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RNA*i* suppression of the morphine biosynthetic gene *salAT* and evidence of association of pathway enzymes

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ABSTRACT

Papaver somniferum L. was transformed with an RNA*i* construct designed to reduce transcript levels of the gene encoding the morphine biosynthetic enzyme, salutaridinol 7-*O*-acetyltransferase (SalAT). RNA *interference* of *salAT* led to accumulation of the intermediate compounds, salutaridine and salutaridinol, in a ratio ranging from 2:1 to 56:1. Along the morphine biosynthetic pathway, salutaridine is stereospecifically reduced by salutaridine reductase (SalR) to salutaridinol, which is subsequently acetylated by SalAT. SalAT transcript was shown by quantitative PCR to be diminished, while *salR* transcript levels remained unaffected. Yeast two-hybrid and co-immunoprecipitation analyses indicated an interaction between SalR and SalAT, which suggested the occurrence of an enzyme complex and provided an explanation for the unexpected accumulation of salutaridine. Decreased concentrations of thebaine and codeine in latex were also observed, while the morphine levels remained constant compared to concentrations found in untransformed control plants.

1. Introduction

Opium poppy (*Papaver somniferum* L.) is one of the oldest known medicinal plants. Laticifers, which pervade the entire plant, contain a plethora of different alkaloids. Some of the alkaloids of economic relevance in *P. somniferum* are the benzylisoquinoline papaverine, and the morphinans thebaine **11**, codeine (14) and morphine **16** (Fig. 1). Papaverine is used as a vasodilator. Thebaine (**11**), although not used therapeutically, is converted into medically useful derivatives including the analgesics oxycodone and buprenorphine (Lednicer and Mitscher, 1990). Codeine (14) is a potent antitussive and morphine (16) is one of the most effective analgesics known. In addition, the biosynthetic intermediate (*S*)-reticuline (3) was found to stimulate hair growth (Nakaoji et al., 1997) and showed potent central nervous system depressant effects (Morais et al., 1998). Chemical synthesis of most of these alkaloids is possible, but not economically feasible (Rice, 1980). The alternative of cell culture production is not possible in all cases. Since alkaloid accumulation is tissue specific (Weid et al., 2004), only selected alkaloids can be produced and accumulated in cell cultures. According to Kutchan et al. (1983), undifferentiated callus of opium poppy produced benzo[*c*]phenanthridines such as sanguinarine, but not the medically important morphinans.

Although the enzymology of morphine (**16**) biosynthesis has almost been unravelled (Kutchan, 1998; Kutchan et al., 2007; Fig. 1), the regulation of the alkaloid biosynthetic processes is largely unknown. Silencing and over-expressing genes encoding enzymes of specific biosynthetic steps can reveal correlations that provide insight into pathway control points. Detailed knowledge of the pathway, including its regulatory properties, offers the possibility of predictive metabolic engineering of alkaloid biosynthesis (Dixon and Steele, 1999; Verpoorte et al., 2000; Hughes and Shanks, 2002).

It is only recently that the production of metabolically engineered opium poppy plants has been reported. Allen and coworkers (Allen et al., 2004) blocked codeinone reductase (COR) (Fig. 1) (Unterlinner et al., 1999) using the RNA*i* technique. The plants accumulated reticuline (3) and several methylated derivatives of reticuline at the expense of morphine (**16**), codeine (14), thebaine (11) and oripavine (**13**). These results were interpreted as indicative of a feedback mechanism that inhibited enzymes early in the morphine-specific biosynthetic pathway (Allen et al., 2004). The transformation of opium poppy with antisense berberine bridge enzyme (*antisense-bbe*) altered the ratio of alkaloids in latex, but not in roots, indicating a regulatory role for enzymes other than BBE (Fig. 1) in the control of sanguinarine biosynthesis (Frick

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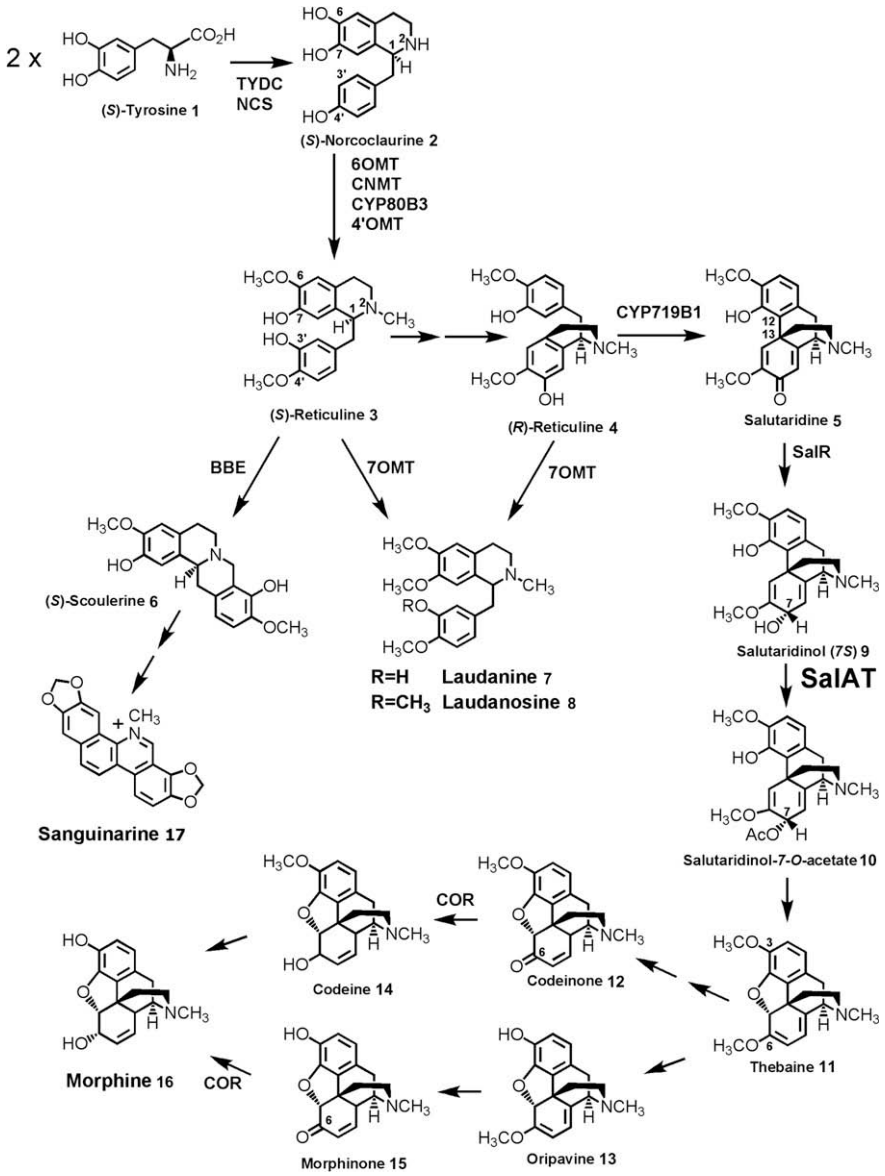

Fig. 1. Biosynthesis of alkaloids in opium poppy. Schematic representation of the biosynthesis of the benzylisoquinoline, benzo[*c*]phenanthridine and morphinan alkaloids in *P. somniferum* including enzymes for which cDNAs have been cloned. Enzyme abbreviations: NCS, (*R*,*S*)-norcoclaurine synthase; TYDC, tyrosine/dopa decarboxylase; 6OMT, (*R*,*S*)-norcoclaurine 6-*O*-methyltransferase; CNMT, (*S*)-coclaurine *N*-methyltransferase; CYP80B3, (*S*)-*N*-methylcoclaurine 3′-hydroxylase; 4′OMT, (*R*,*S*)-3′-hydroxy-*N*-methylcoclaurine 4′-*O*-methyltransferase; BBE, berberine bridge enzyme; 7OMT, (*R*,*S*)-reticuline 7-*O*-methyltransferase; CYP719B1, salutaridine synthase; SalR, salutaridine reductase; SalAT, salutaridinol 7-*O*-acetyltransferase; COR, codeinone reductase. SalAT, used for silencing in this study, is indicated in large print.

et al., 2004; Kutchan et al., 2007). Finally, opium poppy hypocotyls were transformed with a sense and antisense construct of (*S*)-*N*-methylcoclaurine 3′-hydroxylase (CYP80B3) (Frick et al., 2007). This cytochrome P-450-dependent enzyme hydroxylates (*S*)-*N*-methylcoclaurine to form (*S*)-3′-hydroxy-*N*-methylcoclaurine (Fig. 1) (Pauli and Kutchan, 1998; Huang and Kutchan, 2000). Transgenic plants overexpressing *cyp80b3* cDNA showed a 450% increase in the amount of total alkaloid in latex. This enhancement occurred either without changing the ratio of the individual alkaloids, or together with an overall increase in the ratio of morphine. In turn, *antisense-cyp80b3* cDNA expressed in opium poppy caused an 84% reduction of total alkaloid in latex. This decline was accompanied with an alteration in the ratio of individual alkaloids. The relative concentration of morphine (**16**), codeine (14), reticuline, laudanine (7) and laudanosine (8) was increased, whereas thebaine (11) and oripavine (13) were decreased in all *antisense*-plants (Frick et al., 2007).

In this report, we present the analysis of transgenic *P. somniferum* plants in which *salAT* transcript (Fig. 1) (Grothe et al., 2001) has been reduced using RNA*i* technology. Along the morphine-specific biosynthetic pathway in *P. somniferum*, salutaridine (5) is first reduced at the keto group at C-7 by salutaridine reductase (SalR) (Gerardy and Zenk, 1993; Ziegler et al., 2006) to form 7(*S*)-salutaridinol (9), which is subsequently acetylated to salutaridinol-7-*O*-acetate (10) by salutaridinol 7-*O*-acetyltransferase (SalAT). Salutaridinol-7-*O*-acetate (10) is a chemically unstable intermediate that eliminates acetate to form the first pentacyclic morphinan alkaloid of pharmaceutical significance, thebaine (**11**). A reduction in *salAT* transcript and presumably in SalAT enzyme levels in transgenic plants could yield an alkaloid phenotype that would provide insight into the regulation of a specific portion of the morphine biosynthetic pathway. Concurrent to this work, a report was published by Allen et al. (2008) on the over-expression and RNA*i* suppression of *salAT* in opium poppy.

2. Results

Explants of *P. somniferum* were transformed with a *salAT* RNAi vector using an *Agrobacterium*-mediated transformation protocol (Larkin et al., 1999; Chitty et al., 2003). This vector also contained the neomycin phosphotransferase II (nptII) gene for selection of transgenic plants. Using that procedure, 16 phenotypically normal transgenic T_0 plants were regenerated from 125 putatively transformed cell culture lines. Explants were first cultivated in a growth chamber and then transferred to the greenhouse, where they were grown to maturity. Finally, the plants were harvested for analysis. Growth chamber conditions (Frick et al., 2007) and greenhouse conditions (Frick et al., 2005) were described recently.

Eighteen wild type plants of the elite narcotic cultivar *P. somniferum* C058-34 have been analyzed by HPLC (Fig. 2) and LC/ESI-TOFMS (data not shown) (Frick et al., 2005). Although total alkaloid amounts were highly variable (670 ± 250 μg alkaloid/100 μg soluble protein) (Fig. 2C), the overall alkaloid pattern remained constant in all examined plants (Fig. 2B). The main alkaloids observed were thebaine (**11**) (230 ± 100 μg alkaloid/100 μg soluble protein), morphine (16) (280 ± 90 μg alkaloid/100 μg soluble protein) and codeine (14) (90 ± 40 μg alkaloid/100 μg soluble protein) (Fig. 2C). Laudanine (7) (14 ± 6 μg alkaloid/100 μg soluble protein), laudanosine (8) (9 ± 3 μg alkaloid/100 μg soluble protein), (*S*)-scoulerine (6) (11 ± 6 μg alkaloid/100 μg soluble protein), reticuline (4 ± 2 μg alkaloid/100 μg soluble protein) and oripavine (13) (25 ± 11 μg alkaloid/100 μg soluble protein) were also resolved using this method (Fig. 2C). The alkaloids (*S*)-reticuline (3), (*R*)-reticuline (4) and 1,2-dehydroreticuline had the same retention time in chromatograms and could not be separated using the method described. For this reason, the concentration presented herein is likely a mixture of both enantiomers of reticuline 3/4 and of 1,2-dehydroreticuline. They are collectively denoted as "reticuline".

HPLC analysis of the 16 transgenic T_0 plants (Supplementary Fig. S1A) established the occurrence of the intermediate compounds salutaridine (5) and salutaridinol (9) (Fig. 1), which were not observed in wild type plants (Fig. 2). The values for salutaridine (5) varied from 0% to 21.7%, the values for salutaridinol (9) from 0.1% to 3.4% (Supplementary Fig. S1A). Two transgenic plants, 77a38 (Fig. 3; Supplementary Fig. S1A) and 77a30 (Supplementary Fig. S1A), showed especially high amounts of salutaridine (5) (21.7% and 19.5%) and salutaridinol (9) (3.4% and 2.5%). Higher quantities of thebaine (11) were observed in almost all T_0 plants. An exception to this are plants regenerated from cell lines 77a22 and 77a38 (Supplementary Fig. S1A). The latex of the plant regenerated from cell line 99a15 I contained an unusually high amount of (*S*)-scoulerine (6) (14.5%) in comparison to wild type, and also to the other transgenic T_0 plants (Supplementary Fig. S1A).

The T_1 generation was analysed for transgenic plants. Neither the elevated (*S*)-scoulerine (6) level in plants derived from cell line 99a15 I nor the higher thebaine (11) amount in the various T_0 plants was heritable (data not shown). Interestingly, however, all transgenic T_1 plants accumulated the intermediates salutaridine (5) and salutaridinol (9) (data not shown). In Fig. 3, an HPLC analysis of two representative transgenic T_0 plants and their T_1 siblings are shown. The alkaloid concentrations per 100 μg soluble protein determined were for plant 77a38 478 μg morphine (16), 138 μg codeine (14), 212 μg thebaine (11), 34 μg oripavine (13), 24 μg reticuline ((*S*)-reticuline (3), (*R*)-reticuline (4) and 1,2-dehydroreticuline), 14 μg (*S*)-scoulerine (6), 36 μg laudanine (7) and 17 μg laudanosine (8). The plant 99a15 II contained 199 μg morphine (16), 395 μg codeine (14), 4453 μg thebaine (11), 93 μg oripavine (13), 86 μg reticuline ((*S*)-reticuline (3), (*R*)-reticuline (4) and 1,2-dehydroreticuline), 166 μg (*S*)-scoulerine (6), 125 μg laudanine (7) and 46 μg laudanosine (8). Additionally, the alkaloids salutaridinol (9) and salutaridine (5) were detected. In plant 99a15 II (Fig. 3B and D), only a low amount of salutaridine (5) (T_0 7.2%, T_1 1–14% of total alkaloids) and salutaridinol (9) (T_0 1.2%, T_1 0.1–2.1% of total alkaloids) accumulated. In contrast, the plant 77a38 (Fig. 3A), displayed a strong salutaridine/salutaridinol phenotype (21.7% salutaridine (5), 3.4% salutaridinol (9) in T_0). The T_1 progeny (Fig. 3C) contained 7.2–69.3% salutaridine (5) and 1.9–4.8% salutaridinol (9) compared to total alkaloids.

The progeny of line 77a38 (Fig. 3C), accumulated thebaine (11), codeine (14), oripavine (13) and morphine (16). The amount of thebaine (11), however, was dramatically reduced in most plants. In contrast, if the average amount of morphine (16) in these T_1 plants (250 μg alkaloid/100 μg soluble protein) is compared with wild type (280 μg alkaloid/100 μg soluble protein), the results are quite similar. A comparison of the amounts of salutaridine (5) with the quantities of thebaine (11), codeine (14), oripavine (13) and mor-

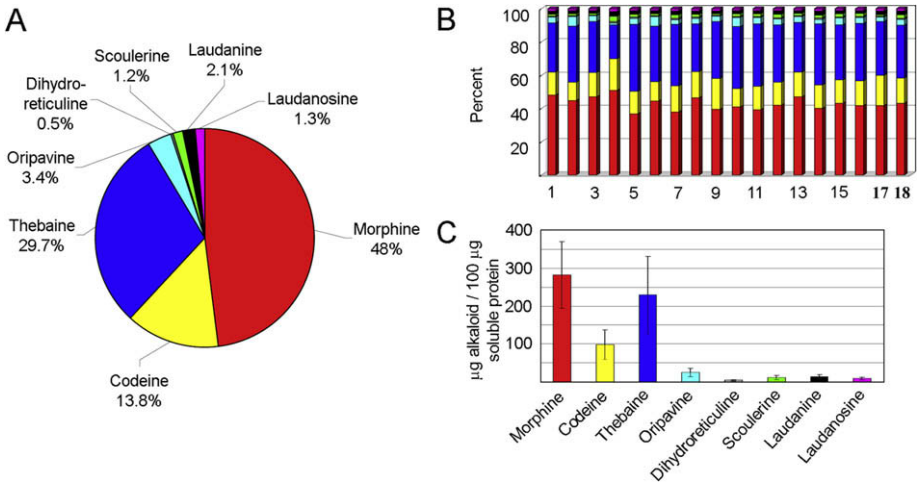

Fig. 2. HPLC analysis of latex alkaloids collected from 18 plants of *P. somniferum* cultivar C058-34 (wild type). (A) Alkaloids in latex were calculated as μg alkaloid/100 μg soluble protein, then normalized to 100% to produce the individual divisions within pie graphs, representing relative ratios of the latex alkaloids. (B) Relative amounts of latex alkaloids of the 18 wild type individuals showing the homogeneous alkaloid pattern shared by all plants. Color code of figures is identical to A. (C) Averaged total amounts of latex alkaloids calculated as μg alkaloid/100 μg soluble protein with standard deviations. The alkaloids (*S*)-reticuline (**3**), (*R*)-reticuline (**4**) and 1,2-dehydroreticuline are collectively denoted as reticuline.

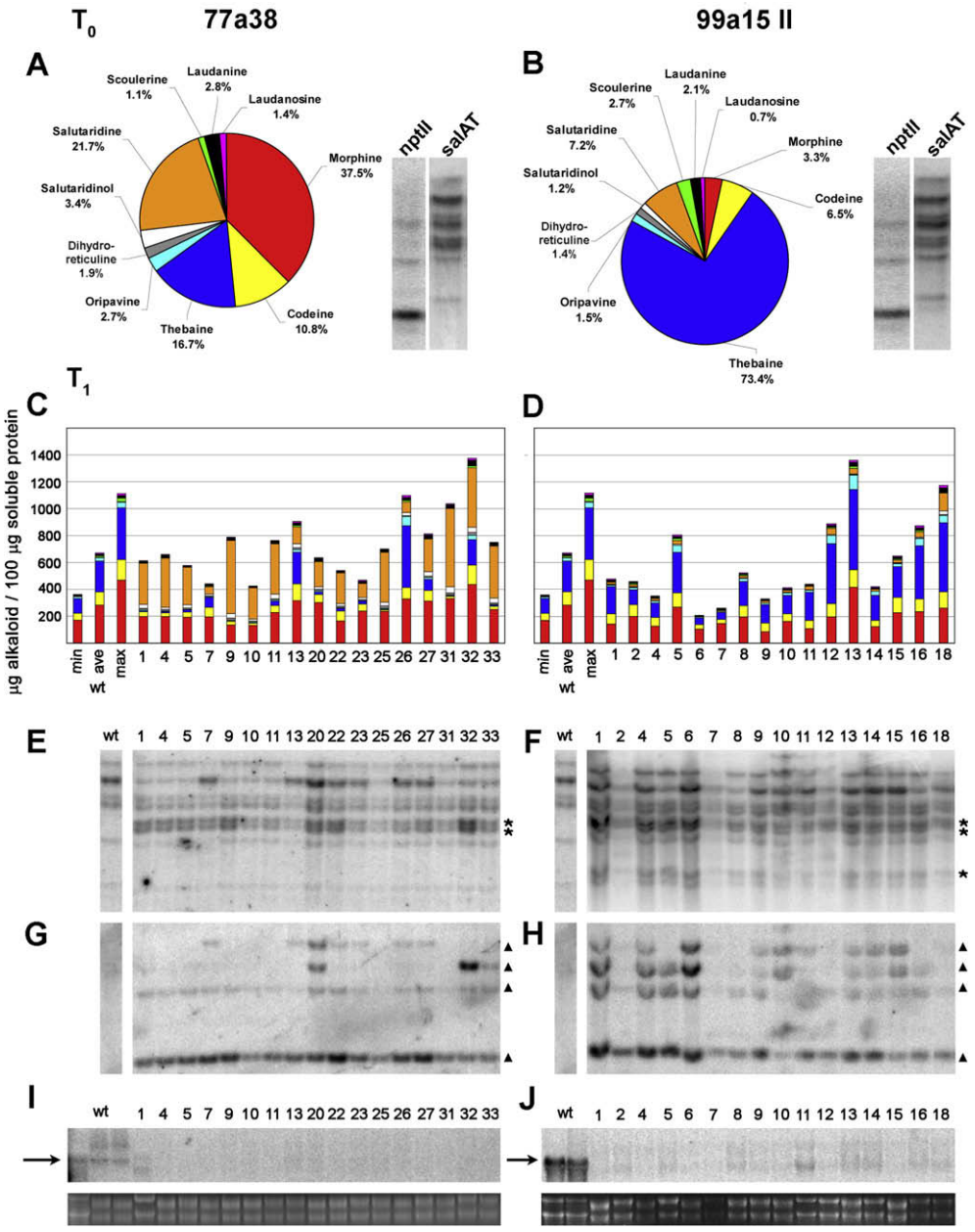

Fig. 3. Analysis of T_0 and T_1 generation of two transgenic plants that contained *salAT* hpRNA. (A + B) Alkaloid pattern and Southern blot analysis of the transgenic T_0 plants 77a38 (A) and 99a15 II (B). (C–J) Analysis of the progeny of the cell lines 77a38 (C, E, G, I) and 99a15 II (D, F, H, J) including HPLC analysis of latex (C + D), Southern blot hybridization labeled with *salAT* probe (E + F) or the selective marker nptII (G + H) and Northern blot of stem RNA hybridized to labeled *salAT* probe including loading control beneath the blots (I + J). Color code of (C) and (D) are identical to (A) and (B). The alkaloids (*S*)-reticuline (**3**), (*R*)-reticuline (**4**) and 1,2-dehydroreticuline are collectively denoted as reticuline. The Southern blots indicate that the regenerated plants are indeed transgenic. Panels E and F show new *salAT* bands (denoted by stars) that are not present in wild type genomic DNA (wt). Panels G and H show nptII bands (denoted by filled triangles) that are completely absent from wt genomic DNA. 77a38 and 99a15 II were not independent transgenics as indicated by the Southern blots in panels A and B, but diverge strongly with respect to SalAT transcript and alkaloid pattern (panels A–D). In the T1 generation of 77a38 and 99a15 II, the siblings split, but the salutaridinol/salutaridine phenotype remains. Northern analysis (panels I and J) demonstrate that SalAT transcript (denoted by an arrow) can be detected in wt plants, but is reduced below the limit of detection in transgenic plants.

phine (16) (linear correlation) yielded an indirect relation with a regression coefficient of $r^2 = 0.998$ (slope $a = -1.005$) (Table 1). This implies that the accumulation of salutaridine (5) is directly proportional to the decrease of alkaloids synthesized in the enzymatic steps *after* SalAT. Table 1 shows linear correlations for the comparison of different alkaloids. As shown by these data, thebaine (11) and codeine (14) have the highest impact on this correlation. The relationship between salutaridine (5) and both oripavine (13) and morphine (16) is much weaker.

To confirm these findings, the relationship of the main alkaloids thebaine (**11**), codeine (14) and morphine (16) with each other were calculated and compared to the wild type data (Table 2). In wild type plants, the quantities of these alkaloids were directly proportional to each other (r^2 0.762–0.8866) (Table 2). In the progeny of 77a38, the proportionality nearly disappeared (r^2 0.0034–0.2602) (Table 2).

The total amount of alkaloids was elevated in the T_0 generation due to the high level of thebaine that was typically observed (Supplementary Fig. S1A). In the T_1 generation, the alkaloid quantity (420–1380 µg alkaloid/100 µg soluble protein) (Fig. 3C and D) was similar to wild type (360–1110 µg alkaloid/100 µg soluble protein).

Table 1
Calculated linear regression coefficients, r^2, showing the relationships between different alkaloids of morphine (16) biosynthesis in the 17 progeny of transgenic plant 77a38. The slopes were negative in all cases.

r^2	Thebaine (11)	Codeine (14)	Oripavine (13)	Morphine (16)	Σ(T,C)	Σ(T,C,M,O)
Salutaridine	0.6596	0.6109	0.368	0.3631	0.822	0.998
Salutaridinol	0.376	0.1538	0.3652	0.0342	0.385	0.3614
Σ(S,Sol)	0.6689	0.6053	0.3814	0.3533	0.8283	0.9981

The individual alkaloid analyses from 17 progeny plants of the transgenic plant 77a38 were used to calculate the regression coefficients. T, thebaine (**11**); C, codeine (**14**); M, morphine (16); O, oripavine (13); S, salutaridine (5); Sol, salutaridinol (9).

Table 2
Calculated linear regression coefficients, r^2, comparing the relationships between the main alkaloids morphine, thebaine and codeine in wild type C058-34 and in the 17 progeny of plant 77a38. The slopes were positive in all cases.

	Morphine	Thebaine	Codeine
Morphine (**16**)		0.8866/0.0034[a]	0.8134/0.2602
Thebaine (**11**)			0.726/0.2255
Codeine (**14**)			

[a] Data presented as r^2 in wild type/r^2 in 77a38 progeny. The individual alkaloid analyses from 17 progeny plants of the transgenic plant 77a38 and eighteen wild type plants were used to calculate the regression coefficients.

The proof of transgenicity was carried out by PCR (data not shown) and Southern blot analysis for all T_0 and T_1 plants. For each test, not only the *salAT* RNA*i* construct, but also the selective nptII marker gene was detected. In Fig. 3A, B and E through H, Southern analysis of two representative transgenic T_0 plants 77a38 (Fig. 3A) and 99a15 II (Fig. 3B) and their T_1 siblings (Fig. 3E–H) are shown. Two to four copies of the transgene were integrated into the plant genome. No association between the amount of salutaridine (5) in latex (Fig. 3A and B; Supplementary Fig. S1A) and the number of transgenic loci (Fig. 3A and B; Supplementary Fig. S1B) was obvious in the T_0 generation. Of all 16 transgenic T_0 plants, only three appeared to be independent (Supplementary Fig. S1B). The segregation indicated that the multiple transgenes were inserted at different loci and inherited as a multiple linkage block. This phenomenon could be observed by labeling the Southern blot with nptII probe (Fig. 3G and H), but not with *salAT* probe (Fig. 3E and F).

The transcript levels of *salAT* messenger RNA of transgenic T_0 and T_1 poppy lines were determined and compared to wild type. A Real Time RT-PCR assay for leaf mRNA was developed for this purpose. A relative comparison of *salAT* message to the reference *tubulin* was used. T_0 plant 99a15 II had 15% and T_0 plant 77a38 only 8% *salAT* message compared to wild type (Supplementary Fig. S1C). In Fig. 4, the transcript levels for the T_1 siblings of the T_0 plants 77a38 (between 1% and 18%, Fig. 4A) and 99a15 II (between 9% and 90%, Fig. 4B) are shown. Real Time RT-PCR results were corroborated for the T_1 generation by Northern blot analysis with stem RNA. All RNA gel blots examined displayed reduced signals from T_1 plant RNA compared to wild type RNA when hybridized with radioactively-labeled *salAT* cDNA (Fig. 3I and J).

To test whether the reduction in *salAT* transcript resulted in a concomitant reduction in SalAT protein, extracts prepared from leaf material was subjected to protein gel blot analysis using antibodies raised against recombinant protein (Weid et al., 2004). SalAT was detected in wild type opium poppy, but the reduced levels of SalAT accumulated in SalAT RNA*i* plants (T_1 progeny of cell lines 77a38 (Fig. 5A) and 99a15 II (Fig. 5B) were below the limit of detection.

To study the influence of RNA *interference* of *salAT* on other genes of morphine (16) biosynthesis, the transcript levels of *salR* and *cor1* were determined for the T_1 progeny of the T_0 plants 77a38 (Fig. 4C and E) and 99a15 II (Fig. 4D and F). SalR stereospecifically reduces salutaridine (5) to salutaridinol (9), which then serves as substrate for the next enzyme in the pathway, SalAT. Since the amount of salutaridine (5), rather than salutaridinol (9), was significantly increased, it was of interest to determine whether *salR* transcript levels had been influenced by the reduction in *salAT* transcript. Real Time RT-PCR showed a high variance in *salR* transcript in transgenic plants with respect to the wild type average (Fig. 4C and D). A direct influence of a reduction in *salAT* transcript level on the transcription of *salR* was not evident. Since *salAT* RNA*i* plants were able to synthesize morphine (16) to levels comparable to that found in wild type plants, the effect of reduced *salAT* expression on *cor1* transcript level was determined. Real Time RT-PCR demonstrated that there was no feed-forward transcriptional activation of *cor1* (Fig. 4E and F).

The CytoTrap® yeast two-hybrid system (Stratagene) was used for detection of cytosolic protein–protein interactions *in vivo* between SalAT and SalR or SalAT and 6OMT (Fig. 6). SalAT (the bait) was cloned into the pSOS vector generating a fusion of the human Ras guanylnucleotide exchange protein SOS (hSOS) and SalAT. SalR or 6OMT (the targets) was cloned into the pMyr vector and expressed as fusion proteins with a myristylation sequence, which anchors the target protein to the yeast membrane. The yeast GAL1 promoter driving the expression of the Myr-target fusion was induced by galactose in the growth medium. Both fusion proteins (pSOS/SalAT and pMyr/SalR or pMyr/6OMT) were co-expressed in the *Saccharomyces cerevisiae* cdc25H strain. The *cdc25* mutation prevents growth at 37 °C, but allows growth at 25 °C. If bait and target proteins interact physically, the hSOS protein is directed to the plasma membrane and complements the *cdc25* defect, activating the Ras-signaling transduction pathway. As a consequence, the cdc25H yeast strain is able to grow at 37 °C.

The *salAT* RNA*i* plants accumulated significantly more salutaridine (5) than salutaridinol (9). Since *salR* transcript levels were not increased in these transgenic plants, a potential physical interaction between SalAT and SalR could cause this accumulation of salutaridine under conditions in which SalAT is reduced (Fig. 6). The growth of the cdc25H yeast strain on galactose media at 37 °C (Fig. 6-1) demonstrated that SalR and SalAT interact in under the conditions used for the yeast two-hybrid analysis. This interaction was not observed between the morphine (16) biosynthesis-specific SalAT and the central tetrahydrobenzylisoquinoline pathway enzyme 6OMT (Fig. 6-2) (Fig. 1). Protein interactions 3 and 4 represent positive (Fig. 6-3) and negative (Fig. 6-4) controls, respectively.

A second line of evidence for the interaction between SalR and SalAT is provided by the co-immunoprecipitation of these two proteins. SalR and SalAT were co-immunoprecipitated *in vitro* by incubating purified His-tagged SalR and untagged SalAT with anti-SalAT antibody (Weid et al., 2004). The protein A-linked agarose bead-associated proteins were resolved by SDS–PAGE and His-tagged SalR was then visualized with anti-polyhistidine-alkaline phosphatase conjugate antibody. Using this method, protein associated with SalAT is bound together with SalAT to the agarose beads. The proteins washed from the beads were visualized on the Western blot with anti-His-tag antibody. Under these conditions, only His-tagged SalR that was associated with SalAT can be visualized (Fig. 7).

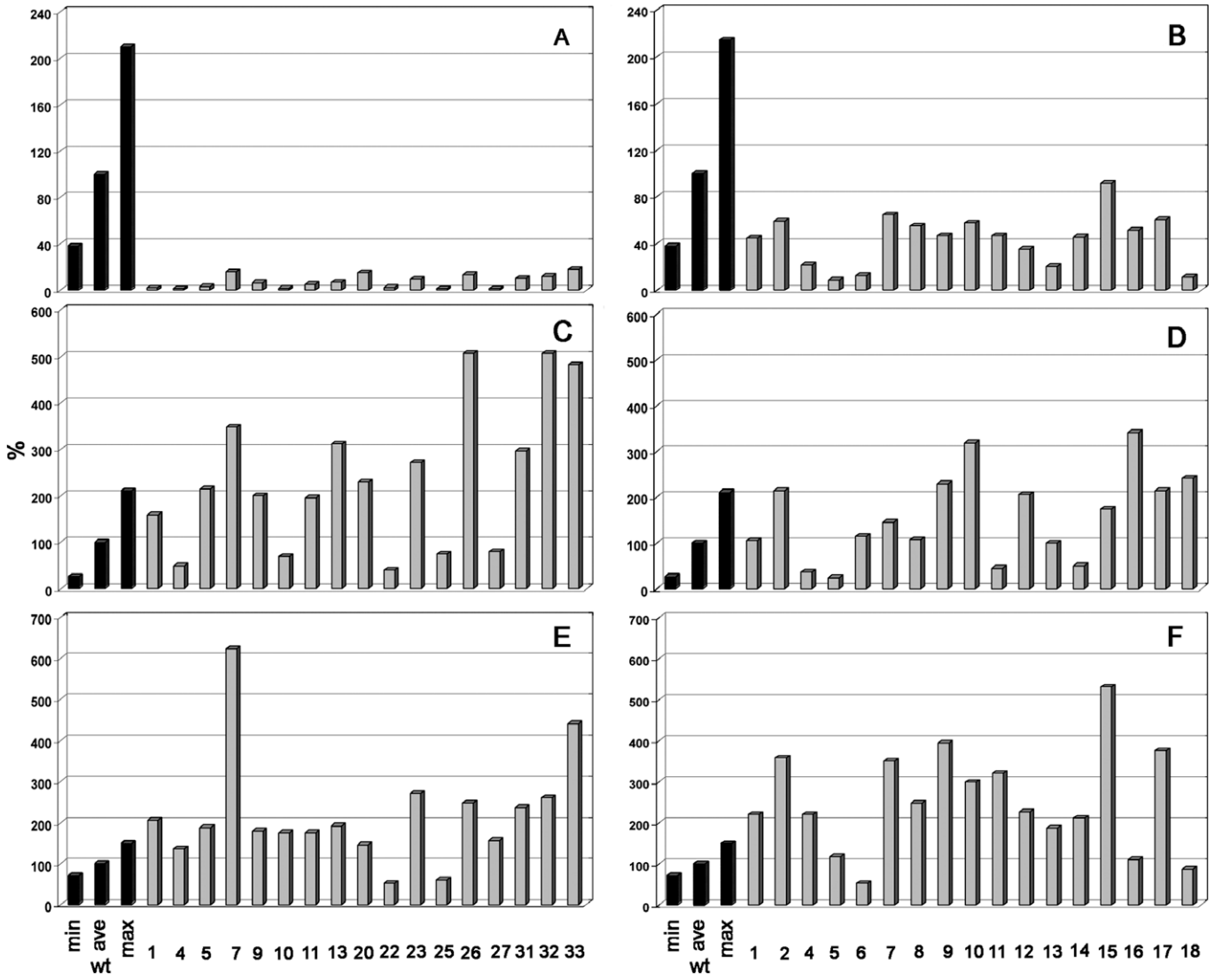

Fig. 4. Real time RT-PCR of T_1 generation leaves of *salAT* RNA*i* plants 77a38 (A, C and E) and 99a15 II (B, D and F) using primers for *salAT* (A and B), *salR* (C and D) and *cor1* (E and F). Three replicates were performed. For analysis of the results, only data with standard deviations equal to or less than 0.5 for the threshold cycle (C_t) were used. C_t values of transgenic plants were compared to the averaged C_t values of six wild type plants.

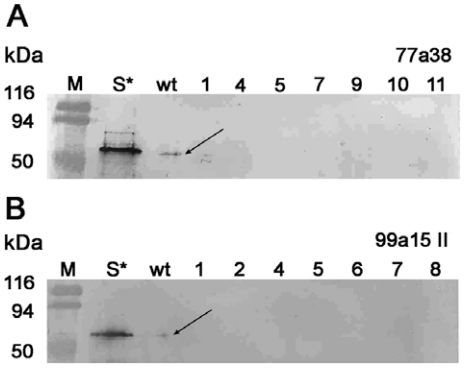

Fig. 5. Western analysis of SalAT protein in leaf extracts. Protein was visualized using antibodies raised against recombinant SalAT and alkaline phosphatase-coupled secondary antibody. (A) T_1 progeny of cell lines 77a38 and (B) T_1 progeny of cell lines 99a15 II. M, molecular weight markers; S∗, recombinant SalAT; wt, wild type opium poppy. 10 μg pure protein or crude protein extract were resolved by 10% SDS–PAGE prior to electroblotting.

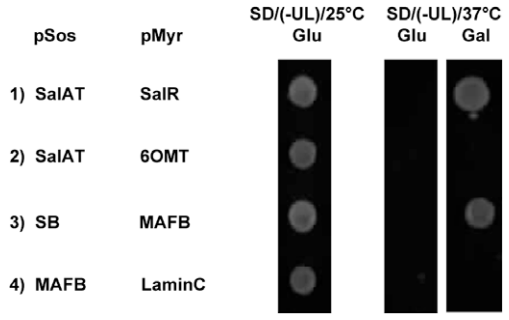

Fig. 6. Detection of protein–protein interaction with the CytoTrap® yeast two-hybrid system (Stratagene). (1) The first lane demonstrates a combination of growth on galactose-containing media at 37 °C and no growth on glucose-containing media at 37 °C as a result of the physical interaction between SalAT and SalR proteins. (2) Lack of growth at 37 °C for colonies harboring SalAT and 6OMT clones indicating that the two proteins failed to interact (3) SB and MAFB represent a positive control (4), MAFB and Lamin C represents a negative control.

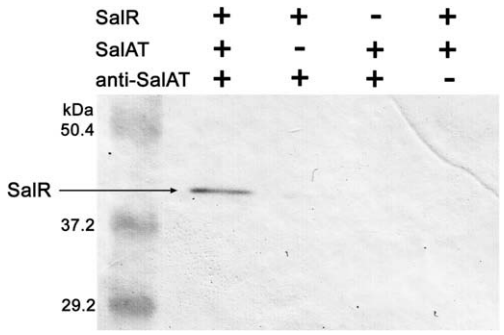

Fig. 7. Co-immunoprecipitation analysis of interaction between SalR and SalAT. Recombinant SalR and SalAT were mixed as indicated above the membrane picture. The mixtures were immunoprecipitated by anti-SalAT antibody. The precipitation pellets were then resolved by SDS–PAGE and analysed by Western blot using an anti-His antibody.

3. Discussion

This study provides a further perspective into the regulation of morphine (16) biosynthesis in opium poppy. A stable transformation with hpRNA was used to reduce *salAT* transcript accumulation, a gene specifically involved in morphine (16) biosynthesis. This RNA *interference* resulted in the accumulation of salutaridine (5) and, to a lower extent, of salutaridinol (9) in latex. Both intermediate alkaloids were undetectable in the latex of wild type plants. This result was notable, since the substrate of SalAT is salutaridinol (9) *not* salutaridine (5). The reduction of salutaridine (5) to salutaridinol (9) is catalyzed by the enzyme SalR and occurs immediately prior to acetylation of salutaridinol (9) by SalAT (Gerardy and Zenk, 1993; Ziegler et al., 2006). An explanation of this observed salutaridine phenotype may be found in the steady-state kinetic parameters of SalR. This oxidoreductase has been shown to catalyze a reversible reaction (Gerardy and Zenk, 1993). The K_m/K_{cat} value determined for recombinant SalR was 0.18 for salutaridine (the physiological forward reaction) and 0.46 for salutaridinol (the physiological reverse reaction) (Ziegler et al., 2006). Under optimal reaction conditions, the physiological reverse reaction, the oxidation of salutaridinol (9) to salutaridine (5), is predominant. It is, therefore, feasible that accumulated salutaridinol (9) is efficiently oxidized by SalR to salutaridine (5), which then accumulates. However, at neutral pH (presumably that of the cytosol), the physiological forward reaction is predominant and the reverse reaction is negligible (Gerardy and Zenk, 1993). It is unlikely that free salutaridinol (9) in the cytosol at neutral pH would be oxidized by SalR to salutaridine (5). The enzyme that catalyzes the formation of salutaridine (5) from (*R*)-reticuline (3) is a *C–C* phenol coupling cytchrome P-450 that under physiological conditions is effectively irreversible, hence salutaridine (5) *can* accumulate into a measurable metabolic pool.

Given the accumulation of salutaridine (5) in the transgenic plants, it was conceivable that SalR and SalAT may be physically associated. To test this hypothesis of an enzyme complex involved in morphine (16) biosynthesis in *P. somniferum*, a yeast two-hybrid system that detects interactions between cytosolic proteins as well as *in vitro* co-immunoprecipitation were used. This approach confirmed that SalR and SalAT interact under the two-hybrid conditions and under the conditions of co-immunoprecipitation. Coordinated action of consecutive enzymes involved in the metabolism of phenylpropanoids has been reported already in the 1970s. In particular, an interaction between PAL and C4H from cotyledons of cucumber *Cucumis sativus* was suggested to occur. Such a multienzyme complex model was attractive because it could explain how the metabolic fate of L-phenylalanine into various phenolic substances could be determined at the amino acid stage (Czichi and Kindl, 1977). The classic example of macromolecular organization, however, is the formation of the cyanogenic glucoside dhurrin in *Sorghum bicolor* (Lindberg-Møller and Conn, 1980). The biosynthesis of dhurrin in *S. bicolor* is now understood to be a highly channeled process catalyzed by two multifunctional cytochromes P-450 (CYP79A1 and CYP71E1) and a soluble UDPG-glucosyltransferase (UGT85B1) that are likely associated in a macromolecular complex at the endomembrane (Tattersall et al., 2001; Kristensen et al., 2005).

Enzyme complex formation in phenylpropanoid metabolism has also been re-visited recently using immunocytochemical localization and the two-hybrid assay. In flavonoid biosynthesis in *Arabidopsis thaliana*, chalcone synthase, chalcone isomerase and dihydroflavonol 4-reductase appear physically associated (Burbulis and Winkel-Shirley, 1999; Winkel, 2004). In the biosynthesis of spermidine in *A. thaliana,* interactions of two sperimidine synthases with spermine synthase were found, suggesting the existence of a metabolon comprising at least the final two steps in this pathway (Panicot et al., 2002). The observed interaction between SalR and SalAT in morphine biosynthesis is consistent with this growing body of evidence for the involvement of macromolecular complexes in the formation of plant secondary metabolites.

In 2004, Allen et al. published results from the study of hpRNA *cor1*/*cor2* transgenic opium poppy. Silenced plants accumulated the central intermediate reticuline (3,4) at the expense of the latter alkaloids in the biosynthesis, such as morphine (16), thebaine (11), codeine (14) and oripavine (13). It was argued that three regulatory processes could be responsible for this phenotype: the occurrence of a negative substrate feedback on either the enzyme or the transcript level or the presence of a macromolecular enzyme complex were all discussed (Allen et al., 2004). The latter possibility cannot be supported by the results obtained herein. If a complex of all enzymes between reticuline (3/4) and codeine (14) did indeed occur, *salAT* RNA*i* plants would also have accumulated reticuline (3/4) rather than salutaridine (5) and salutaridinol (9). Although an interaction between SalR and SalAT appears to exist, not all enzymes of the morphinan branch are necessarily involved in such a macromolecular complex. An additional complication in interpretation of the results obtained by Allen et al. (2004) is that the hairpin construct used to transform opium poppy was a hybrid of *cor1*, which encodes COR of morphine biosynthesis, and *cor2*, which is a cDNA 70% identical to the *cor1* family, but of unknown function (Unterlinner et al., 1999).

Alkaloids downstream of salutaridinol-7-*O*-acetate (10) accumulated in *salAT* RNA*i* plants. In particular, morphine (16) accumulated to levels comparable to those found in wild type plants. An indirectly proportional correlation of the accumulation of salutaridine (5) and both thebaine (11) and codeine (14) was observed. The influence on morphine (16) synthesis in contrast was much lower. Real Time RT-PCR showed, however, no enhanced transcript level of COR in *salAT* RNA*i* plants. Since Weid et al. (2004) showed the localization of COR in laticifer cells and SalAT in phloem parenchyma, it is reasonable that *salAT* transcript levels do not effect *cor1* transcription. The localization of SalR has not yet been experimentally determined, but if at least two cell types are involved in morphine (16) biosynthesis, a transport of the biosynthetic intermediates salutaridinol-7-*O*-acetate (10) or thebaine (11) from parenchyma cells into laticifers must be presumed. An up-regulation of these transport mechanisms could be involved in the accumulation of high amounts of morphine (16) in *salAT* RNA*i* plants. A recent publication (Allen et al., 2008) reports that morphine (16), codeine (14) and thebaine (11) levels are increased in both *salAT* over-expressing and *salAT* RNA*i* plants. A detailed quantitation is presented for *salAT* over-expressing plants, but only HPLC chromatograms of the alkaloids morphine (16), codeine (14) and

thebaine (11) are shown for *salAT* RNA*i* plants. The lack of quantitative data precludes a direct comparison with results obtained herein. The intermediate salutaridine (5) is reported accumulated *salAT* RNA*i* plants, but in contrast to our results, salutaridinol (9) was not detected by Allen et al. Both our data and the data reported in Allen et al. (2008) are not consistent with results reported on the *cor1*/*cor2* RNA*i* opium poppy (Allen et al., 2004). The results reported on *salAT* RNA*i* plants (Allen et al., 2008) do, however, provide an independent example of an alkaloid profile that could be consistent with a physical interaction of SalR and SalAT.

4. Conclusion

Although not all of the effects expressed by the transgenic plants can currently be explained in detail, this study provides new insights into the field of regulation of the biosynthesis of morphine (16). The findings that *salAT* RNA*i* plants accumulated much more salutaridine than salutaridinol (9), and that the morphine (16) biosynthetic enzymes SalAT and SalR interact in yeast two-hybrid assays and in co-immunoprecipitation experiments cast a new light on our view of the complexity of alkaloid formation in opium poppy.

5. Experimental

5.1. Plant material

The genotype of *P. somniferum* L. used was the narcotic cultivar C058-34 obtained from Tasmanian Alkaloids Pty Ltd., Westbury, Australia.

5.2. Vector construction

The *salAT* RNA*i* vector was constructed by ligation of fragments of *salAT* cDNA (Grothe et al., 2001) in sense and antisense direction into the vector pHANNIBAL, which contains the 35S promoter and OCS terminator (Wesley et al., 2001). Restriction sites were added to a 656 bp long sequence of *salAT* using amplification (cycle, 94 °C, 3 min, 30 cycles of 94 °C, 30 s; 55 °C, 30 s; 72 °C, 1 min, cycle 72 °C, 7 min, finally, the reaction was cooled to 4 °C) with appropriate primers (sense: 5′-TCT AGA CTC GAG CAT CTG TAC GTG AAA AGT TGC-3′ and antisense: 5′-GGA TCC GAA TTC TCA CTT AGG TGA AGC TCG AA-3′). This fragment corresponded to the cDNA of *salAT* from nucleotide 752 through 1406. The PCR product was digested with XhoI and EcoRV (sense) or XbaI and BamHI (antisense) and consecutively cloned in either orientation into the vector pHANNIBAL surrounding the PDK intron. The construct was then digested with *Not*I and the resulting expression cassette was subsequently ligated into the binary vector pART27 (Gleave, 1992) in front of the nptII selection cassette.

5.3. Plant transformation

The stable *Agrobacterium*-mediated transformation of opium poppy was carried out according to Larkin et al. (1999) and Chitty et al. (2003). The pART27 *salAT* RNA*i* vector was transferred into *Agrobacterium tumefaciens* strain AGL1 (Lazo et al., 1991) *via* electroporation (Biorad; puls 2 kV with 400 Ω and 50 μF). Seeds of *P. somniferum* L. line C058-34 were sterilized (Larkin et al., 1999) and grown on B5O media (Larkin et al., 1999; Chitty et al., 2003). Hypocotyls of 7-day-old seedlings were co-cultivated with the agrobacteria for 3–5 days, washed and placed on fresh 19D media. Within several weeks, pre-embryogenic callus was formed and grown on hormone-free B5O media (Larkin et al., 1999; Chitty et al., 2003). After about one year, the first embryos appeared. Explants were cultivated as described recently (Frick et al., 2007). Plantlets were then transferred to the greenhouse where they were grown to maturity. Plants were grown as previously described (Frick et al., 2005).

5.4. DNA and RNA extraction

Plant genomic DNA and total RNA were isolated from the two upper leaves harvested 3 days after pollination and extracted using standard protocols (Maniatis et al., 1982). An extraction buffer containing 10 mM Tris–HCl pH 7.5, 50 mM sodium chloride, 1% (w/v) SDS, 4% (w/v) PVPP and 14 mM 2-mercaptoethanol was used, followed by phenol–$CHCl_3$ extraction and precipitation with i-ProH and NaOAc. RNA was isolated from this nucleic acid mixture by precipitation with LiCl overnight.

Total RNA for Northern blots was extracted from upper stems using TRIZOL® Reagent (Invitrogen), a single-step RNA isolation method developed by Chomczynski and Sacchi (1987).

5.5. Southern, Northern and Western blot analysis

Genomic DNA (10 μg) were digested with EcoRV (Invitrogen) and the fragments were resolved on a native agarose gel. Total RNA (10 μg) were incubated 5 min at 65 °C and resolved on a 1.2% formaldehyde denaturing gel. The electrophoretically resolved DNA or RNA was transferred onto a nylon membrane (Biodyne B Membrane, 0.45 μm; Pall) as described by the manufacturer. Blots were hybridized with the appropriate [α-^{32}P]-labeled cDNAs (Megaprime DNA Labeling System, Amersham). For Southern blot analysis, the cDNAs was either full-length *salAT* (Grothe et al., 2001) or a partial nptII cDNA. The *salAT* cDNA used for hybridization to Northern blots contained only part of the full-length sequence (3–751 bp) that was not used for RNA*i* vector design. Hybridization was performed at 68 °C for Northern blots and at 60 °C for Southern blots. Western blot analysis was carried out according to Weid et al. (2004).

5.6. HPLC and LC/ESI-TOFMS analysis of latex

Latex was harvested from poppy plants by incision of capsules 3 days after pollination. The harvest and extraction protocol of alkaloids is described in Frick et al. (2005). HPLC analysis was performed on an LC 1100 series Agilent system (Agilent Technologies, Waldbronn, Germany) using the reversed phase column Lichrospher 60 RP-select B (4 × 250 mm, 5 μm; Merck) and UV detection at 210, 282 and 490 nm. The separation was achieved using a gradient from solvent A [CH_3CN:H_2O (2:98; v/v) + 0.01% (v/v) phosphoric acid] to solvent B [CH_3CN:H_2O (98:2; v/v) + 0.01% (v/v) phosphoric acid] under the following conditions: flow 1 ml/min; 0–25 min: 0–46% B; 25–26 min: 46–100% B; 26–33 min:100% B; 33–35 min: 100–0% B, 35–40 min: 0% B (wash). Peaks were routinely identified from their UV spectra and by comparison of their retention times to those of authentic standards. The identity of the peaks was confirmed by LC–MS. LC/ESI-TOFMS analysis has been described in detail elsewhere (Frick et al., 2005).

5.7. Polymerase chain reaction analysis of transformation

PCR was performed: (a) for nptII: as described recently (Frick et al., 2004) (b) for sense fragment: primer sequences, sense: 5′-ACA ATC CCA CTA TCC TTC G-3′ and antisense: 5′-CAC TTA ACT ATT TTA TAC TAA AAG G-3′ and (c) for antisense fragment: primer sequences, sense: 5′-GTC GAA CAT GAA TAA ACA AGG-3′ and antisense: 5′-TAC AAC GTG CAC AAC AGA AT-3′ cycle, 94 °C, 3 min, 30 cycles of 94 °C, 30 s; 55 °C, 30 s; 72 °C, 1 min, cycle 72 °C, 7 min. Finally, the reaction was cooled to 4 °C.

5.8. Reverse transcriptase Real Time PCR

For analysis of *salAT*, *cor1*, *salR* and *tubulin* transcript levels in leaves, a reverse transcriptase RT-PCR method was used. Contaminating DNA was removed by DNase digestion with Fisher Optizyme Recombinant DNase I followed by phenol–$CHCl_3$ extraction. RNA was checked for remnant DNA prior to use. cDNA was prepared from 5 μg of RNA using SuperScript III First-Strand Synthesis System for RT-PCR with oligo dT primers (Invitrogen). Real Time PCR was performed with an iQ5 Real Time PCR Detection System from BioRad. Reactions were carried out with Brilliant SYBR Green QPCR Master Mix (Stratagene) in accordance to the manufacturer's instructions. Cycling conditions were as follows: 95 °C, 10 min, 40 cycles of 95 °C, 30 s; 60 °C, 1 min; 72 °C, 1 min. At the end of the cycling, the samples were heated to 95 °C and subjected to a melting curve program of 55–95 °C in 0.5 °C increments with a 10 s hold at each temperature. Primers for amplification of *salAT* transcript were (forward) 5′ - CTT GAG GTT TGA CGG AGC CAT - 3′ and (reverse) 5′ - ACG ACG AGA GCA TGA AGT GGA - 3′. Primers for amplification of *cor1* were (forward) 5′ - TCA CTC TCA GTT CCG GCA TTC - 3′ and (reverse) 5′ - CGC TTT CAA AAA CGC CAA TT - 3′. Primers for amplification of *salR* were (forward) 5′ - TCA ACT TGA TGT TAC GGA TCC AA - 3′ and (reverse) 5′ - CCC CAG CAT TGT TTA CCA AGA - 3′. Primers for amplification of *tubulin* were (forward) 5′ - GCC GAT GCA GCT ACA ATG AGT A - 3′ and (reverse) 5′ - CCG AGT CGA AAG AAG GAA TCA A - 3′. Samples were analysed in triplicate. For analysis of the results, only data with standard deviations equal to or less than 0.5 for the threshold cycle (C_t) were used. Relative quantification was performed without efficiency correction (Pfaffl, 2004). C_t values of transgenic plants were compared to the averaged C_t values of six wild type plants.

5.9. Yeast two-hybrid

The CytoTrap™ XR Library Construction Kit (Stratagene) was used for the preparation of the bait protein and the cDNA interactors. PCR was performed to amplify the cDNA encoding SalAT (Grothe et al., 2001). The primer sequences were: forward primer 5′-CT TTG GAT CCA TGG CAA CAT GTA TAG TGC T-3′ and reverse primer 5′-ATT AGC GGC CGC TCA AAT CAA TTC AAG GAT TTC AC-3′; cycle, 94 °C, 3 min, 30 cycles of 94 °C, 30 s; 55 °C, 30 s; 72 °C, 1.5 min, cycle 72 °C, 10 min. Finally, the reaction was cooled to 4 °C. The *Nco*I/*Not*I digested fragment was cloned into the pSOS vector (Stratagene). DNA encoding SalR (Ziegler et al., 2006) and 6OMT (Ounaroon et al., 2003) were amplified with PCR as described above. Primer sequences for *salR* were pMyr-16B1-EcoRI: 5′-CA GAA TTC ATG CCT GAA ACA TGT CC-3′ and pMyr-16B1-XhoI: 5′-CA CTC GAG ATA ACT CAA AAT GCA GAT AGT TCT G-3′. Primer sequences for *6omt* were pMyr-6OMT-EcoRI: 5′-CA GAA TTC ATG GAA ACA GTA AGC AAG ATT GAT CAA C-3′ and pMyr-6OMT-XhoI: 5′-CA CTC GAG TTA ATA AGG GTA AGC CTC AAT TAC-3′. SalR and *6omt* were subsequently digested with *Eco*RI/XhoI and unidirectionally inserted into the pMyr XR vector (Stratagene). Restriction enzyme digest and sequence analysis verified the expression of the genes in frame with the SOS or Myr domain. Both target clones (pMyr XR/SalR and pMyr XR/6OMT) were independently introduced into cdc25Hα *S. cerevisiae* competent cells by co-transformation with the bait plasmid construct pSOS/SalAT according to the manufacturer's instructions. Each transformation was plated on 100 mm SD/glucose (-UL) plates and incubated at 25 °C for 48 h. The co-transformants were then plated on a galactose-based media and on a glucose-based media for GAL1 promoter repression and incubated at 37 °C for 48 h. "Interactor candidates" were subjected to two serial interaction tests (primary and secondary) on galactose-containing plates at 37 °C for 48 h each to verify the interaction.

5.10. Protein expression and purification

Papaver somniferum SalR was amplified by PCR from the recombinant plasmid SalR/pQE-30 (Ziegler et al., 2006) using the primer 5′-CCCGCTAGCATGCCTGAAACATGTCCAAATACTGT-3′ as the forward primer and 5′-CCCGAATTCTCAAAATGCAGATAGTTCTGAACAATC-3′ as the reverse primer. The 0.9-kb DNA fragment was ligated into an NheI/EcoRI-digested pET28a expression vector (Novagen, Madison, WI). *Papaver somniferum* SalAT cDNA was amplified by PCR from the recombinant plasmid SalAT/pCR T7NT/TOPO (Weid et al., 2004) using the primer 5′-CCCCCATATGGCAACAATGTATAGTGCTGCTGTTG-3′ as the forward primer and 5′-CCCGGATCCTCAAATCAATTCAAGGATTTCACTTAGGTG-3′ as the reverse primer. The 1.4-kb DNA fragment was ligated into an NdeI/BamHI-digested pET28a expression vector (Novagen).

The SalR/pET28a expression construct was transformed into *Escherichia coli* BL21(DE3)RIL. Transformed *E. coli* was grown at 37 °C in Luria–Bertani broth containing 50 μg ml^{-1} kanamycin until A_{600} reached ~0.8. After protein induction with 1 mM isopropyl-β-D-thiogalactopyranoside, the culture was grown at 16 °C for 16 h. Cells were harvested by centrifugation (8000*g*, 10 min) and resuspended in an extraction buffer of 50 mM Tris–HCl pH 7.5, 100 mM NaCl, 5 mM 2-mercaptoethanol and 10% (w/v) glycerol in H_2O. After sonication and centrifugation (20,000*g*, 20 min), the His-tagged SalR was purified with a cobalt affinity resin (Talon, Clontech, Mountain View, CA) according to the manufacture's instruction. The enzyme was dialyzed at 4 °C against the extraction buffer, then against 20 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1 mM DTT and 30% (w/v) glycerol. The enzyme was loaded onto a gel filtration column (HiLoad 16/60 Superdex 75 prep grade, GE Healthcare) with a buffer of 20 mM Tris–HCl pH 7.5, 150 mM NaCl and 0.1 mM DTT at a flow rate of 0.5 ml/min using an Äkta Purifier (GE Healthcare). The SalR-containing fractions were combined, concentrated with a Centriprep YM-10 (Millipore, Billerica, MA) and stored at −80 °C. To check enzyme activity of purified SalR, a total volume of enzyme solution (60 μl) including 9 μmol potassium phosphate buffer pH 6.0, 30 nmol NADPH and 6 nmol salutaridine (5) were incubated for 1 h at 30 °C. The subsequent steps were the same as described in Gerardy and Zenk (1993) except for using a Lichrospher 60 RP-select B HPLC column (250 × 4 mm, 5 μm; Merck, Darmstadt, Germany).

The SalAT/pET28a expression construct was transformed into *E. coli* BL21(DE3)RIL. Transformed *E. coli* was grown at 37 °C in Luria–Bertani broth containing 50 μg ml^{-1} kanamycin until A_{600} reached ~0.8. After protein induction with 1 mM isopropyl-β-D-thiogalactopyranoside, the culture was grown at 16 °C for 16 h. Cells were harvested by centrifugation (8000*g*, 10 min) and resuspended in an extraction buffer of 50 mM Tris–HCl pH 7.5, 100 mM NaCl, 5 mM 2-mercaptoethanol and 10% (w/v) glycerol in H_2O. After sonication and centrifugation (20,000*g*, 20 min), the His-tagged SalAT was purified with a cobalt affinity resin (Talon, Clontech) according to the manufacture's instruction. The enzyme was incubated with thrombin overnight at 4 °C with concomitant dialysis against the extraction buffer. Dialyzed protein was incubated with a mixture of the cobalt resin and a benzamidine–sepharose resin (GE Healthcare) to remove both uncleaved protein and thrombin. The protein was then dialyzed against buffer Q consisting of 20 mM Tris–HCl pH 7.5, 5 mM 2-mercaptoethanol and 10% (w/v) glycerol. The protein was loaded onto an ion exchange column (HiTrap 1 ml Q HP, GE Healthcare) at a flow rate of 1.0 ml/min using an Äkta Purifier (GE Healthcare). Retained SalAT was washed with 50 mM NaCl in buffer Q then eluted with 150 mM

NaCl in buffer Q. The enzyme was then loaded onto a gel filtration column (HiLoad 16/60 Superdex 75 prep grade, GE Healthcare) with a buffer of 20 mM Tris–HCl pH 7.5, 150 mM NaCl and 0.1 mM DTT at a flow rate of 0.5 ml/min using an Äkta Purifier (GE Healthcare), then concentrated with Centriprep YM-10 (Millipore, Billerica, MA) and stored at −80 °C. To check enzyme activity of purified SalAT, a total volume of enzyme solution (60 µl) including 13.2 µmol tricine–NaOH buffer pH 8.0, 18 nmol acetyl-CoA and 6 nmol salutaridinol (9) were incubated for 15 min at 47 °C. The subsequent procedure was the same as that for SalR as described above.

5.11. Co-immunoprecipitation

Co-immunoprecipitation *in vitro* was performed by incubating purified His-tagged SalR (0.2 nmol) and purified untagged SalAT (0.2 nmol) with 6 µl of anti-SalAT antibody (Weid et al., 2004) overnight at 4 °C. The immunoprecipitate in 1 ml of binding buffer (10 mM Tris–HCl pH 7.5, 150 mM NaCl and 0.1% (v/v) Triton-X-100) was mixed with equilibrated protein A-linked agarose beads (25 µl, Invitrogen, Carlsbad, CA) and incubated for 30 min. After centrifugation (2000*g*, 2 min), the supernatant was removed and the pellet was washed four times with 1 ml binding buffer. The pellet was suspended and boiled in 30 µl of SDS–PAGE loading buffer. Protein samples were separated by SDS–PAGE and transferred onto an Hybond-ECL nitrocellulose membrane (GE Healthcare). His-tagged SalR was then detected by anti-polyHistidine-alkaline phosphatase conjugate antibody (Sigma, St. Louis, MO).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2009.03.002.

References

Allen, R.S., Millgate, A.G., Chitty, J.A., Thisleton, J., Miller, J.A.C., Fist, A.J., Gerlach, W.L., Larkin, P.J., 2004. RNAi-mediated replacement of morphine with the nonnarcotic alkaloid reticuline in opium poppy. Nat. Biotechnol. 22, 1526–1527.

Allen, R.S., Miller, J.A., Chitty, J.A., Fist, A.J., Gerlach, W.L., Larkin, P.J., 2008. Metabolic engineering of morphinan alkaloids by over-expression and RNAi suppression of salutaridinol 7-*O*-acetyltransferase in opium poppy. Plant Biotech. J. 6, 22–30.

Burbulis, I.E., Winkel-Shirley, B., 1999. Interactions among enzymes of the *Arabidopsis* flavonoid biosynthetic pathway. Proc. Natl. Acad. Sci. USA 96, 12929–12934.

Chitty, J.A., Allen, R.S., Fist, A.J., Larkin, P.J., 2003. Genetic transformation in commercial Tasmanian cultivars of opium poppy, *Papaver somniferum*, and movement of transgenic pollen in the field. Funct. Plant Biol. 30, 1045–1058.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal. Biochem. 162, 156–159.

Czichi, U., Kindl, H., 1977. Phenylalanine ammonia lyase and cinnamic acid hydroxylases as assembled consecutive enzymes on microsomal membranes of cucumber cotyledons: cooperation and subcellular distribution. Planta 134, 133–143.

Dixon, R.A., Steele, C.L., 1999. Flavonoids and isoflavonoids – a gold mine for metabolic engineering. Trends Plant Sci. 4, 394–400.

Frick, S., Chitty, J.A., Kramell, R., Schmidt, J., Allen, R.S., Larkin, P.J., Kutchan, T.M., 2004. Transformation of opium poppy (*Papaver somniferum* L.) with antisense berberine bridge enzyme gene (*anti-bbe*) via somatic embryogenesis results in an altered ratio of alkaloids in latex but not in roots. Transgenic Res. 13, 607–613.

Frick, S., Kramell, R., Schmidt, J., Fist, A.J., Kutchan, T.M., 2005. Comparative qualitative and quantitative determination of alkaloids in narcotic and condiment *Papaver somniferum* cultivars. J. Nat. Prod. 68, 666–673.

Frick, S., Kramell, R., Kutchan, T.M., 2007. Metabolic engineering of a morphine biosynthetic P450 in opium poppy surpasses breeding. Metab. Eng. 9, 169–176.

Gerardy, R., Zenk, M.H., 1993. Purification and characterization of salutaridine: NADPH 7-oxidoreductase from *Papaver somniferum*. Phytochemistry 34, 125–132.

Gleave, A.P., 1992. A versatile binary vector system with a T-DNA organisational structure conducive to efficient integration of cloned DNA into the plant genome. Plant Mol. Biol. 20, 1203–1207.

Grothe, T., Lenz, R., Kutchan, T.M., 2001. Molecular characterization of the salutaridinol 7-*O*-acetyltransferase involved in morphine biosynthesis in opium poppy *Papaver somniferum*. J. Biol. Chem. 276, 30717–30723.

Huang, F.C., Kutchan, T.M., 2000. Distribution of morphinan and benzo[*c*]phenanthridine alkaloid gene transcript accumulation in *Papaver somniferum*. Phytochemistry 53, 555–564.

Hughes, E.H., Shanks, J.V., 2002. Metabolic engineering of plants for alkaloid production. Metab. Eng. 4, 41–48.

Kristensen, C., Morant, M., Olsen, C.E., Ekstrøm, C.T., Galbraith, D.W., Møller, B.L., Bak, S., 2005. Metabolic engineering of dhurrin in transgenic Arabidopsis plants with marginal inadvertent effects on the metabolome and transcriptome. Proc. Natl. Acad. Sci. USA 102, 1779–1784.

Kutchan, T.M., 1998. Molecular genetics of plant alkaloid biosynthesis. In: Cordell, G.A. (Ed.), The Alkaloids: Chemistry and Pharmacology, vol. 50. Academic Press, San Diego, pp. 257–316.

Kutchan, T.M., Ayabe, S., Krueger, R.J., Coscia, E.M., Coscia, C.J., 1983. Cytodifferentiation and alkaloid accumulation in cultured cells of *Papaver bracteatum*. Plant Cell Rep. 2, 281–284.

Kutchan, T.M., Frick, S., Weid, M. 2007. Engineering plant alkaloid biosynthetic pathways – progress and prospects. In: Bohnert, H.J., Nguyen, H.T. (Eds.), Advances in Plant Biochemistry and Molecular Biology. In: Nes, W.D., Lewis, N.G. (Eds.), Bioengineering and Molecular Biology of Plant Pathways, vol. 1. Elsevier Science Ltd., Oxford, pp. 281–308.

Larkin, P.J., Chitty, J.A., Brettell, R.I.S. 1999. Methods for Plant Transformation and Regeneration. International Patent Publication Number WO 99/34663.

Lazo, G.R., Stein, P.A., Ludwig, R.A., 1991. A DNA transformation-competent *Arabidopsis* genomic library in *Agrobacterium*. Bio-Technol. 9, 963–967.

Lednicer, D., Mitscher, L.A., 1990. The Organic Chemistry of Drug Synthesis, vol. 4. John Wiley and Sons Canada Ltd., New York.

Lindberg-Møller, B., Conn, E.E., 1980. The biosynthesis of cyanogenic glucosides in higher plants. J. Biol. Chem. 255, 3049–3056.

Maniatis, T., Fritsch, E.F., Sambrook, J. (Eds.), 1982. Molecular Cloning: A Laboratory Manual, second ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Morais, J.M., Barbosa-Filho, J.M., Almeida, R.N., 1998. Central depressant effects of reticuline extracted from *Oceta duckei* in rats and mice. J. Ethno. Pharmacol. 62, 57–61.

Nakaoji, K., Nayeshiro, H., Tanahashi, T., 1997. Norreticuline and reticuline as possible new agents for hair growth acceleration. Biol. Pharm. Bull. 20, 586–588.

Ounaroon, A., Decker, G., Schmidt, J., Lottspeich, F., Kutchan, T.M., 2003. (R, S)-Reticuline 7-*O*-methltransferase and (R, S)-norcoclaurine 6-*O*-methltransferase of *Papaver somniferum* – cDNA cloning and characterization of methyl transfer enzymes of alkaloid biosynthesis in opium poppy. Plant J. 36, 808–819.

Panicot, M., Minguet, E.G., Ferrando, A., Alcazar, R., Blazquez, M.A., Carbonell, J., Altabella, T., Koncz, C., Tiburcio, A.F., 2002. A polyamine metabolon involving aminopropyl transferase complexes in *Arabidopsis*. Plant Cell 14, 2539–2551.

Pauli, H.H., Kutchan, T.M., 1998. Molecular cloning and functional heterologous expression of two alleles encoding (*S*)-*N*-methylcoclaurine 3′-hydroxylase (CYP80B1), a new methyl jasmonate-inducible cytochrome P-450 dependent monooxygenase of benzylisoquinoline alkaloid biosynthesis. Plant J. 13, 793–801.

Pfaffl, M.W., 2004. Quantification strategies in real-time PCR. In: Bustin, S.A. (Ed.), A–Z of Quantitative PCR. No. 5. International University Line, La Jolla, pp. 87–120.

Rice, K.C., 1980. Synthetic opium alkaloids and derivatives. A short total synthesis of (+)-dihydrothebainone, (+)-dihydrocodeinone, and (+)-nordihydrocodeinone as an approach to a practical synthesis of morphine, codeine, and congeners. J. Org. Chem 45, 3135–3137.

Tattersall, D.B., Bak, S., Jones, P.R., Olsen, C.E., Nielsen, J.K., Hansen, M.L., Høj, P.B., Møller, B.L., 2001. Resistance to an herbivore through engineered cyanogenic glucoside synthesis. Science 293, 1826–1828.

Unterlinner, B., Lenz, R., Kutchan, T.M., 1999. Molecular cloning and functional expression of codeinone reductase: the penultimate enzyme in morphine biosynthesis in the opium poppy *Papaver somniferum*. Plant J. 18, 465–475.

Verpoorte, E., van der Heijden, R., Memelink, J., 2000. Engineering the plant cell factory for secondary metabolite production. Trans. Res. 9, 323–343.

Weid, M., Ziegler, J., Kutchan, T.M., 2004. The roles of latex and the vascular bundle in morphine biosynthesis in the opium poppy *Papaver somniferum*. Proc. Natl. Acad. Sci. USA 101, 13957–13962.

Wesley, S.V., Helliwell, C.A., Smith, N.A., Wang, M., Rouse, D.T., Liu, Q., Gooding, P.S., Singh, S.P., Abbott, D., Stoutjesdijk, P.A., Robinson, S.P., Gleave, A.P., Green, A.G., Waterhouse, P.M., 2001. Construct design for efficient, effective and high-throughput gene silencing in plants. Plant J. 27, 581–590.

Winkel, B.S.J., 2004. Metabolic channeling in plants. Annu. Rev. Plant Biol. 55, 85–107.

Ziegler, J., Voigtländer, S., Schmidt, J., Kramell, R., Miersch, O., Ammer, C., Gesell, A., Kutchan, T.M., 2006. Comparative transcript and alkaloid profiling in *Papaver* species identifies a short chain dehydrogenase/reductase involved in morphine biosynthesis. Plant J. 48, 177–192.