneration protocol for homozygous paramutated tissue (see the Materials and methods) allowed us to conduct such a suppressor screen by exposing sterile leaf explants to regeneration medium on a large scale. As tissue culture conditions themselves are mutagenic (a phenomenon also referred to as somaclonal variation), no additional mutagenesis treatment was done. In this screen, a mutant cell line was isolated that appeared as a dark green sector on a regenerating shoot (Fig. 6A). The sector was excised and uniformly green plantlets could be recovered by regeneration (Fig. 6B). Unlike the *sulfurea* mutant, these green plantlets were capable of growing in the absence of exogenously added auxin. However, they grew very slowly on synthetic medium (Fig. 6C, D) and were stunted, providing preliminary evidence for the mutant not being a revertant, but rather a suppressor of *sulf*.

When the putative suppressor mutant was grown in soil, the phenotype was even more striking. Mutant leaves displayed strong epinastic growth (Fig. 6E–H), a phenotype demonstrated previously to be induced by the presence of excess amounts of auxin (Kawano *et al*., 2003; Zhao, 2008). This pronounced phenotype unequivocally established that the mutant does not represent a revertant of *sulfurea*, but instead is a true suppressor mutant. It was therefore named *SOSU1* (*SUPPRESSOR OF SULFUREA 1*).

Floral development in *SOSU1* was strongly affected with approximately 90% of the flowers showing severe phenotypic aberrations, most of which represented homeotic transformations of floral organs (Fig. 6I, J). *SOSU1* flowers were male and female sterile making it necessary to propagate the mutant vegetatively.

The SUPPRESSOR OF SULFUREA *mutant overaccumulates auxin*

The *SOSU1* mutation suppressed the two most striking aspects of the *sulfurea* phenotype: the pigment deficiency and the arrested growth and development. In order to analyse the restoration of photoautotrophic growth in greater detail, photosynthetic electron transport was measured spectroscopically (Table 2). Although *SOSU1* plants had slightly lower chlorophyll contents than the wild type, the accumulation of the components of the photosynthetic electron transport chain was nearly identical (if calculated on a chlorophyll basis; Table 2). Most importantly, the severe photosystem I deficiency seen in *sulfurea* (Table 1) was fully restored to wild-type levels (Table 2).

The stunted growth of *SOSU1* plants, the severely disturbed floral development and the strongly epinastic leaf growth are most readily interpretable as an auxin overaccumulation phenotype. To test if the suppressor mutant indeed synthesizes excess amounts of auxin, the levels of free and conjugated IAA were measured by GC-MS. These analyses revealed that *SOSU1* plants accumulated greatly elevated levels of free IAA, whereas the amount of conjugated IAA was not significantly different from that in the wild type (Fig. 7). This demonstrates that the *SOSU1* mutant harbours a suppressor mutation that conditions enhanced accumulation of auxin.

Consistent with an auxin overproduction phenotype, decapitation of *SOSU1* plants did not result in the fast outgrowth of lateral meristems, as it did in the wild type. In addition to this substantial delay in lateral shoot formation, the number of lateral shoots developing per plant was strongly reduced (not shown).

Discussion

In this work, it is shown that the tomato *sulfurea* mutant, one of the classical mutants that led to the discovery of paramutation in plants, suffers from partial auxin auxotrophy. This auxotrophy seems to be restricted to a block in the tryptophan-independent pathway of auxin biosynthesis, while the tryptophan-dependent pathway is functional. As the two pathways are highly regulated in a tissue-specific and developmental stage-specific manner (Ljung *et al*., 2005; Woodward and Bartel, 2005; Zhao, 2008), it is unsurprising that the auxin auxotrophy in *sulfurea* is restricted to those tissues and developmental processes that are particularly dependent upon the tryptophan-independent pathway, such as the shoot meristems. Interestingly, induction of the tryptophan-dependent pathway (e.g. by wounding; Sztein *et al*., 2002) or the external application of IAA promoted the survival of *sulfurea* seedlings, but was insufficient to overcome the mutant phenotype entirely (Fig. 3). This is consistent with the idea that, although wounding and auxin supplementation help by providing additional auxin, these treatments cannot restore the proper patterns of tissue-specific synthesis and local distribution of auxin. Also, in line with a defect in the tryptophan-independent pathway, feeding of intermediates in the tryptophan-dependent pathway did not significantly alleviate the growth phenotype. In addition, to confirm the faithful provision of precursors to the tryptophan-dependent pathway, a set of preliminary metabolite-profiling experiments was conducted with wild-type and *sulfurea* seedlings. These analyses revealed only moderate quantitative changes in the levels of a few metabolites between the wild type and the mutant (B Ehlert and R Bock, unpublished results). Most importantly, all sugars and amino acids were readily

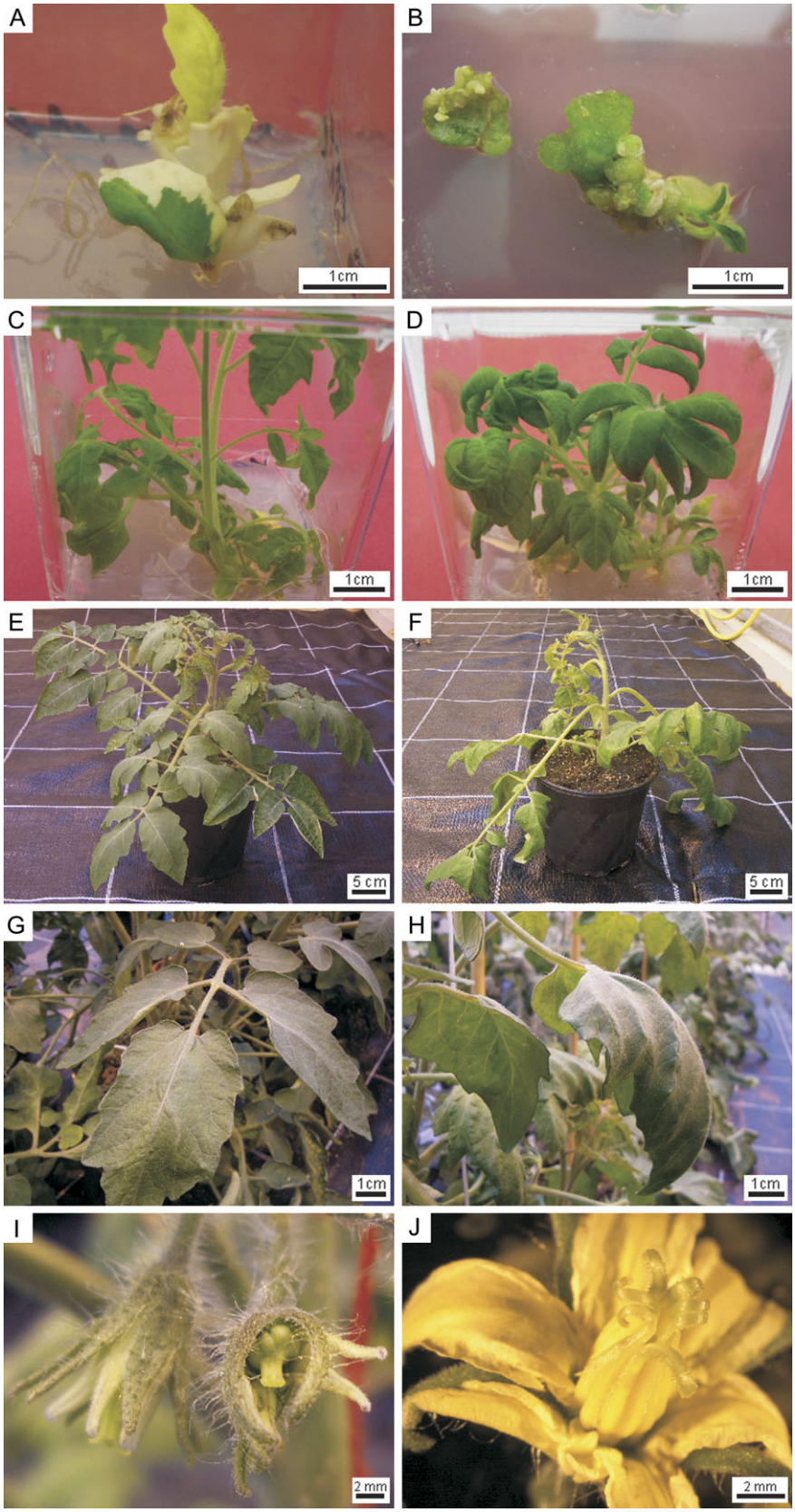

Fig. 6. Phenotype of the isolated *SUPPRESSOR OF SULFUREA 1* (*SOSU1*) mutant. (A) Isolation of *SOSU1* from a suppressor screen in tissue culture employing somaclonal variation. (B) Regeneration of *SOSU1* plants from the green leaf sector. (C, D) Phenotypic comparison of a wild-type

Table 2. *Spectroscopic analysis of photosynthesis in leaves of wild-type tomato plants and the isolated* SUPPRESSOR OF SULF *mutant* (SOSU1)

Plants were grown under standard greenhouse conditions. For abbreviations, see Table 1. Higher growth light intensities explain the elevated plastocyanin content and chlorophyll *a*:*b* ratio compared to the wild type in Table 1.

	Wild type	*SOSU1*
F_v/F_m	0.77±0.00	0.77±0.01
PC ($\Delta I/I \times 10^{-3}$ mg^{-1} Chl)	15.1±1.3	16.4±4.9
P_{700} ($\Delta I/I \times 10^{-3}$ mg^{-1} Chl)	47.1±6.3	46.4±4.0
PC/P_{700}	1.30±0.09	1.41±0.37
Chlorophyll (mg m^{-2})	800.9±167.9	561.2±102.9
Chlorophyll *a*/*b*	3.52±0.08	3.51±0.03

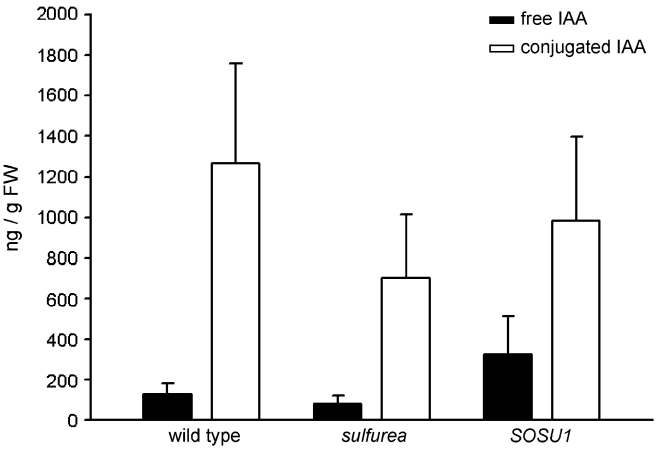

Fig. 7. Auxin overaccumulation in the *SOSU1* suppressor mutant. Accumulation of free and conjugated IAA was quantitated in wild-type plants, paramutated *sulfurea* plants, and *SOSU1* plants. IAA contents are given in ng g^{-1} fresh weight (FW). Since the *SOSU1* plants did not produce seeds, auxins could not be extracted from seedlings (as in Fig. 5), but were extracted from leaves of greenhouse-grown plants in this experiment.

detected in *sulfurea* seedlings, including the three aromatic amino acids (tryptophan, phenylalanine, and tyrosine) synthesized via the same biochemical pathway as auxin, the Shikimate pathway (Woodward and Bartel, 2005).

Auxin-deficient phenotypes can be caused by a variety of genetic disturbances, including mutations affecting auxin transport (Bennett *et al*., 2006; Leyser, 2006; Petrasek *et al*., 2006; Teale *et al*., 2006; Boutté *et al*., 2007), signal transduction (Paciorek and Friml, 2006; Szemenyei *et al*., 2008), biosynthesis (Cohen *et al*., 2003), degradation (Woodward and Bartel, 2005; Zhao, 2008) or the genetic regulation of any of these processes. Several lines of evidence suggest that, in the *sulfurea* mutant, auxin biosynthesis is affected. First, both our agar block experiments and our grafting experiments with transplanted stem sections indicate that auxin transport and signalling pathways are generally intact in *sulfurea*. Second, as discussed above, several aspects of the *sulfurea* phenotype suggest that the mutant suffers from a specific defect in tryptophan-independent auxin biosynthesis, while the tryptophan-dependent pathway appears to be intact. Third, IAA overproduction in the isolated suppressor mutant *SOSU1* and the typical symptoms of an auxin overaccumulation phenotype provide strong evidence for biosynthesis being affected rather than auxin transport or signalling.

An interesting and not yet fully resolved aspect of auxin metabolism is its subcellular compartmentalization. While tryptophan conversion to IAA via tryptamine and indole-3-acetaldehyde takes place in the cytosol, the localization of the tryptophan-independent pathway is still unknown. Circumstantial evidence suggests that tryptophan-independent IAA synthesis occurs in the plastid (Rapparini *et al*., 1999, 2002). If this is indeed the case and if there is a function of the plastid-produced IAA inside the chloroplast, this could explain the pigment-deficient phenotype of the *sulfurea* mutant. The pale-green phenotype is most probably a secondary consequence of the auxin deficiency, which is also supported by our finding that restoration of auxin synthesis in the *SOSU1* suppressor mutant also restored pigmentation to nearly wild-type levels. The photosystem I deficiency in the *sulfurea* mutant was investigated in greater detail by conducting transcript profiling analyses using the commercially available potato microarrays. Although these analyses did not provide a strong candidate gene for *SULF*, they revealed a set of genes that were strongly down-regulated in the *sulfurea* mutant. Interestingly, the most strongly down-regulated gene was *ATAB2* (B Ehlert and R Bock, unpublished results). ATAB2 is a conserved nuclear-encoded protein that is required for translation of the *psaB* mRNA (Dauvillée *et al*., 2003; Barneche *et al*., 2006). As *psaB* encodes a reaction centre protein of photosystem I, it seems conceivable that the drastic down-regulation of *ATAB2* conditions the PSI deficiency in *sulfurea*. It is therefore speculated that *ATAB2* is a target of *SULF* in that its expression may be under the control of one of the signal transduction pathways regulated by tryptophan-independent auxin biosynthesis. *ATAB2* cannot be identical with *SULF*, because (i) T-DNA knockout mutants of *ATAB2* grow normally under heterotrophic conditions (Barneche *et al*., 2006; B Ehlert and R Bock, unpublished results) and (ii) low-level *ATAB2* expression is still readily

plant and a *SOSU1* plant in sterile culture on sucrose-containing medium. Note that the suppressor mutant in (D) is approximately three times older than the wild-type plant in (C). (E, F) Phenotypic comparison of soil-grown wild-type (E) and *SOSU1* (F) plants. (G, H) Close-up of leaves of the plants shown in (E) and (F). (I, J) Examples of mutant phenotypes of *SOSU1* flowers showing multiple homeotic transformations of floral organs.

detectable in the *sulfurea* mutant both by microarray hybridization and RT-PCR (B Ehlert and R Bock, unpublished results).

At present, it is not yet possible to distinguish between the *SULF* gene encoding an enzyme directly involved in IAA synthesis or *SULF* encoding a regulator of the tryptophan-independent pathway (e.g. a specific transcription factor). The *SULF* gene was mapped to the centromeric heterochromatin of tomato chromosome 2 (Hagemann, 1993), a region that lacks any other genetic marker in close proximity (Tanksley *et al*., 1992). Although this makes the isolation of the *SULF* gene very difficult, the rapidly progressing tomato genome sequencing project (http://www.sgn.cornell.edu/) is expected to reveal candidate genes in the foreseeable future. This will hopefully help in solving some of the enigmas surrounding tryptophan-independent auxin biosynthesis and its physiological functions. Identification of the *SULF* gene will also allow the molecular mechanism of paramutation in tomato to be studied, for example, by searching for small RNAs derived from the locus and testing for changes in chromatin structure and/or DNA methylation patterns (Alleman *et al*., 2006; Hale *et al*., 2007; Henderson and Jacobsen, 2007).

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