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Characterisation of recombinant epithiospecifier protein and its over-expression in *Arabidopsis thaliana*

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Abstract

Epithiospecifier protein (ESP) is a protein that catalyses formation of epithionitriles during glucosinolate hydrolysis. In vitro assays with a recombinant ESP showed that the formation of epithionitriles from alkenylglucosinolates is ESP and ferrous ion dependent. Nitrile formation in vitro however does not require ESP but only the presence of Fe(II) and myrosinase. Ectopic expression of ESP in *Arabidopsis thaliana* Col-5 under control of the strong viral CaMV 35S promoter altered the glucosinolate product profile from isothiocyanates towards the corresponding nitriles.

Keywords: *Arabidopsis thaliana*; Glucosinolate; Epithiospecifier protein; Myrosinase

1. Introduction

Glucosinolates (thioglucosides) are a class of sulphur containing secondary metabolites present in all cruciferous plants (Underhill, 1981). Glucosinolates and their degradation products are important bioactive compounds in the diet and health of animals and humans (Mithen, 2001). Myrosinase (E.C. 3.2.1.147) catalyses the hydrolysis of glucosinolates to give rise to an unstable thiohydroximate-*O*-sulphonate (Fig. 1), which can further rearrange to an isothiocyanate. Alternatively, in the presence of epithiospecifier protein (ESP) and Fe(II), epithionitriles are produced. ESP is unique in as much as it has no activity towards the initial substrate but only with the unstable thiohydroximate-*O*-sulphonate intermediate (Bones and Rossiter, 1996).

ESP has been independently purified to homogeneity (Bernardi et al., 2000; Foo et al., 2000) and has been shown for the first time to be a stable protein present in several isoforms (Foo et al., 2000). An anti-ESP antibody reacted with both the 39 and 35 kDa ESP forms in *Brassica napus* and also with a polypeptide corresponding to the 35 kDa ESP in *Crambe abyssinica* but is not present in all cruciferous plants (Foo et al., 2000). Partial amino acid sequencing of the 39 kDa ESP isoform revealed high similarity (81% identity) with a hypothetical 37 kDa protein from *Arabidopsis thaliana* (GenBank Accession Number H37255) and several jasmonate inducible proteins (Bernardi et al., 2000) while more recently ESP has been shown to be induced in the anthers of *Arabidopsis* by jasmonate (Mandaokar et al., 2003).

It has been suggested (Taipalensuu et al., 1996) that ESP is the previously identified myrosinase associated

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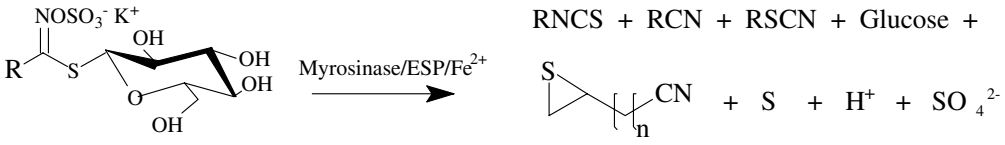

Fig. 1. A generalised scheme of glucosinolate hydrolysis by myrosinase and ESP.

protein (MyAP), a glycoprotein, purified from *B. napus*. MyAP is wound and methyl jasmonate-inducible and has strong similarity to an early nodulin. However, we have recently shown that ESP does not require any specific myrosinase, as ESP is also active with myrosinase isolated from the aphid *Brevicoryne brassicae* (Foo et al., 2000; Jones et al., 2001).

A mechanism for the generation of epithionitriles via Fe(II) has been proposed (Foo et al., 2000). However, the Fe(II) dependency of ESP has recently been questioned (Lambrix et al., 2001). These authors expressed *A. thaliana* ESP in *Eschericia coli* and found no Fe(II) dependence. In *A. thaliana* ecotypes which do not express ESP a 10-bp deletion in the *ESP* promoter sequence was detected (Lambrix et al., 2001) and this deletion strongly correlates with a loss of ESP activity.

A. thaliana accession Col-5 has a glucosinolate profile similar to that of broccoli (*Brassica oleracea* var. *italica*) and contains glucoraphanin (4-methylsulfinylbutylglucosinolate) and on hydrolysis gives sulforaphane (1-isothiocyanato-4-(methylsufinyl)butane) as the major product. 1-Isothiocyanato-4-(methylsufinyl)butane has been shown to be an important anticarcinogen by inducing quinone reductase, a phase II detoxification enzyme in Hepa1c1c7 cell cultures (Zhang et al., 1992) and since this report many other studies supporting these findings have been published (Zhang et al., 1994; Gamet-Payrastre et al., 2000; Chiao et al., 2002; Fahey et al., 2002; Petri et al., 2003). Thus expression of ESP will be important in determining the bioactive profile of Brassica food crops.

We have set out to explore the possibility of using ESP to modify plant glucosinolate degradation profiles using *Arabidopsis* as a model. We use recombinant ESP to demonstrate an in vitro Fe(II) requirement for ESP activity in the formation of the thiirane ring.

2. Results and discussion

2.1. Activity of recombinant ESP: substrate specificity and effect of ferrous ions

The *B. napus* ESP peptide sequence was used to search for homology in the *Arabidopsis* genome using BLASTP. The predicted *A. thaliana* open reading frames were then aligned by ClustalW using their designated NCBI Accession Numbers. Based upon *B. napus* peptide sequence, NCBI Accession Number AAD25776 was predicted to encode ESP since it alone contains the unique insertions of 6 and 9 amino acids between positions 149–154 and 206–214, respectively, of the expected gene product.

A corresponding *ESP* EST clone (GenBank Accession Number H37255) was cloned into the prokaryotic expression vector pTWIN1 to produce recombinant ESP comprising the 341 aa ESP protein (37 kDa) and an *N*-terminal chitin binding domain-intein tag (25.5 kDa). SDS–PAGE analysis of the bacterial pellet after IPTG induction verified the presence of a product of ~62 kDa (predicted 62.5 kDa; Fig. 2).

The substrate specificity of ESP and the effect of ferrous ions on its activity were assayed by incubating the recombinant ESP protein with different glucosinolates and a range of ferrous ions concentrations (0–2 mM) in the presence of myrosinase from *Sinapis alba*.

From 2-propenylglucosinolate (Fig. 3(a)), ESP catalysed the formation of 2-(thiiran-2-yl)acetonitrile in a ferrous ion dependent manner. Myrosinase and ferrous ions alone increase the nitrile proportion which reaches over 90% at 2 mM (Fig. 3(b)).

When (*R*)-2-hydroxybut-3-enylglucosinolate (Fig. 4(a)) is used as the glucosinolate in the assay in the presence of ESP, 3-hydroxy-3-(thiiran-2-yl)propanenitrile and trace amounts of 3-hydroxy-4-pentenenitrile are detected. As is the case with 2-propenylglucosinolate the

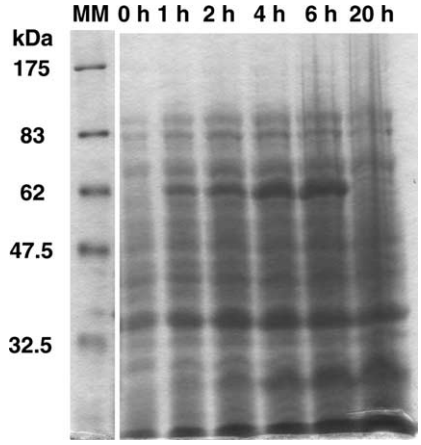

Fig. 2. SDS–PAGE analysis of the IPTG induction of recombinant ESP in *E. coli*. Transformed cells were pelleted and analysed on a 10% (w/v) polyacrylamide vertical slab gel. MM: Prestained Protein Marker (New England Biolabs); aliquots were analysed after 0, 1, 2, 4, 6 and 20 h of induction at 28 °C with 0.5 mM IPTG.

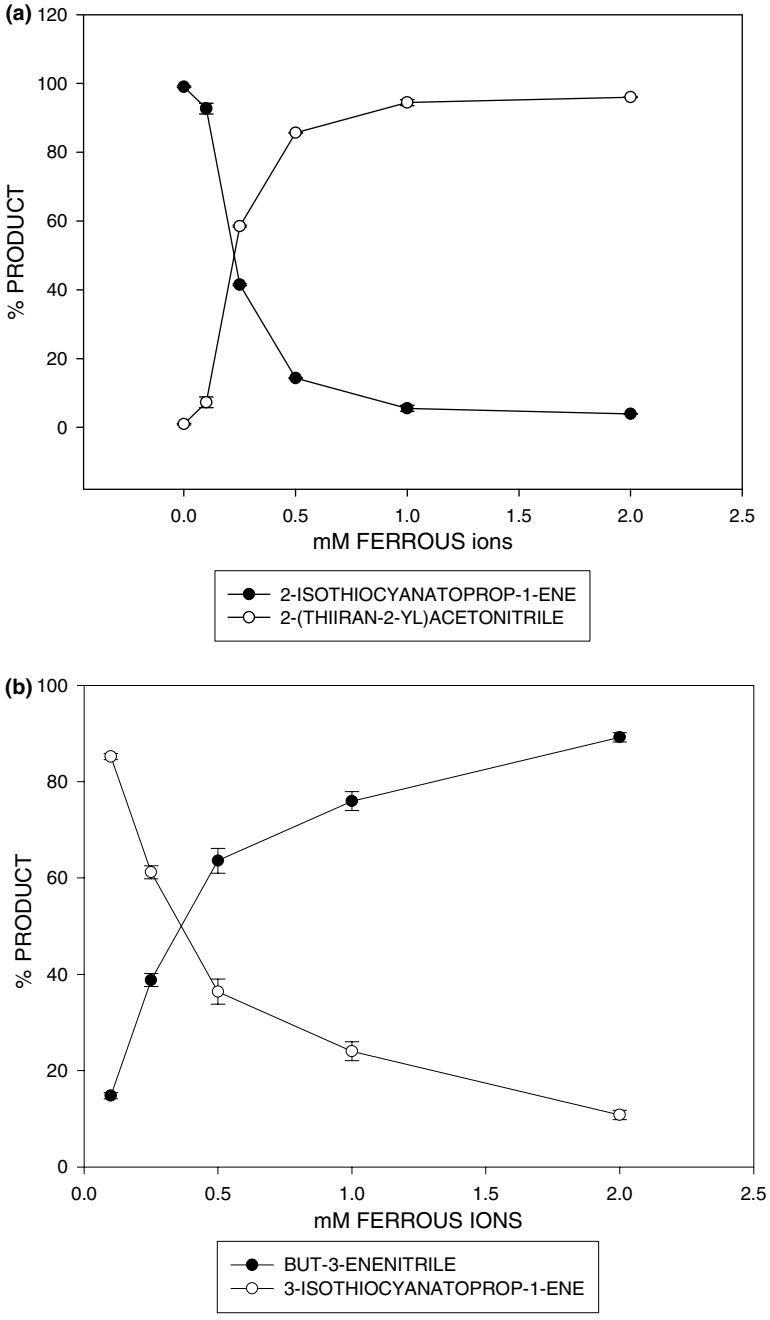

Fig. 3. Assays of recombinant ESP activity on 2-propenylglucosinolate. Effect of ferrous ion concentration (0–2.0 mM) on the proportion of 2-(thiiran-2-yl)acetonitrile, 3-isothiocyanatoprop-1-ene and but-3-enenitrile on hydrolysis of 2-propenylglucosinolate by myrosinase in the presence (a) and absence (b) of recombinant ESP.

production of the corresponding epithionitriles from (*R*)-2-hydroxybut-3-enylglucosinolate is ferrous ion dependent, showing a strong increase of their proportion between 0.1 and 0.25 mM (Fig. 4(a)). The major product formed by ESP in the absence of ferrous ions is 5-vinyloxazolidine-2-thione with small amounts of 3-hydroxy-3-(thiiran-2-yl)propanenitrile. 5-Vinyloxazolidine-2-thione is also the major product formed in the absence of both ESP and ferrous ions (Fig. 4(b)), while the addition of ferrous ions results in an increase in the corresponding nitrile.

In a third series of ESP activity assays 4-methylsulfinylbutylglucosinolate was used. In the absence of ferrous ions the only product detected is 1-isothiocyanato-4-(methylsulfinyl)butane. Its proportion decreases, however, with increasing concentrations of ferrous ions and 5-(methylsulfinyl)pentanenitrile is formed (Fig. 5). With 4-methylsulfinylbutylglucosinolate

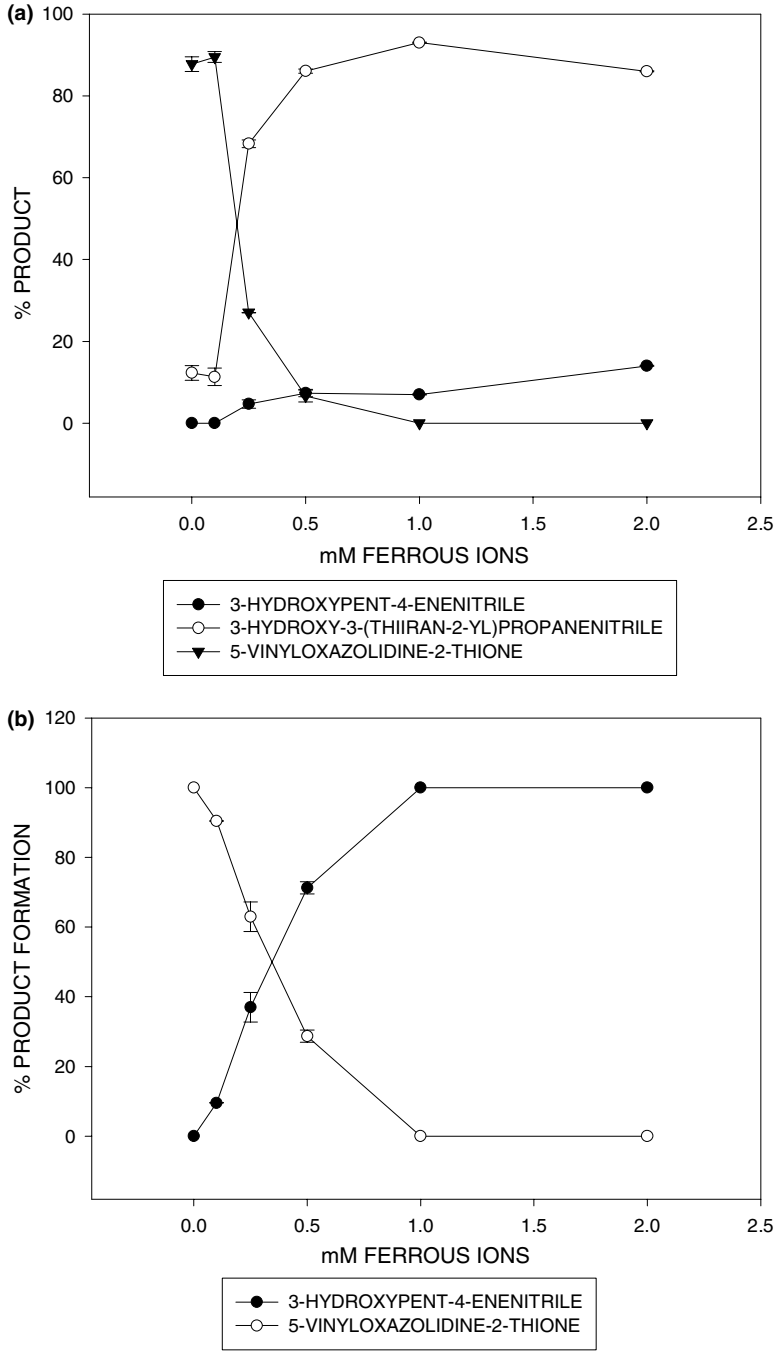

Fig. 4. Assays of recombinant ESP activity on (*R*)-2-hydroxybut-3-enylglucosinolate. Effect of ferrous ion concentration (0–2.0 mM) on the proportion of 3-hydroxy-3-(thiiran-2-yl)propanenitrile, 5-vinyloxazolidine-2-thione and 3-hydroxy-4-pentenenitrile formation after hydrolysis of (*R*)-2-hydroxybut-3-enylglucosinolate by myrosinase in the presence (a) and absence (b) of recombinant ESP.

the presence of recombinant ESP has no influence on the nature of the hydrolysis product formed indicating that the unstable thiohydroximate-*O*-sulphonate intermediate generated by myrosinase activity upon 4-methylsulfinylbutylglucosinolate is not a substrate for recombinant ESP.

The assays with recombinant ESP indicate clearly that activity is ferrous ion dependent (Figs. 3(a), 4(a) and 5). Moreover ESP only has activity towards thiohydroximate-*O*-sulphonate intermediates generated by myrosinase activity on alkenylglucosinolates (2-propenylglucosinolate and (*R*)-2-hydroxybut-3-enylglucosinolate in our assays) but not on alkylglucosinolates (4-methylsulfinylbutylglucosinolate). In addition, the production of nitriles with the three different glucosinolates in the absence of recombinant ESP shows that nitrile formation is ESP independent. This is in disagreement with an earlier report (Lambrix et al., 2001) where the

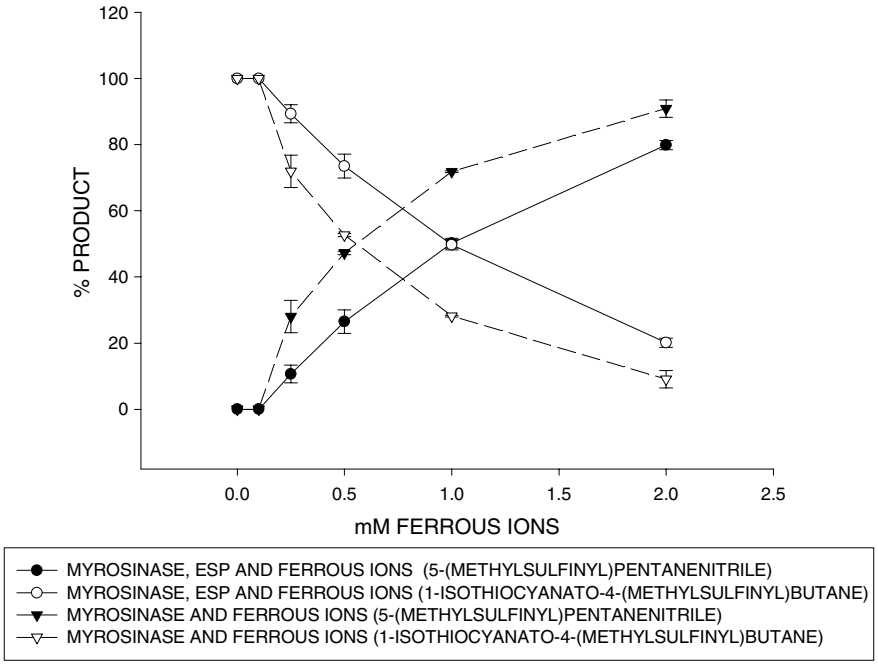

Fig. 5. Assays of recombinant ESP activity on 4-methylsulfinylbutylglucosinolate. Effect of ferrous ion concentration (0–2.0 mM) on the proportion of 1-isothiocyanato-4-(methylsufinyl)butane and 5-(methylsulfinyl)pentanenitrile formation after hydrolysis of 4-methylsulfinylbutylglucosinolate by myrosinase in the presence or absence of recombinant ESP.

authors described the capability of recombinant ESP from *A. thaliana* to convert 3-hydroxypropylglucosinolate and 4-methylsulfinylbutylglucosinolate into their respective nitriles. Whereas they only observed isothiocyanate production in the absence of ESP (Lambrix et al., 2001), in our assays the sole presence of myrosinase and ferrous ions is sufficient for nitrile production, and in the case of 4-methylsulfinylbutylglucosinolate the presence of ESP even decreases the proportion of nitrile to isothiocyanate at a given ferrous ion concentration (Fig. 5). The reasons for the discrepancies between these two recombinant ESP expression systems remain to be elucidated.

2.2. Ectopic expression of ESP

The *A. thaliana* EST encoding ESP was cloned into a binary vector behind the strong viral promoter CaMV *35S* and introduced into Col-5 plants. We first determined the levels of ESP expression in leaf material of *S. alba* (Sa), *B. napus* (Bn), *Raphanus sativum* (Rs) and *A. thaliana* Col-5 (At) using the *Arabidopsis ESP* cDNA as a probe (Fig. 6(a)). As expected (Foo et al., 2000) *ESP* was absent in radish, Col-5 and *S. alba* but present in *B. napus* (Fig. 6(a)). The integrity of selected transformed lines was verified by sequencing transgene specific PCR products. ESP expression levels in *35S::ESP* plants were determined by RNA blot analysis (Fig. 6(b)). A number of different transgenic lines expressing high levels of *ESP* transcripts were selected and assessed for ESP activity by grinding leaf tissue and analysing the degradation products by GC–MS. One transgenic line (ESPS20) on the basis of its consistent production of nitriles was taken to the T2 generation and analysed in detail for its hydrolysis profile (Table 1). Compared to

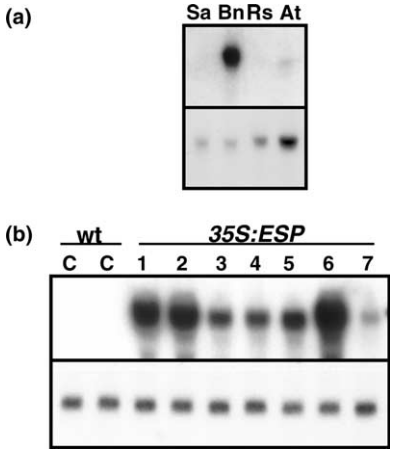

Fig. 6. RNA blot analysis of *ESP* transcript levels. (a) Comparison of steady state levels of *ESP* in leaves (5 week old) of *S. alba* (Sa), *B. napus* (Bn), *Raphanus sativum* (Rs) and *A. thaliana* Col-5 (At). 10 μg of total RNA was loaded in each lane with the exception of *A. thaliana*, where 20 μg was loaded to visualise the endogenous *ESP* levels. The lower panel shows the same blot probed with the *A. thaliana Actin2* gene probe. (b) Verification of *35S::ESP* transgenic lines. Total RNA (15 μg/lane) extracted from leaves of untransformed *A. thaliana* was compared to RNA extracted from 4 independent *35S::ESP* transgenic lines (lanes 1 and 2 = ESPS5, lanes 3 and 4 = ESPS20, lanes 5 and 6 = ESPS19, lane 7 = ESPS18, lanes c = wild type Col-5).

Table 1
The hydrolysis profile of wild type and epithiospecifier protein over-expressing Col-5 plants and the influence of the addition of EDTA in the absence and presence of (*R*)-2-hydroxybut-3-enylglucosinolate

Plant	3-OH-NIT	3-OH-ETN	GOITRIN	4-MSB-NIT	4-MSB-ITC
ESPS20	na	na	na	67.8 ± 5.2	32.2 ± 5.2
ESPS20 + PG	9.4 ± 0.4	90.6 ± 0.4	0	66.6 ± 6.3	33.4 ± 6.3
ESPS20 + PG +EDTA	2.7 ± 1.4	21.7 ± 10.9	74.6 ± 10.2	28.1 ± 9.6	71.9 ± 9.6
WT-COL-5	na	na	na	8.6 ± 1.8	91.4 ± 1.8
WT-COL-5 + PG	27.3 ± 4.9	0	72.7 ± 4.9	6.9 ± 0.1	93.1 ± 0.1
WT-COL-5 + PG + EDTA	5.24 ± 1.0	0	94.76 ± 1.0	0	100

ESP activity assays were performed on 300 mg of fresh leaf tissue of wild type Col-5 (WT-COL-5) and ESP over-expressing (ESPS20) plants (6 weeks after pricking out) in the absence or presence of (*R*)-2-hydroxybut-3-enylglucosinolate (1 mg) and/or EDTA (5 mM), extracted with dichloromethane and analysed by GC–MS. Results are expressed as a percentage of hydrolysis products from each glucosinolate (i.e., (*R*)-2-hydroxybut-3-enylglucosinolate and 4-methylsufinylbutylglucosinolate). Data are means ± SE ($n = 3$). PG = (*R*)-2-hydroxybut-3-enylglucosinolate; 3-OH-NIT = 3-hydroxypent-4-enenitrile; 3-OH-ETN = 3-hydroxy-3-(thiiran-2-yl)propanenitrile; GOITRIN = 5-vinyloxazolidine-2-thione; 4-MSB-NIT = 5-(methylsulfinyl)pentanenitrile; 4-MSB-ITC = 1-isothiocyanato-4-(methylsulfinyl)butane.

the wild type Col-5 which produces mostly 1-isothiocyanato-4-(methylsulfinyl)butane with some 5-(methylsulfinyl)pentanenitrile the transformed plant produces significant amounts of the nitrile (Table 1). To further assess the role of ferrous ions on the ESP activity in planta (*R*)-2-hydroxybut-3-enylglucosinolate was added to fresh tissue of wild type Col-5 and ESP over-expressing plants (ESPS20) with or without the iron chelator EDTA (Table 1). The addition of (*R*)-2-hydroxybut-3-enylglucosinolate to the assays leads to the formation of 5-vinyloxazolidine-2-thione and the corresponding nitrile in a ratio of approximately 2.5:1 but no 3-hydroxy-3-(thiiran-2-yl)propanenitrile for the wild type plant. In the ESPS20 assay however, the hydrolysis profile is made up of 90.6% 3-hydroxy-3-(thiiran-2-yl)propanenitrile and 9.4% 3-hydroxypent-4-enenitrile, revealing the ESP activity in these plants. If EDTA is added to the assays, in order to lower the ferrous ion concentration, the proportion of nitriles derived from both the added (*R*)-2-hydroxybut-3-enylglucosinolate and the endogenous 4-methylsufinylbutylglucosinolate decreases. As expected, this is accompanied by an increase of the proportion of 5-vinyloxazolidine-2-thione and 1-isothiocyanato-4-(methylsulfinyl)butane, respectively, showing that the nitrile formation is favoured by high ferrous ion concentrations i.e. EDTA chelates ferrous ions. In addition, for the ESPS20 tissue the epithionitrile proportion also decreases, confirming the ferrous ion dependency of ESP activity already observed in the recombinant ESP activity assays.

As mentioned before, during the course of this study a paper describing ESP from the *A. thaliana* accession Ei-2 emerged (Lambrix et al., 2001). Critically, ESP activity was assayed in vitro from crude extracts of bacteria expressing Ei-2 ESP. These assays showed no dependency on Fe(II) for ESP activity. To examine whether our recombinant ESP differs significantly from Ei-2 ESP we compared the predicted amino acid sequences of the two ESP alleles (Fig. 7). There are only three amino acid differences between the sequences, one conservative $T_{18}S$ and two nonconservative changes, $G_{13}E$ and $A_{308}P$, at the amino and carboxyl termini. No predicted iron binding sites could be found by searching available pattern databases suggesting the differences in iron dependency were not due to primary sequence divergence.

The divalent cations, Mn^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, Mo^{2+}, and Co^{2+} were all tested at 0.6 mM for their effects on ESP/myrosinase hydrolysis of 2-propenylglucosinolate. Unlike Fe(II) which directs degradation exclusively to the epithionitrile, presence of these metal ions produced only isothiocyanates and no detectable amounts of nitrile or epithionitrile (results not shown). Our data strongly support Fe(II) as the only metal involved in the ESP reaction mechanism.

In planta iron concentration was estimated at a concentration of 0.14 mM in the assays for myrosinase/ESP activity. However, although this represents total and not available Fe(II) and most iron in the leaf is likely to be in the Fe(II) form, then it is possible that concentrations would be sufficient for ESP function in the transformed *A. thaliana* plants. Previous work (MacLeod and Rossiter, 1986, 1987) has shown that myrosinase will generate nitriles in Fe(II) concentrations as low as 0.093 mM. However, although unlikely the possible existence of a Fe(II) independent mechanism for ESP cannot be ruled out, nor the possibility of a bound form of Fe(II) that interacts with ESP and myrosinase.

Therefore, the question of Fe(II) dependency for nitrile and epithionitrile formation in the plant remains an open question. Thus our data indicate previous hypotheses predicting ESP as being solely involved in the formation of nitriles alone require further examination since sulphur insertion into the alkenyl group is clearly enzymatic and dependent on Fe(II).

In the transgenic plants 100% nitrile was never achieved suggesting that either concentrations of Fe(II) are not sufficiently high or that the 4-methylsulfinylbutylglucosinolate is not a substrate for ESP.

Fig. 7. Comparison of predicted ESP proteins from accessions Ei-2 and Col-0 ClustalW alignment of predicted ESP proteins derived from accession Ei-2 (Lambrix et al., 2001) or Col-0 (this paper). Variant amino acid residues are shaded grey.

The different assays described here, using both recombinant ESP and ESP over-expressing plants, show clearly the ferrous ion dependency of the conversion of alkenyl glucosinolates to epithionitriles by ESP. They also show that, at least at the conditions used, nitrile formation from alkenyl and nonalkenyl glucosinolates does not require the presence of ESP activity and that 4-methylsulfinylbutylglucosinolate is not a substrate for recombinant ESP. It has been suggested that there may be a protein that modifies the activity of epithiospecifier protein (Lambrix et al., 2001) which may explain our results although it seems unlikely that ESP can function without ferrous ions.

Our experiments provide proof that by using a targeted transgenic approach, it is possible to alter the chemistry of plants without any noticeable phenotypic effect on the plant. We lay the foundation to extend these studies towards manipulation of glucosinolate degradation profiles in *Arabidopsis*. Thus by showing it is possible to manipulate ESP in *Arabidopsis* it will be possible to use this approach to alter the bioactive components of glucosinolate profiles which will be of importance in investigating the importance of nitriles versus isothiocyanates in plant–insect interactions and in studies examining biological interactions.

3. Experimental

3.1. Chemical standards

1-Isothiocyanato-4-(methylsufinyl)butane were obtained from LKT Laboratories Inc and 5-(methylsulfinyl)pentanenitrile was synthesised. Mass spectra of hydrolysis products were compared to known data (Spencer and Daxenbichler, 1980). 2-Hydroxybut-3-enylglucosinolate was isolated from *B. napus* seed and 4-methylsulfinylbutylglucosinolate was obtained by a chemoenzymatic route (Iori et al., 1999) and 2-propenylglucosinolate from Sigma–Aldrich.

3.2. Ectopic expression of ESP in A. thaliana

A *35S::ESP* fusion in a T-DNA binary vector was constructed by ligating the *ESP* cDNA as an *Xba*I filled, *Nco*I restriction fragment into pCambia 3201 (http://www.cambia.org) digested with *Nco*I and *Pml*I . The construct, *35S::ESP*, was verified by sequencing and introduced into *Agrobacterium tumefaciens* GV3101-(pRK190) by electroporation. *A. thaliana* Col-5 plants were transformed by a simple dip transformation (Clough and Bent, 1998). Primary transformants were selected after germination on soil watered with glufosinate

ammonium (150 mg l^{-1}, Hoechst). The integrity of primary transgenes was verified by sequencing *35S::ESP* PCR products using *CaMV 35S* and *ESP* specific primers, and by RNA blot analysis. Homozygous *35S::ESP* plants were selected from the T2 generation.

3.3. Growth of plants and treatments

A. thaliana accession Col-5 seeds were sown in Levington F2 compost and vernalised for 2 days at 4 °C. Plants were grown under short day conditions in a controlled environment chamber (12 h light, 100–150 μmol m^{-2} s^{-1} at 22 °C day, 20 °C night) for 5–6 weeks before use.

3.4. RNA blot analysis

Total leaf RNA was isolated by guanidinium hydrochloride extraction as described (Logemann et al., 1987). Total RNA (14 μg) was fractionated on denaturing 1.5% formaldehyde agarose gels (Sambrook et al., 1989) and transferred to Hybond-N nylon membrane (Amersham). RNA loading was determined by visualising the ethidium bromide-stained ribosomal RNA content and/or hybridisation to ^{32}P *A. thaliana actin 2* (GenBank Accession Number AY064043). ^{32}P labelled ESP probes were prepared using a random primer labelling kit (Stratagene). Washes of the ESP blots were carried out at a final stringency of 0.02X SSC, 0.1% SDS for 30 min at 62 °C.

3.5. Prokaryotic expression of ESP

A full length *A. thaliana ESP* cDNA clone (At1g54040) was identified in the *A. thaliana* EST database. The *ESP* cDNA was excised as an *Eco*RI filled, *Nco*I restriction fragment of ~1.4 kb and ligated into *Eco*RI filled, *Nco*I digested pTWIN1 (IMPACT-TWIN; New England Biolabs). This leads to an N-terminal fusion protein with a theoretical molecular mass of 62.5 kDa.

This pTWIN-ESP construct was, after sequence verification, transformed into Epicurian Coli BL21-CodonPlus (DE3)-RIL cells (Stratagene). ESP expression was verified by monitoring the accumulation of recombinant protein over time by SDS–PAGE.

3.6. Activity assays of the recombinant ESP protein

The BL21-CodonPlus cells transformed with pTWIN-ESP were inoculated into 100 ml of LB medium and grown at 37 °C to an OD_{600} of 0.6 before induction with 0.5 mM IPTG (isopropyl β-D-thiogalactopyranoside, Sigma) at 28 °C. After 6 h the bacteria were pelleted, re-suspended in imidazole buffer (0.1 M, pH 6.5) and pelleted again. The bacterial pellet was re-suspended in 2.5 ml imidazole buffer, disrupted by French Press and pelleted. The clear supernatant was desalted on a Bio-Rad Econo-Pac 10 DG column.

Assays were performed using 4-methylsulfinylbutylglucosinolate, 2-propenylglucosinolate and (*R*)-2-hydroxybut-3-enylglucosinolate (10 mM) in 100 μl of 0.1 mM imidazole buffer containing 16.5 μg partially pure myrosinase from *S. alba*, 80 μl (375 μg) of desalted ESP supernatant and a range of Fe(II) ion (ferrous ammonium sulphate) concentrations. The assays were incubated for 30 min, extracted with dichloromethane and ESP activity was determined (Foo et al., 2000) by measuring the resulting hydrolysis products by capillary GC.

Samples were analysed by GC–MS on a Hewlett–Packard 6890 GC linked to a 5973 MSD. Injections were made onto a HP-5MS 5% Phenylmethylsiloxane (30 m × 250 mm) column in the pulsed splitless or split mode using a temperature programme: inlet temp 225 °C; initial temperature 40 °C, 5 min; 5 °C min^{-1} until 180 °C; 10 °C min^{-1} until 280 °C; hold 10 min. All compounds were identified by using either standards or by their mass fragmentation patterns.

3.7. Analysis of hydrolysis products in fresh leaf tissue

Leaves from single plants (300 mg fresh weight) were ground in a homogenisation tube and incubated for 5 min 30 °C. The homogenate was extracted with dichloromethane (2 × 2 ml) and the combined organic phases dried with anhydrous $MgSO_4$, filtered and carefully concentrated using a flow of argon gas. Samples were analysed by GC–MS as previously described.

3.8. Analysis of iron

The analysis of iron was carried out using a standard acid digestion method followed by flame photometry (Allen, 1989).

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