Abstract
In order to define its possible involvement in production of stearic acid for wax biosynthesis, the presence of 3-ketoacyl acyl synthase II (KAS II) activity was investigated in different tissues of leek (Allium porrum L.) leaves. KAS II activity was identified in sheath and lamina epidermis, as well as in underlying parenchyma. In all three tissues, activity was inhibited by $50 \mu M$ cerulenin, and showed an absolute requirement for acyl-ACP substrates. More interestingly, the different tissues did not display similar KAS II substrate specificities. Parenchyma and lamina epidermis tissues presented typical KAS II activities, since C$_{18,0}$-ACP was the exclusive product. In contrast, in sheath epidermis, KAS II activity resulted in the synthesis of acyl-chains up to 22 carbons in length, suggesting the existence in this tissue of an unusual KAS II. This activity was sufficient to elongate all of the palmitoyl-ACP produced by the fatty acid synthase, suggesting that C$_{18,0}$ is the substrate of the microsomal elongases involved in wax biosynthesis.

Introduction
Plant aerial surfaces are covered by epicuticular waxes, complex mixtures of very long (C$_{20}$–C$_{34}$) fatty acids, alkanes, aldehydes, ketones and esters. All of these components are derived from the saturated very-long-chain fatty acids (VLCFAs). Leek epidermis, which is responsible for the entire wax synthesis in leek, has been for a long time a suitable system for both genetic and biochemical studies. Using this material, Liu and Post-Beittenmiller [1] discovered an epidermis-specific thioesterase with a strong specificity towards stearoyl-acyl carrier protein (ACP), which could be involved in wax biosynthesis. Fatty acids up to stearic acid (C$_{18,0}$) are synthesized as ACP thioesters in the stroma of the plastid by fatty acid synthase (FAS). Whereas in leaves most of the C$_{18,0}$-ACP is desaturated to C$_{18,1}$ before being hydrolysed and exported out of the plastid for further desaturation and/or elongation, in epidermal cells the specific stearoyl-ACP activity could provide the large quantity of C$_{18,0}$ saturated fatty acids required by the microsomal elongase involved in wax biosynthesis. However, as $\beta$-ketoacyl synthase (KAS) II was previously not detected in leek epidermis [2], it has been speculated that palmitic acid (C$_{16,0}$) rather than C$_{18,0}$ fatty acids may be exported out of the plastid to serve as substrates for these elongases. As Rhee et al. [3] recently determined the region of onset of wax accumulation along the leek leaf, we re-investigated the presence of KAS II activity in different parts of the leaf and attempted to define its possible involvement in the production of stearic acid for wax biosynthesis.
Enzymic assays

Routine enzyme tests were performed for 1 h in the presence of 1 mM dithiothreitol, 1 mM NADH, 1 mM NADPH, 1 mM (v/v) Triton X-100 and 2 μM ACP in a final volume of 50 μl of 100 mM Tris/HCl buffer, pH 8.0. For FAS assays, the reaction mixture contained in addition 5 μM acetyl-CoA + 10 μM [1-14C]malonyl-CoA. For KAS assays, the reaction mixture contained in addition 100 μM cerullenin and 0.3 μM palmitoyl-ACP + 10 μM [2-14C]malonyl-CoA or 1.5 μM [1-14C]acyl-ACPs (palmitoyl or stearoyl-ACP) + 10 μM malonyl-CoA. The reactions were stopped with 16 μl of 4 M KOH in 80% methanol and the lipids were saponified for 30 min at 70 °C. After acidification with 150 μl of 1 M HCl, the fatty acids were extracted, transmethylated and separated on KC18 reversed-phase TLC plates (Whatman), which were developed in acetonitrile/methanol/water (130: 70: 1, by vol.) and quantified on a PhosphoImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Results and discussion

In this study, leek leaves were divided into two parts, the leaf base below the ligule (sheath) and the top of the leaf (lamina), and the epidermis was peeled from both sides. Protein extracts were prepared from the sheath and lamina epidermis as well as from the underlying parenchyma and used to measure FAS and KAS II activities. Both activities were 2-fold higher in the sheath epidermis and parenchyma compared with the lamina epidermis. KAS II activity was very low and sometimes barely measurable in lamina epidermis, whereas it was repetitively high in the two other tissues. These different KAS II activities along the leaf and the difficulties encountered in obtaining systematically very active fractions can explain why previous studies have failed to measure KAS II activity in epidermis [2]. In sheath epidermis, KAS II activity was at least twice as high as FAS activity, suggesting that C18-ACP fatty acids could be the substrate of the microsomal elongases involved in wax biosynthesis.

The presence of a KAS II-type activity was confirmed by investigating the effect of increasing concentrations of cerullenin and testing different fatty acid primers. In all three tissues, KAS II activity was only 50%, inhibited by 100 μM cerullenin whereas FAS activity (primarily driven by KAS I) was very sensitive to cerullenin (10 μM leading to 90% inhibition). No KAS II activity was detected in the absence of primer and in the presence of palmitic acid or palmitoyl-CoA, very low activities were detected. The relative sensitivity to cerullenin and the absolute requirement for acyl-ACP substrates strongly supported the presence of a KAS II-type activity.

When the labelled products were analysed on reversed-phase TLC (Figure 1), the different tissues did not display similar KAS II substrate specificities. In parenchyma and lamina epidermis, FAS activity mainly led to the synthesis of C16,0 (30-40%) and C18,0 (40-50%). These tissues presented typical KAS II activities because C16,0-ACP was the exclusive product. In contrast, KAS II activity in sheath epidermis resulted in the synthesis of acyl-chains up to 22 carbons in length, suggesting the existence in this tissue of an unusual KAS II enzyme that is able to accept both C18,0- and C20,0-ACPs as substrates. Under the different conditions used to measure FAS and KAS II activities using sheath epidermis protein extracts, C20,0-ACP and C22,0-ACP represented respectively up to 21 and 6% of the products (Figure 1).

The synthesis of acyl-ACPs with more than 18 carbon atoms was of great interest since lipid metabolism in plastids has previously been reported to be limited to acyl chains of up to C18,0. In fact, the presence of these VLCFAs has been reported by Shimakata and Stumpf [4], who observed the formation of C20,0 fatty acids when studying KAS activities from spinach leaf in vitro as well as in reconstitution experiments using purified KAS I and KAS II enzymes. The presence of VLCFAs in our assays was not due to the presence of microsomal contaminants, as our enzyme sources did not contain any elongation activity. In fact, the only primer leading to label incorporation in elongation assays was stearoyl-ACP, again supporting the existence in sheath epidermis of a particular KAS II-type enzyme. The presence of such KAS II activity only in sheath epidermis could be related to wax biosynthesis because Rhee et al. [5] have shown that this region of the epidermis, i.e. the base of the leaf, was responsible for wax deposition. In this region, FAS and KAS II activities lead to the synthesis of C18,0-ACP, which is hydrolysed by the epidermis-specific stearoyl-ACP, so that C18,0 is exported out of the plastid to serve as a substrate for the microsomal elongase. In such a pathway for wax biosynthesis, KAS II’s ability to elongate C16,0- and C20,0-ACPs found in sheath epidermis is becoming futile, as the presence of a normal KAS II activity would be sufficient.
Nevertheless, a situation similar to that found in *Cuphea* seeds, where the accumulation of medium-chain fatty acids relies on the synergistic effect of thioesterase and condensing enzymes specific for medium-chain fatty acids [5,6], could occur in epidermis.

The results presented in this study definitively demonstrate the existence of KAS II activity in epidermal tissues, supporting the hypothesis that stearic acid is the substrate of the microsomal elongases involved in wax production. Moreover, the ability of the sheath epidermis KAS II enzyme to accept C$_{18,9}$- and C$_{20,0}$-ACPs as substrates suggests the existence in this tissue of a particulate KAS II enzyme that is probably related to wax biosynthesis.

**Figure 1**

*Product analysis of FAS and KAS II activities from parenchyma, lamina epidermis and sheath epidermis protein extracts*

Proteins (25 µg) were incubated for 1 h in the presence of different substrates and the products were extracted, transmethylated and separated as reported in the Enzymic assays section.
Modulation of fatty acid biosynthesis by antisense β-keto reductase expression

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Abstract
Plants with an antisense construct to β-ketoacyl-acyl carrier protein reductase have reduced seed and leaf fatty acid contents and show distinct phenotypes, including leaf curl, stunted growth and wrinkled seeds. The carbon resource allocation is altered in both of these tissues. In leaves there is an inability to synthesize transient starch and in seeds embryogenesis is foreshortened and storage proteins accumulate prematurely.

Introduction
We have used an antisense approach to down-regulate β-ketoacyl-acyl carrier protein reductase (KR), a component of fatty acid synthase. The aims of this programme are to investigate what effects a reduction in fatty acid synthesis has upon seed and leaf metabolism and in particular the effects on resource allocation between lipids, proteins and carbohydrates.

Two different promoters were used to drive the antisense constructs to ensure activity in both of the target tissues: the cauliflower mosaic virus 35 S (CaMV) promoter shows activity in a range of tissues including leaf, and the acyl carrier protein (ACP) promoter used was known to be active in embryos.

Antisense plants
The plants used in this study were all homozygous for a single antisense insert: each line contains a novel insertional event and the phenotypes displayed are stable throughout several generations. The leaves of many antisense lines are curled and are slightly smaller at maturity than those of the Westar parent. They also show differences in development, including altered time to bolting, prolonged flowering and reduced numbers of seeds set. Severely affected lines show a high rate of pre-germination, although this does not compromise full germination in a proportion of the seeds. Seeds are also wrinkled and altered in pigmentation.

KR activity measurements
We have used a novel radiochemical assay to determine the levels of KR activity in individual leaves of Westar and antisense plants. The CaMV-driven antisense line CaMV4 shows an approx. 35% reduction in activity and a similar reduction in fatty acid levels in 4-day-old leaves, which is when the maximum activity is detected in Westar.

In seed material the levels of KR activity are sufficiently high to be measured spectrophotometrically, following the acetoacetyl-CoA-stimulated oxidation of NADPH. The activity measured in Westar during development is typical of that seen for fatty acid synthase components, rising steadily throughout early embryogenesis to reach a plateau at 29–35 days after flowering (DAF). Typically, ACP-promoter antisense lines, such as ACP3, show a significant reduction in KR activity in early embryos, but the activity recovers to wild-type levels in older tissue. Overall, the ACP3 line exhibits a reduction of approx. 50% in KR activity between days 23 and 35 (the period of maximum fatty acid synthesis), which results in a