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Epithiospecifier protein activity in broccoli: The link between terminal alkenyl glucosinolates and sulphoraphane nitrile

David J. Williams [a,b,*], Christa Critchley [a], Sharon Pun [b], Stephen Nottingham [b], Timothy J. O'Hare [c]

[a] The University of Queensland, School of Integrative Biology, Brisbane, Qld 4072, Australia
[b] Department of Primary Industries and Fisheries, 19 Hercules Street, Hamilton, Qld 4007, Australia
[c] Department of Primary Industries and Fisheries, Gatton, Qld 4343, Australia

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ABSTRACT

The chemical nature of the hydrolysis products from the glucosinolate-myrosinase system depends on the presence or absence of supplementary proteins, such as epithiospecifier proteins (ESPs). ESPs (non-catalytic cofactors of myrosinase) promote the formation of epithionitriles from terminal alkenyl glucosinolates and as recent evidence suggests, simple nitriles at the expense of isothiocyanates. The ratio of ESP activity to myrosinase activity is crucial in determining the proportion of these nitriles produced on hydrolysis. Sulphoraphane, a major isothiocyanate produced in broccoli seedlings, has been found to be a potent inducer of phase 2 detoxification enzymes. However, ESP may also support the formation of the non-inductive sulphoraphane nitrile. Our objective was to monitor changes in ESP activity during the development of broccoli seedlings and link these activity changes with myrosinase activity, the level of terminal alkenyl glucosinolates and sulphoraphane nitrile formed. Here, for the first time, we show ESP activity increases up to day 2 after germination before decreasing again to seed activity levels at day 5. These activity changes paralleled changes in myrosinase activity and terminal alkenyl glucosinolate content. There is a significant relationship between ESP activity and the formation of sulforaphane nitrile in broccoli seedlings. The significance of these findings for the health benefits conferred by eating broccoli seedlings is briefly discussed.

1. Introduction

Glucosinolates are sulphur containing glycosides found in cruciferous plants (Rodman et al., 1998) including agriculturally important crop plants such as crambe (*Crambe abyssinica)* and *Brassica* vegetables, e.g. broccoli (*Brassica oleracea* var. *italica*), as well as the model plant *Arabidopsis thaliana*. They consist of a thioglucoside moiety linked to a variety of amino-acid derived side chains and may be classified into chemical families according to these side chains (Fenwick et al., 1986; Quinsac, 1993), which include alkyl, alkenyl, methylthioalkyl, methylsulphinylalkyl, and indole groups. The composition and content of glucosinolates vary widely between different species and developmental stages within a given species (Macfarlane Smith and Wynne Griffiths, 1988; McGregor, 1988; Porter et al., 1991; Li et al., 1999; Petersen et al., 2002; Brown et al., 2003). At present limited information is available on these variations during early plant development in cruciferous plants, with most studies focussing on differences between seeds and mature plants (Kjaer, 1974; Fahey et al., 1997; Fahey and Stephenson, 1999; West et al., 2004) or in the organs of cabbage seedlings (Rosa, 1997) or the model plant *A. thaliana* (Petersen et al., 2002; Brown et al., 2003).

When cells of these plants are damaged (e.g. in food preparation, chewing or pest attack), the glucosinolates are hydrolysed by the endogenous plant enzyme myrosinase. The extent of hydrolysis and the products formed depends largely upon the activity and source, i.e. endogenous or exogenous of the myrosinase, presence of supplementary proteins, e.g. epithiospecifier protein (ESP) and hydrolysis conditions such as pH and temperature (Fenwick et al., 1983; Ludikhuyze et al., 2000; Burow et al., 2006). The principle hydrolysis products formed are isothiocyanates and nitriles (see also Fig. 6a). Additionally, glucosinolates with terminal alkenyl groups in the presence of ESP are capable of hydrolysing to form epithionitriles (see also Fig. 6b). Kaoulla et al. (1980) and MacLeod and Rossiter (1985) established a link between ESP activity and the level of these terminal alkenyl glucosinolates. This prompted MacLeod and Rossiter (1985) to speculate that activity is absent from those species lacking glucosinolates with terminal alkenyl side chains. This speculation was supported by Lambrix et al. (2001) who found that the presence of a functional ESP in *Arabidopsis* ecotypes coincided with the accumulation of alkenyl glucosinolates. Since these studies little research has been conducted into this relationship, despite potential for decreasing ESP activity

* Corresponding author. Address: Department of Primary Industries and Fisheries, 19 Hercules Street, Hamilton, Qld 4007, Australia. Tel.: +61 7 34068586; fax: +61 7 34068665.
E-mail address: williad1@dpi.qld.gov.au (D.J. Williams).

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through traditional breeding technology. A major health benefit from reducing ESP activity could be vegetables with increased levels of isothiocyanates.

From a human nutritional perspective, isothiocyanates are the most important of these breakdown products. Isothiocyanates are inducers of phase 2 detoxification enzymes, which protect against carcinogens and other toxic electrophiles (Zhang et al., 1992, 1994; Mithen et al., 2003). Glucoraphanin (4-methylsulphinylbutyl glucosinolate) is the predominant glucosinolate in most broccoli varieties and upon hydrolysis it forms the isothiocyanate sulphoraphane (4-methylsulphinylbutyl isothiocyanate) and sulphoraphane nitrile (5-methylsulphinylpentane nitrile) (see also Fig. 6a). Sulphoraphane has been identified as a particularly potent inducer of these phase 2 detoxification enzymes in mammalian cell cultures and rodents (Zhang et al., 1992; Faulkner et al., 1998). Sulphoraphane nitrile is ineffective as an inducer of these enzymes in cell cultures (Matusheski and Jeffery, 2001; Basten et al., 2002; Mithen et al., 2003). Several recent studies showed that ESP has a broader role in addition to directing epithionitrile formation. It also promotes the formation of simple nitriles and in broccoli, sulphoraphane nitrile is formed from the endogenous glucoraphanin (Lambrix et al., 2001; Matusheski et al., 2004). However, this proposal was recently challenged by de Torres Zabala et al. (2005). Using recombinant *Arabidopsis* ESP they found that production of nitriles was ESP independent. In contrast to this result Matusheski et al. (2006) found that the recombinant broccoli protein directed the hydrolysis of glucoraphanin to form sulforaphane nitrile. These authors did not offer a reason for the discrepancy between theirs and the earlier study. If ESP can indeed influence the formation of nitriles, then its activity is of significance if the potential health benefits of these plants are to be realised.

To date investigations into ESP have focussed on seeds (Kaoulla et al., 1980; MacLeod and Rossiter, 1985; Bernardi et al., 2000; Foo et al., 2000), 5 day old seedlings (Matusheski et al., 2004) or mature plants (Lambrix et al., 2001; Matusheski et al., 2004; Burow et al., 2006). Information on changes in ESP activity during the early stages of plant development is scarce which is surprising as there is extensive literature on the potential health benefits of consuming seedlings or to use the commercial term for sprouted seeds, sprouts (Fahey et al., 1997; Nestle, 1997; Shapiro et al., 2001; Lee and Lee, 2006; Bennett et al., 2007). This information would be especially valuable as most broccoli seedlings are eaten raw in a bid to maximise any potential health benefits thus providing no physical hindrance, i.e. cooking to ESP activity (Fahey et al., 1997; Nestle, 1997; Oswald and Oswald, 2002; O'Hare et al., 2008). Our objectives in this study were to provide a detailed analysis of ESP activity in several cultivars of broccoli during early development as well as identifying factors that may influence ESP activity. Also the study aims to provide additional evidence for the expanded role of ESP.

2. Results and discussion

2.1. Development from seed to seedling

As part of this study we investigated the transition from seed to seedling in detail using seeds germinated on wetted paper sheets held in the dark at 25 °C. One day after placement in the sheets there were no visible changes except for seed hydration. However, two days after planting the radicles emerged with shoots on average 10 mm long and 15 mm roots. By the end of the 14 day test period the length of the shoots had grown steadily to 70 mm with the root length increasing to about 100 mm. The moisture content and the results of the germination test are shown in Table 1. The high germination rate for all the seeds tested suggests any increases in glucosinolate content or ESP activity were not due to high levels of non-viable old seeds which have characteristically lower levels of glucosinolates and ESP activity (Cole, 1980; Rangkadilok et al., 2002).

Table 1
Mean moisture contents (g/100 g) ($n = 3$) and germination (%) ($n = 3$) in three cultivars of broccoli and crambe during seedling development

Plant age in days	Mean moisture contents (g/100 g) and Germination (%) Plant			
	Calabrese	DeCicco	Romanesco	Crambe
Seed	4.6 (89%)	7.8 (93%)	3.6 (85%)	6.7 (86%)
2	82.4	66.5	76	68.9
3	89.2	84.5	86.4	87.7
4	92.1	91.3	91.9	89.2
5	93.3	93.3	93	90.9
6	94	93.9	94.1	93.7
7	94.7	94.4	94.5	93
10	95.4	95.7	95.6	nt
12	96	95.6	96	95.5
14	95.4	96.2	95.7	nt

nt, not tested.

2.2. Glucoraphanin in broccoli cultivars during seedling development

The glucoraphanin content of seeds and seedlings of the three commercial broccoli cultivars increased markedly during the first days of development (Table 2; Fig. 1), dropped after day 2 and were stable from day 4 to 14. The cultivar DeCicco investigated was also represented in an earlier study (West et al., 2004), where seeds from 34 cultivars of broccoli were examined for glucoraphanin content. Our result (31.7 μmol/g FW) was in close agreement with the 34.5 μmol/g FW given by these authors. Further comparisons were limited as there is little information on glucoraphanin level changes during early development. Pereira and co-workers (2002) studied the effects of temperature on levels of glucosinolates in developing broccoli seedlings. They found glucoraphanin levels of 49.5 μmol/g DW in 6 day old cv. Marathon seedlings grown at 30/15 °C day/night temperatures. This value and the levels found in this experiment are much higher than the 10.3 μmol/g DW reported by Matusheski et al. (2004) for 5 day old cv. Majestic seedlings. This large variation in glucoraphanin content among different broccoli cultivars has been observed by many investigators (Fahey et al., 1997; Pereira et al., 2002; Matusheski et al., 2004; West et al., 2004). Using the model plant *A. thaliana*, Brown et al.

Table 2
Glucoraphanin content (μmol/g DW) in three cultivars of broccoli during seedling development

Plant age in days	Glucoraphanin content [± S.D., μmol/g DW] Cultivar					
	Calabrese		DeCicco		Romanesco	
Seed	34.1 ± 2.09	a	34.4 ± 2.42	a	30.5 ± 0.92	a
2	97.7 ± 4.90	b	88.3 ± 2.93	b	77.5 ± 1.73	b
3	56.4 ± 3.38	cd	63.2 ± 4.65	c	52.2 ± 4.51	cd
4	67.0 ± 1.65	e	79.3 ± 3.00	de	60.4 ± 2.38	ef
5	65.6 ± 1.47	ef	82.0 ± 5.42	d	51.4 ± 0.72	d
6	60.0 ± 0.43	gc	77.0 ± 1.15	de	64.4 ± 1.58	ge
7	60.3 ± 3.39	fgc	75.0 ± 3.65	e	58.1 ± 2.83	efc
10	54.3 ± 5.22	d	79.5 ± 5.04	de	55.8 ± 8.14	fcd
12	65.0 ± 3.31	efg	61.3 ± 1.57	c	67.5 ± 5.50	g
14	65.0 ± 2.69	efg	60.0 ± 2.54	c	60.0 ± 3.46	ef

Within each cultivar means followed by a common letter are not significantly different at $P = 0.050$.

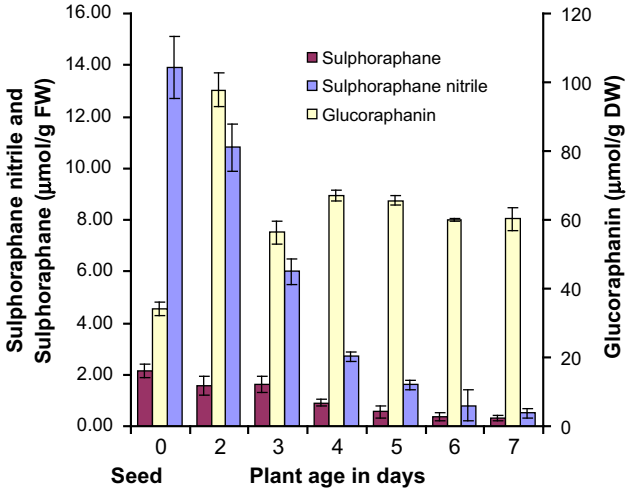

Fig. 1. Effect of seedling age on glucoraphanin concentration (µmol/g DW) and subsequent formation of sulphoraphane and sulphoraphane nitrile (µmol/g FW) in the broccoli cultivar Calabrese.

(2003) investigated glucosinolate concentrations during germination. Our findings closely follow the pattern shown by these authors who reported a general increase in total glucosinolates during the first two days and then a decrease of 30% between days 2 and 8. In the present study, glucoraphanin loss from day 2 to day 7 was 38% for cv Calabrese, 15% for cv. DeCicco and 25% for cv. Romanesco. As the losses were substantial Brown and his co-workers (2003) suggested the disappearance of glucosinolates may be a consequence of catabolic processes occurring in the intact plants.

2.3. Changes in terminal alkenyl glucosinolates during seedling development

Fig. 2 shows changes in the levels of terminal alkenyl glucosinolates during plant development of the broccoli cultivar Calabrese which was deemed typical of the three cultivars tested. The major alkenyl glucosinolate in all three cultivars was progoitrin (2(*R*)-2-hydroxy-3-butenyl glucosinolate), with smaller amounts of sinigrin (allyl glucosinolate) and gluconapin (3-butenyl glucosinolate). This is in agreement with studies of Kushad et al. (1999) and West et al. (2004) who found substantial amounts of progoitrin in the seeds of the broccoli cultivar DeCicco. Progoitrin, sinigrin and gluconapin contents were much lower than the glucoraphanin levels but the changes over time followed a similar pattern to glucoraphanin in all three cultivars examined. This pattern agrees with changes in progoitrin levels presented by MacGregor (1988) in a study into glucosinolate content of developing rapeseed *(Brassica napus* L. 'Midas') seedlings. The author found the progoitrin levels relative to the total glucosinolates measured increased to day 6 and then decreased thereafter. The changes in the levels of alkenyl glucosinolates after day 2 in the present study were more dramatic than those shown by glucoraphanin. The minor glucosinolates were reduced by 57% (cv. Calabrese), 51% (cv. DeCicco) and 58% (cv. Romanesco). The extent of the losses provides supporting evidence that glucosinolates may be undergoing catabolism. Also the larger losses experienced by the alkenyl glucosinolates agree with the suggestion of Brown and co-workers (2003) that this degradative system has some specificity. As changes in the full array of glucosinolates and glucosinolate breakdown products were not monitored in this experiment more detailed studies are needed to evaluate the contributions of synthesis and degradation to these variations in the seeds and seedlings of these plants.

2.4. ESP activity of broccoli cultivars during seedling development

Similar to the pattern exhibited by terminal alkenyl glucosinolate concentration, ESP activity increased dramatically after germination, showing a maximum in day 2 seedlings, decreasing rapidly by day 4 and then levelling to the end of the experiment (Fig. 2). This pattern was repeated for the other two broccoli cultivars tested. As far as we are aware this is the first time that changes in ESP activity have been reported for any broccoli cultivars during early plant development. The extent of these variations is even more surprising when results of ESP activity changes in seeds and seedlings of crambe are considered (Fig. 3). The ESP activity of the crambe seeds and seedlings showed the more conventional pattern with seeds possessing the highest activity and a steady slow decrease with plant age. There have been abundant studies into ESP activity of brassicaceous seeds (Tookey, 1973; Cole, 1978; Kaoulla et al., 1980) and mature plants (Lambrix et al., 2001; Matusheski et al., 2004, 2006). The results of these studies

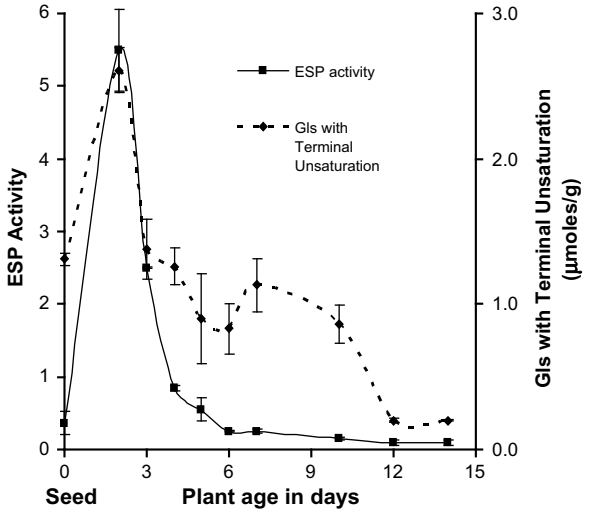

Fig. 2. Effect of seedling age on ESP activity (defined as the ratio of CHEB/CHB) and the levels of terminal alkenyl glucosinolates (Gls) (µmoles/g DW) in the broccoli cultivar Calabrese.

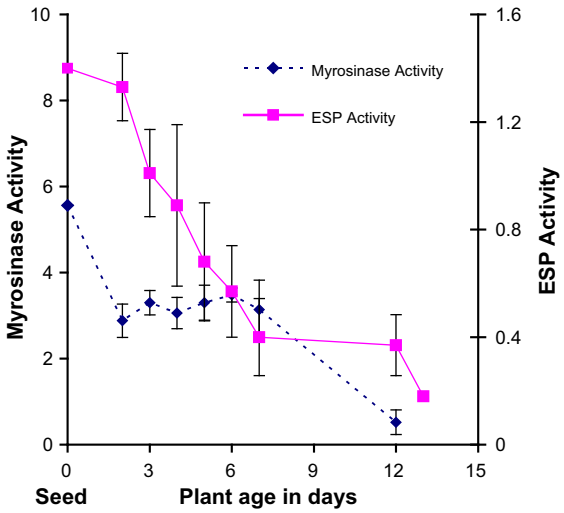

Fig. 3. Effect of seedling age on ESP activity (defined as the ratio of CHEB/CHB) and myrosinase activity (expressed as µg sinigrin consumed/min/g FW) in *Crambe abyssinica* seeds and sprouts.

have unanimously suggested that ESP activity is at its highest in seeds followed by a steady decline to the reduced levels in mature plants.

Not since the studies of Cole (1978, 1980) have any investigations into autolysis product changes of brassicaceous vegetables during ontogeny and how they relate to ESP activity been reported. In 1978, Cole studied changes in autolysis products of seeds and seedlings of turnip. This investigation found a steady decrease in products to day 4. Interestingly, if the levels of epithionitriles (an indication of ESP activity) obtained in Cole's study are summed and then divided by the total autolysis products formed, a maximum at day 1 emerges. The ratio so calculated is essentially similar to the ratio calculated to determine ESP activity in the present study (Fig. 6b) as formation of goitrin (5-vinyl-1,3-oxazolidine-2-thione), the other hydrolysis product in the model assay employed, is precluded by the assay conditions (Tookey, 1973). The significance of our results to the potential health benefits of consuming broccoli seedlings is considerable. Even though the results show ESP activity at a maximum in day 2 seedlings, the activity at days 3, 4 and 5 is still on a par with the activity shown by the seeds. Therefore the full benefit of glucoraphanin hydrolysis to sulphoraphane may be seriously compromised by the enhanced ESP activity in seedlings harvested between days 2 and 5.

2.5. Myrosinase activity of broccoli cultivars during seedling development

Myrosinase activity measured on the broccoli extracts increased similarly to the pattern exhibited by terminal alkenyl glucosinolate concentration and ESP activity (Fig. 4). The increase to day 2 and the subsequent decrease was not as dramatic as that exhibited by ESP activity but the overall curve shape was the same for all three cultivars tested. The myrosinase activity measured on the developing crambe seedlings (Fig. 3) shows a different pattern to those of broccoli but interestingly they conform to a similar pattern as shown by their ESP activities except the sharp reduction appears to be delayed until day 6. Numerous studies have shown that myrosinase activity varies with plant species, organ and stage of development (Henderson and McEwen, 1972; Phelan et al., 1984; Bones, 1990; James and Rossiter, 1991). Using isoelectric focussing Phelan et al. (1984) found myrosinase activity in radish appeared highest in the seed and decreased during growth. A similar pattern was shown by the crambe seeds and seedlings (Fig. 3) in this study. A later investigation by Bones (1990) found the myrosinase activity in the hypocotyls (organ containing the majority of activity) of developing *Brassica napus* seedlings gave a maximum value 2 days after germination followed by a nearly exponential decrease over the next 18 days. An increase in total myrosinase activity, most notably after 2 days was noted by James and Rossiter (1991) during early development of *B. napus*; however, a myrosinase activator, ascorbic acid, was added to their assay.

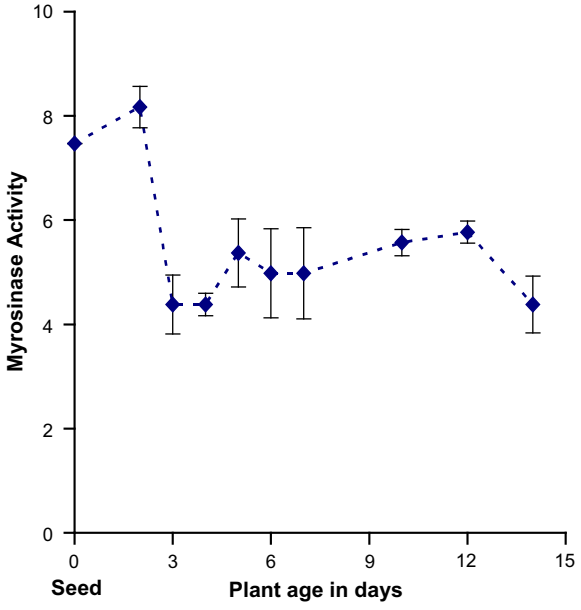

Fig. 4. Effect of seedling age on myrosinase activity (expressed as μg sinigrin consumed/min/g FW) in the broccoli cultivar Calabrese.

Ever since Tookey (1973) separated ESP from crambe myrosinase, researchers have been interested in the association between these proteins (Petroski and Tookey, 1982; Petroski and Kwolek, 1985; Petroski, 1986; Burow et al., 2006). To assess the extent of this association, we compared the two activities measured in the seeds and seedlings of the three broccoli cultivars and crambe. A weak correlation ($p = 0.116$, $R^2 = 0.279$) between these two parameters was observed for the Calabrese broccoli cultivar which was typical of the other two cultivars. Interestingly the removal of the seed data strengthened the relationship to give a significant association ($p = 0.032$, $R^2 = 0.503$). Similar strengthening was exhibited by the other two cultivars. Crambe only showed a weak relationship ($p = 0.165$, $R^2 = 0.293$) for the seed and seedling activities. The removal of seed data for crambe did not have any effect on the relationship ($p = 0.224$, $R^2 = 0.278$). To understand the myrosinase activity changes and the link between ESP activity in broccoli seedlings and the lack of a relationship exhibited by the broccoli seeds and crambe, a complete assessment of the isoenzyme profiles and their changes with development would be needed.

2.6. Relationship between ESP activity and the level of terminal alkenyl glucosinolates

The relationship between ESP activity and terminal alkenyl glucosinolates has intrigued researchers for many years. After Tookey's original study (1973) in which the author presented evidence that ESP was required in the formation of epithionitriles from alkenyl glucosinolates, other investigators have provided results indicating that the relationship is even closer than Tookey envisaged. Kaoulla et al. (1980) in a study of ESP activity in the seeds of cabbage and watercress suggested that when no terminal alkenyl glucosinolates were present, neither was ESP. To substantiate this, a later study by MacLeod and Rossiter (1985) evaluated ESP activity in seeds of several crucifers. Their results agreed with the findings of previous authors, although they extended their conclusions to propose that only trace amounts of susceptible glucosinolates were needed for appreciable ESP activity to be present. Little research into this relationship occurred until Lambrix et al. (2001) analysed the hydrolysis products of leaf samples collected from 122 *Arabidopsis* ecotypes. This study indicated that ecotypes that accumulated alkenyl glucosinolates had high frequencies of ESP gene expression. In examining the ecotype *Ler*, the authors noted that this plant had no alkenyl glucosinolates but still expressed a functional ESP. However, a closer inspection of the glucosinolate profiles of *Arabidopsis* ecotypes presented by Kliebenstein et al. (2001) showed that while alkenyl glucosinolates were not detected in the leaves of *Ler*, substantial amounts of sinigrin were present in the seeds. The results from our study in conjunction with the earlier investigations (Kaoulla et al., 1980: MacLeod and Rossiter, 1985) suggest that a definitive relationship exists between ESP activity and the level of terminal alkenyl glucosinolates and it may originate in the seeds of these plants.

There is also genetic evidence that such a relationship exists. In a study into inheritance in *A. thaliana*, Mithen and co-workers (Mithen et al., 1995) obtained results that indicated alleles at a single locus (*GS-AOP*) regulated the conversion of methylsulphinyl- alkyl glucosinolates into alkenyl glucosinolates. In a review Kliebenstein et al. (2005) suggested the locus is conserved across most *Brassicacea*, with *GS-AOP* directing alkenyl production in other *Brassicas* as well as in *Arabidopsis*. In the same review, the authors confirmed the close relationship of ESP with terminal alkenyl glucosinolates by noting that the *ESP* locus interacts epistatically with *GS-AOP* to influence plant defence against herbivore damage.

The present study demonstrates a relationship between ESP activity and terminal alkenyl glucosinolate level. However, as the levels of the non-terminal alkenyl glucosinolate, glucoraphanin followed a similar pattern in this study clearly more research is needed before stating definitively that ESP activity is absent from plants with no terminal alkenyl glucosinolates and that only trace amounts of these compounds in the seeds are sufficient for ESP activity. If this link can be substantiated there is potential for reducing ESP activity by reducing or preferably removing these glucosinolate substrates, thus increasing the health benefits of these vegetables.

2.7. Relationship between ESP activity and sulphoraphane nitrile formation

Sulphoraphane nitrile concentrations varied between cultivars, ranging from 0.5 ± 0.5 to 13.9 ± 0.4 μmol/g FW in cv. Calabrese 0.9 ± 0.1 to 17.8 ± 1.3 μmol/g FW in cv. DeCicco and 1.1 ± 0.2 to 8.0 ± 0.4 μmol/g FW in cv. Romanesco. These results agree with the findings of Matusheski et al. (2001) who found that cultivars of broccoli have distinct patterns of hydrolysis product formation. Preliminary experiments monitoring pH changes associated with the thawing of the hydrolysis extracts indicated no significant pH changes and therefore it was concluded that sulphoraphane nitrile production was not attributable to pH variation (Matusheski et al., 2004). When ESP activity and sulphoraphane nitrile levels were compared in seeds and seedlings at day 2, 3, 4, 5, 6 and 7 a weak relationship was observed (Fig. 5a–c). This agrees with the studies of Matusheski et al. (2006) into ESP activity and the extent of sulphoraphane formation in the florets of 20 commercial broccoli cultivars, where a significant negative correlation ($p = 0.012$, $R^2 = 0.305$) between these parameters was detected. From these results the authors concluded that ESP played a role in the formation of sulforaphane nitrile at the expense of sulphoraphane. If the seed data are removed from the present study, a remarkable correlation between activity and sulphoraphane nitrile formation is observed (Fig. 5a–c). The removal of the seed measurements can be justified as it is well documented that seeds can have altered ratios of glucosinolate hydrolysis products compared to the whole vegetable of the same species (Saarivirta, 1973; Matusheski and Jeffery, 2001). The results in the present study support this observation.

A study by Lambrix and co-workers (2001) using recombinant *Arabidopsis* ESP found the protein was capable of directing the hydrolysis of glucoraphanin, the principal glucosinolate in a number of *Arabidopsis* ecotypes, to sulphoraphane nitrile. From these results the authors suggested that ESP may regulate nitrile formation during non-alkenyl glucosinolate hydrolysis in addition to epithionitrile formation from alkenyl glucosinolates.

In 2004 Matusheski and co-workers demonstrated that ESP activity tracked closely with sulphoraphane nitrile formation in broccoli florets and 5 day old seedlings. A later report by de Torres Zabala et al. (2005) also using recombinant ESP from *Arabidopsis* cast doubt on the previous findings by showing that simple nitrile formation did not require ESP, only the presence of Fe^{2+} and myrosinase. Interestingly these authors observed that ESP enhanced sulforaphane nitrile formation in transgenic plants overexpressing ESP, compared to wild type plants that did not express ESP. A recent study using recombinant broccoli ESP (Matusheski et al.,

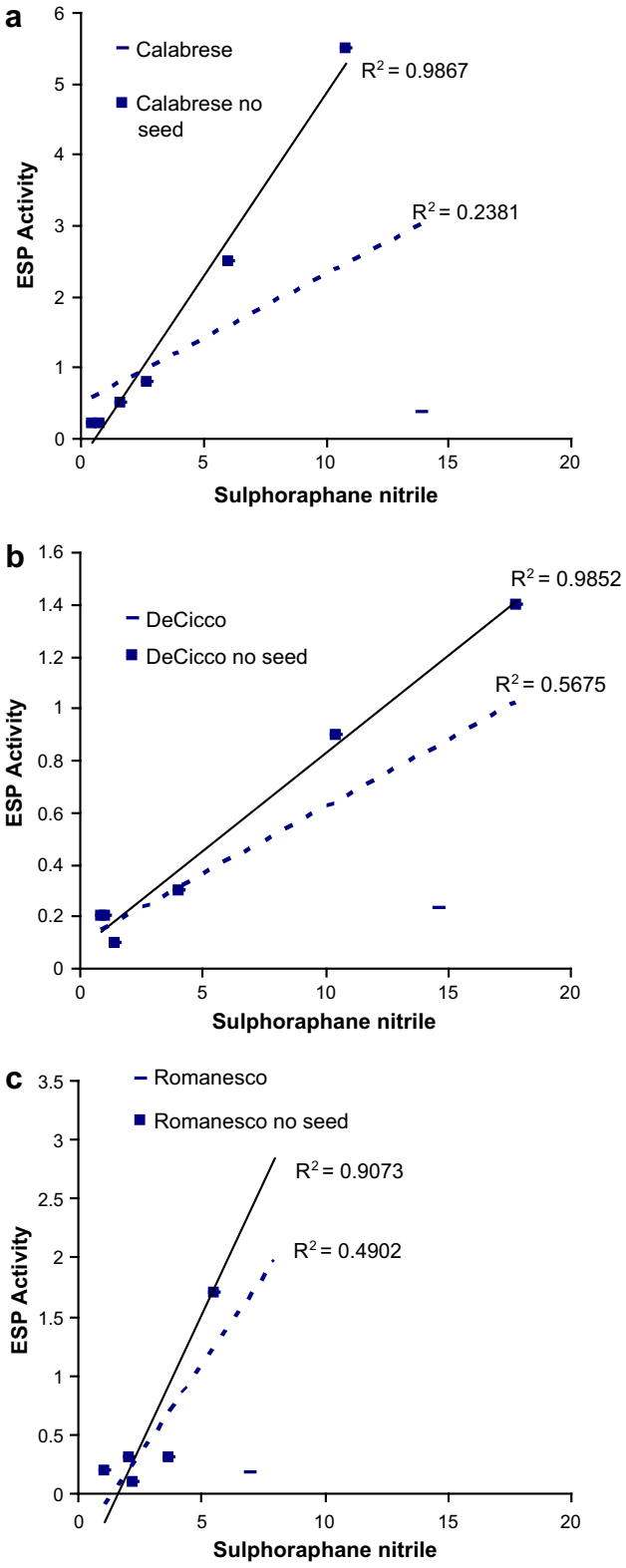

Fig. 5. Relationship between ESP activity (defined as the ratio of CHEB/CHB) and sulphoraphane nitrile levels (μmol/g FW) in three broccoli cultivars with ($n = 7$) and without ($n = 6$) seed values (a) cv. Calabrese (b) cv. DeCicco and (c) Romanesco.

2006) demonstrated that the protein not only directed myrosinase dependent metabolism of the alkenyl glucosinolate epi-progoitrin (2(*S*)-2-hydroxy-3-butenyl glucosinolate) toward epithionitrile formation, but also directed the hydrolysis of the glucosinolate, glucoraphanin, to form sulforaphane nitrile. No explanation was offered in these studies for the discrepancy between the results which suggested an ESP role in simple nitrile formation and the conclusions reported by de Torres Zabala et al. (2005). The results presented in this study also show a close relationship between ESP activity and the formation of sulforaphane nitrile in the three broccoli cultivars tested. In fact both parameters are so highly correlated in the seedlings that these data provide convincing proof that sulforaphane nitrile formation in broccoli is under ESP control.

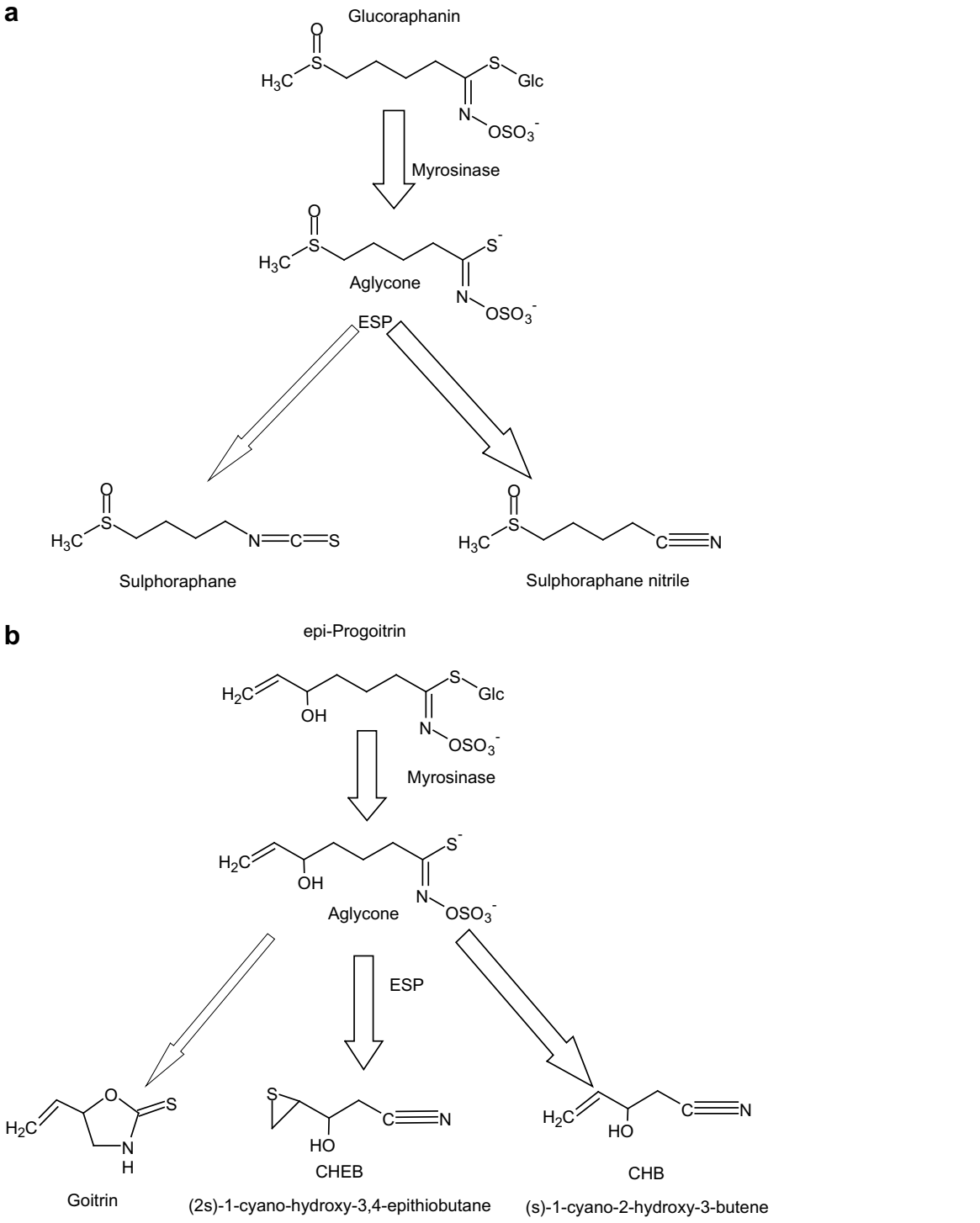

Fig. 6. Model of glucosinolate hydrolysis of (a) glucoraphanin and (b) epi-progoitrin. Myrosinase catalysed hydrolysis of glucosinolates yields unstable aglycones which spontaneously re-arrange to isothiocyanates or oxazolidine-2-thiones, if a β-hydroxy group is present eg. epi-progoitrin. In presence of ESP, hydrolysis reaction re-directed towards the formation of corresponding nitriles. If a terminal alkenyl group is in the side chain eg. epi-progoitrin an epithionitrile is formed. For this study width of arrows proportional to amount of hydrolysis products formed in the 2 day old seedlings.

It has been documented that some cruciferous plants form nitriles as their principal hydrolysis products while others produce primarily isothiocyanates (Gil and MacLeod, 1980; Hasapis and MacLeod, 1982; Lambrix et al., 2001). Sulphoraphane nitrile was the principal hydrolysis product measured in each broccoli cultivar tested (Fig. 1; Fig. 6a), agreeing with several other studies (Mithen et al., 2003; Matusheski et al., 2004, 2006). From Fig. 1 the highest percent contribution of sulphorahane to total hydrolysis products for the broccoli cultivar Calabrese was 38% at day 7 and it occurred when the ESP activity was at its lowest level for the analysis period. According to the results presented here and results from these previous investigations one could confidently state that broccoli is a nitrile forming plant at all stages of development.

In this study we demonstrated that ESP activity increases in the early days of development, with a maximum at day 2. A close association between ESP and myrosinase activities for broccoli seedlings was confirmed. We also showed that the levels of terminal alkenyl glucosinolates track closely with changes in ESP activity supporting speculation that ESP is absent from plants lacking these glucosinolates. Our results offer further evidence that broccoli is a nitrile forming plant and that sulphoraphane nitrile formation is under ESP control.

Now that ESP activity and its links with simple nitrile formation and to a limited extent terminal alkenyl glucosinolates in early broccoli development have been described the stage is set for removing or reducing ESP activity in the seedlings of these plants. Identifying specific broccoli cultivars that express decreased levels of ESP or possess very low levels of alkenyl glucosinolates may allow optimal conversion of glucoraphanin to sulphoraphane.

3. Experimental

3.1. Materials

The broccoli seeds (cv. Calabrese) were a gift from OptiGrow Pty. Ltd. The other broccoli seeds were purchased from Eden Seeds (cv. DeCicco) and Royston Seeds (cv. Romanesco). Crambe seeds used for seeding and preparation of epi-progoitrin were a gift from the Centre for Legumes in Mediterranean Agriculture, The University of Western Australia. Organic solvents (HPLC grade) were purchased from Biolab (Australia) Pty. Ltd. Purified glucoraphanin, progoitrin and gluconapin were purchased from Dr. Jens Sorenson at the Bioraf Denmark Foundation (Copenhagen, Denmark). Purified epi-progoitrin was obtained by adapting the protocol reported by Rochfort et al. (2006) to isolate this glucosinolate from crambe seeds. Isothiocyanates, nitriles and epithionitriles for GC calibration were purified from broccoli or crambe seeds using previously described extraction and purification methods and verified by mass spectrometry (Matusheski et al., 2001; Niedoborski et al., 2001). All other chemical reagents, including phenyl/ benzyl isothiocyanates and sinigrin were purchased from Sigma Aldrich (Australia).

3.2. Preparation of broccoli seedlings

Seeds were germinated in accordance with the International Seed Testing Association (ISTA, 2003) recommendations. Seeds were positioned between paper sheets, wetted with water, rolled in a wet towel and placed in a germinator at 25 °C without light. Preliminary experiments indicated a germination rate of greater than 85% or above for all three cultivars of broccoli and crambe. Seedlings with no defect on the cotyledon shoot and root system were classified as a normal seedling and used for the analysis. If any defects (as in ISTA rules) were found, the seedlings were classified as abnormal and discarded.

3.3. Formation of sulphoraphane, sulphoraphane nitrile and preparation of ESP, myrosinase activity extracts from broccoli/crambe seeds and seedlings

Samples of the dry seeds and harvested seedlings were homogenised with 1 volume of distilled deionised water in triplicate. Homogenates were immediately squeezed through cheesecloth and the filtrate centrifuged at 4 °C and 10,000*g* for 15 min. The supernatants were collected, pH, ESP and myrosinase activities measured and the remainder of the extract stored at -20 °C (Matusheski et al., 2004). The extracts were thawed for 12 h at room temperature, and pH measured. The hydrolysed extracts were analysed for sulphoraphane and sulforaphane nitrile by GC.

3.4. Analysis of sulphoraphane and sulphoraphane nitrile

Three replicated hydrolysis samples of broccoli seeds and seedlings were analysed for sulphoraphane and sulphoraphane nitrile formation using the method of Matusheski et al. (2004), but modified by utilising a 5 μl methylene chloride extract injected into a 1:30 split Varian 3900 GC system. The flowpath consisted of a 4 mm ID single gooseneck liner with nitrogen as the carrier gas. The GC was calibrated using standard curves of 1–100 mg/ml benzyl isothiocyanate (internal standard), purified sulphoraphane and sulphoraphane nitrile in methylene chloride (Matusheski et al., 2001).

3.5. Analysis of glucosinolates

To 1 g of broccoli seeds and seedlings, 10 ml of boiling water was added and the mixture boiled for 5 min. The bulk of the water was decanted and the heated broccoli samples were transferred to a mortar and pestle with 5 ml of water. The samples were ground to a paste. The resultant slurry was transferred to a 20 ml volumetric flask and sonicated for 5 min. The extract was filtered through Whatman No. 4 filter paper, and then made to the mark with the decanted water (Rochfort et al., 2006). The samples were then stored at −20 C until HPLC analysis. The levels of glucoraphanin and terminal alkenyl glucosinolates were determined by HPLC as previously described by West et al. (2002).

3.6. Measurement of ESP activity

The activity of ESP was measured by incubating the glucosinolate epi-progoitrin with purified myrosinase enzyme in the presence of the extract under study as previously described by Matusheski et al. (2004). Epithiospecifier protein activity was defined as the ratio of epithionitrile [(2*S*)-1-cyano-2-hydroxy-3,4-epithiobutane] (CHEB) to simple nitrile [(*S*)1-cyano-2-hydroxy-3-butane] (CHB) formed in the presence of excess myrosinase and Fe (Fig. 6b). The GC was calibrated using standard curves of 1–100 mg/ml phenyl isothiocyanate (internal standard), CHB and CHEB in methylene chloride.

3.7. Measurement of myrosinase activity

The activity of the enzyme myrosinase present in the extract was measured by the hydrolysis of a known amount of sinigrin added to the extracts as previously described by Verkerk and Dekker (2004). The amount of convertible sinigrin was calculated after 20 min of exposure to the extracts. Myrosinase activity was expressed as μg sinigrin consumed/min/g (FW).

3.8. Statistical analysis

Statistical analysis for the glucosinolate content and ESP activity during plant development for each cultivar was performed with

ANOVA and Fisher's protected LSD ($\alpha = 0.05$). The relationships between ESP activity, myrosinase activity, terminal alkenyl glucosinolate level and sulphoraphane nitrile formation were determined using linear regression for estimates of the co-efficient of determination (R^2).

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