DISSERTATION

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Effect of Se-fertilization on the growth and S-metabolism of Broccoli (*Brassica oleracea var. italica*)

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SUMMARY

Broccoli (*Brassica oleracea* var. italica) has been proposed as a functional food for cancer prevention, based on its high glucosinolate (GSL) content and capacity for selenium (Se)-accumulation. However, as selenate and sulfate share the initial assimilation route, Se-fertilization may interfere with GSL accumulation. Indeed, previous studies have shown that selenate-fertilization may impinge on plant growth and compromise GSL accumulation. To reevaluate the potentially adverse effects of Se-fertilization, I have performed a comprehensive study on sand-grown young broccoli plants (weekly selenate applications of 0.8 μmol plant^{-1} via the root) and field-grown adult broccoli plants during head formation (single selenate application via leaf spray: 25.3 or 253 μmol plant^{-1}). The results show that selenate-application did not affect growth, concentrations of cysteine, glutathione, total GSL and glucoraphanin as a major aliphatic GSL, or the expression of *BoMYB28*, encoding a master regulator for aliphatic GSL biosynthesis. Conversely, due to changed expression of sulfate transporters (*BoSULTR1;1, 1;2, 2;1*, and *2;2*), sulfate and total S concentrations increased in the shoot of young plants but decreased in the root. In summary, broccoli can be fertilized with Se without reduction in GSL content, even with Se accumulation significantly above recommended levels for human consumption.

ZUSAMMENFASSUNG

Brokkoli (*Brassica oleracea* var. italica) wurde wegen seinem hohen Glucosinolatgehalt (GSL-Gehalt) und seiner Kapazität zur Anreicherung von Selen (Se) seit einiger Zeit als ein Functional Food zur Krebsprävention diskutiert.

Da sich jedoch Selenat und Sulfat zu Beginn den gleichen Assimilationsweg teilen, könnte Se-Düngung die GSL-Akkumulation stören. Tatsächlich haben vorherige Studien gezeigt, dass Selenatdüngung auf das Pflanzenwachstum Einfluß nehmen und die GSL Akkumulation beeinträchtigen könnte. Um die potentiell nachteiligen Effekte der Se-Düngung neu zu beurteilen, wurde eine umfassende Studie an jungen Brokkolipflanzen in Sandkultur (mit wöchentlichen Selenatverabreichungen von 0,8 µmol Pflanze^{-1} über die Wurzel) und an adulten Brokkolipflanzen während der Blütenstandsbildung (eine einzige Selenatbehandlung mittels Sprühapplikation auf Blätter: 25,3 oder 253 µmol Pflanze $^{-1}$) durchgeführt. Die Ergebnisse zeigen, dass das Verabreichen von Selenat weder das Pflanzenwachstum noch den Gehalt an Cystein, Glutation, Gesamt-GSL und an Glucoraphanin (als Hauptglucosinolat), noch die Expression von *BoMYB28* (der einen Hauptregulator der aliphatischen GSL-Biosynthese kodiert) beeinflusste. Im Gegenzug stiegen aufgrund einer veränderten Expression der Sulfattransporter (*BoSULTR1;1*, *1;2*, *2;1*, und *2;2*) die Sulfat- u. Gesamtschwefelkonzentrationen im Spross junger Pflanzen, während sie in deren Wurzel abnahmen. Die Ergebnisse zeigen, dass Brokkoli mit Se gedüngt werden kann, ohne eine Verringerung des GSL-Gehalts zu verursachen, selbst dann, wenn die erreichte, Se-Akkumulation, deuthlich über der für den menschlichen Verzehr empfohlenen Menge lag.

1. INTRODUCTION

Among the diverse spectrum of higher plant secondary metabolites, the glucosinolates (GSL) of the *Brassicaceae* have attracted particular attention. Not only could GSL biosynthesis and metabolism be studied in the model plant *Arabidopsis thaliana*, thus allowing its molecular dissection in great detail, but some of these GSL have also been proposed to exhibit a cancer-preventive potential when consumed regularly in the human diet (Keck & Finley, 2004; Finley, 2005; Verkerk *et al*., 2008). In addition, some chemical elements, like selenium (Se), are important and essential micronutrients for human health. Broccoli (*Brassica oleracea* var. italica), which is a common vegetable, has high GSLs content and can accumulate high amount of Se. Therefore, in this thesis, the effect of Se-fertilization on S-metabolism in broccoli was investigated on the purpose to improve the nutrient-value of broccoli for human consumption.

1.1 Glucosinolates

Glucosinolates (GSLs) are nitrogen- and sulfur-containing metabolites derived from a variety of amino acids (Grubb & Abel, 2006; Halkier & Gershenzon, 2006). *In planta*, the degradation of GSLs by the enzyme myrosinase upon destruction of cellular structures (e.g. wounding), results in the formation of several distinct products, with the exact composition of the reaction product spectrum depending on various factors, including the presence of different product-specifying proteins (Wittstock & Burow, 2007). Prominent among the reaction products are isothiocyanates, which have been implicated in plant defense against pathogens and herbivores (Brader *et al.*, 2001; Mikkelsen *et al.*, 2003; Wittstock *et al.*, 2004; Mewis *et al.*, 2006; Shroff *et al.*, 2008; Bednarek *et al.*, 2009; Clay *et al.*, 2009), and, when consumed by mammals,

have been shown to act as dietary inducers of detoxification enzymes that favorably modify carcinogen metabolism (Munday & Munday, 2004; Paolini *et al.*, 2004; Verkerk *et al.*, 2008). A strong focus of recent research has been on the various effects of the glucoraphanin-derived isothiocyanate sulforaphane, as this compound was reported to exhibit a high potential for cancer prevention (Chiao *et al.*, 2002; Fahey *et al.*, 2002; Munday & Munday, 2004; Paolini *et al.*, 2004; Cornblatt *et al.*, 2007)

1.1.1 GSLs biosynthesis

The formation of GSLs can be conveniently divided into three separate stages (Fig. 1.1). First, certain aliphatic and aromatic amino acids are elongated by inserting methylene groups into their side chains. Second, the amino acid moiety itself, whether elongated or not, is metabolically reconfigured to give the core structure of GSLs. Third, the initially formed glucosinolates are modified by various secondary transformations.

The major GSLs are derived from methionine, tryptophan and phenylalanine. Some genes in different stages of GSLs biosynthesis also have been identified. For example, methylthioalkylmalate (MAM) synthases catalyze the condensation reaction for the first side chain elongation step (Kroymann *et al*., 2001; Field *et al*., 2004; Textor *et al*., 2007). Cytochromes P450 monooxygenases family are known for the oxidation step in the core structure formation (Bak & Feyereisen, 2001; Bak *et al*., 2001; Hansen *et al*., 2001; Naur *et al*., 2003). Secondary modification of the side chain is generally considered to be the final stage in GSLs synthesis. The substantial natural variation of aliphatic GSLs in *Arabidopsis* has expedited identification of two α-ketoglutarate-dependent dioxygenases, encoded by the tightly linked and duplicated AOP2 and AOP3 genes, which control production of alkenyl and hydroxyalkyl GSLs,

respectively (Kliebenstein *et al*., 2001b).

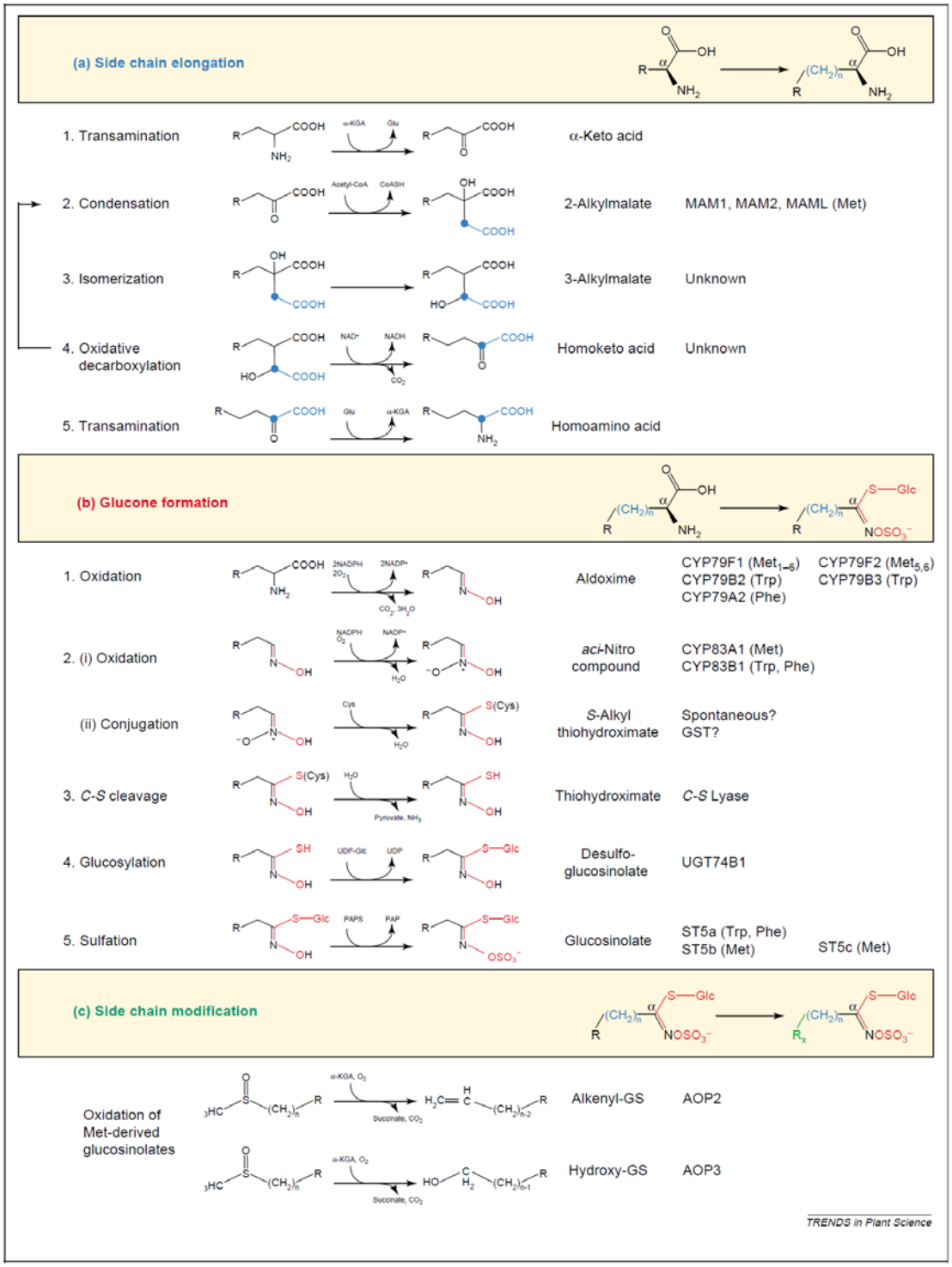

Fig. 1.1 Stages of glucosinolate biosynthesis (from Grubb & Abel, 2006).

1.1.2 MYB transcription factors

MYB factors represent a family of proteins that include the conserved MYB DNA-binding domain. In contrast to animals, plants contain a MYB-protein subfamily that is characterised by the R2R3-type MYB domain. 'Classical' MYB factors, which are related to c-Myb, seem to be involved in the control of the cell cycle in animals, plants and other higher eukaryotes. In plants, MYB proteins can be classified into three subfamilies depending on the number of adjacent repeats in the MYB domain (one, two or three) (Rosinski & Atchley 1998; Jin & Martin 1999). *MYB* genes containing two repeats (i.e. *R2R3-MYB*) constitute the largest *MYB* gene family in plants. In *A. thaliana*, more than 100 *R2R3-MYB* genes have been found and categorised into 22 subgroups on the basis of conserved amino-acid sequence motifs present carboxyterminal to the MYB domain (Fig. 1.2; Kranz *et al*., 1998; Stracke *et al*., 2001). No functional data are available for most of the R2R3-type *AtMYB* genes. However, systematic searches for knockouts have been initiated recently and the number of *AtMYB* genes for which functional information has become available has grown significantly during the past year (Meissner *et al*., 1999). R2R3-type *MYB* genes have been shown to regulate phenylpropanoid metabolism in *A. thaliana*. Overexpression of *AtMYB75/PAP1* (*PRODUCTION OF ANTHOCYANIN PIGMENT1*) and *AtMYB90/PAP2* results in accumulation of anthocyanins (Borevitz et al., 2000), and *AtMYB4* represses the synthesis of sinapoyl malate (Jin et al., 2000). Another important function for R2R3-type MYB factors is the control of development and determination of cell fate and identity. *AtMYB0/GLABROUS 1* (*GL1*) and *AtMYBB66/WEREWOLF* (*WER*) are involved in epidermal cell patterning (Oppenheimer *et al*., 1991; Lee *et al*., 1999). R2R3-type MYB factors also participate in plant responses to environmental factors and in mediating hormone actions. *AtMYB2* has been found to regulate the *AtADH1* (*ALCOHOL DEHYDROGENASE1*)

gene promoter, and it might also be involved in the response to low oxygen (Hoeren *et al*., 1998). *AtMYB30* expression is strongly correlated with cell death during the hypersensitive response upon pathogen attack or elicitor treatment (Daniel *et al*., 1999). Recently, the role of R2R3-MYB transcription factors as master regulators of GSL biosynthesis has been reported. *AtMYB28* acts as the major positive regulator of genes encoding the enzymes of aliphatic GSL biosynthesis (Gigolashvili *et al.*, 2007b; Hirai *et al.*, 2007) and *AtMYB29* is an accessory factor in response to methyl jasmonate signaling and a positive regulator of aliphatic GSL biosynthesis (Hirai *et al.*, 2007; Gigolashvili *et al.*, 2008). *AtMYB34* and *AtMYB51* both regulate indolic GSL biosynthesis (Celenza *et al*., 2005; Gigolashvili *et al.*, 2007a).

1.1.3 GSLs and plant defense

GSLs constitute a large family of secondary metabolites with over 120 different chemical structures known (Fahey *et al*., 2001). All GSLs have a core structure, composed of a β-thioglucose and an N-hydroxyiminosulphate group, and an aglycone side-chain, which is structurally highly diverse (Fig. 1.3). Upon tissue disruption (e.g. during herbivory), GSLs (which are stored in the plant vacuole) are mixed with myrosinase, a glucosidase that is spatially separated from its substrate (Kelly *et al*., 1998). The myrosinase activates the glucosinolates by removal of the glucose moiety. This results in the production of nitriles and isothiocyanates, that are toxic and deterrent to generalist insect herbivores. This plant-defense system is also called the mustard oil bomb. Most GSLs breakdown products have biocidal activities, mainly because their functional groups have an electrophilic carbon center.

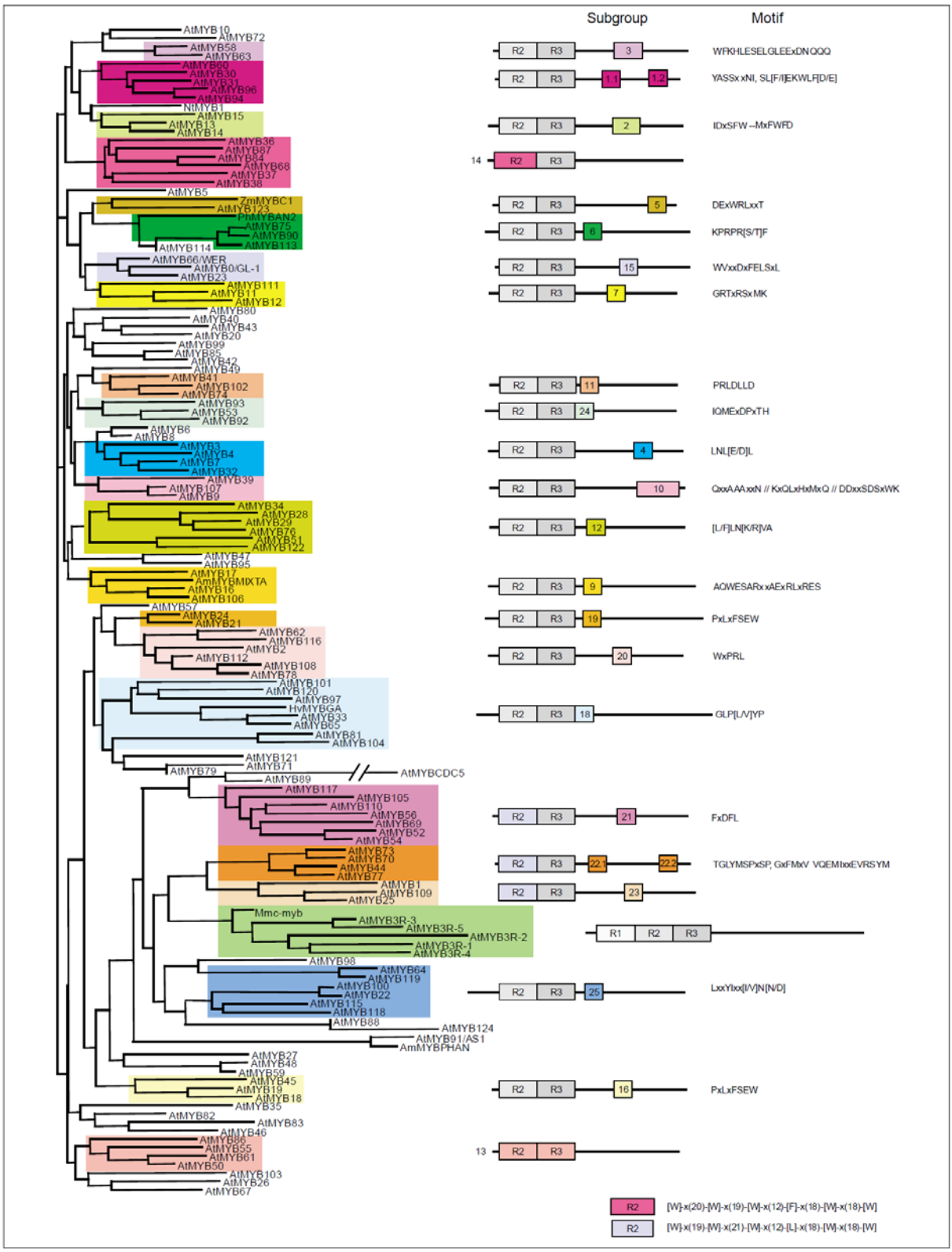

Fig. 1.2 Relationship of *A. thaliana* MYB proteins that have two or three repeats (from Stracke *et al.*, 2001).

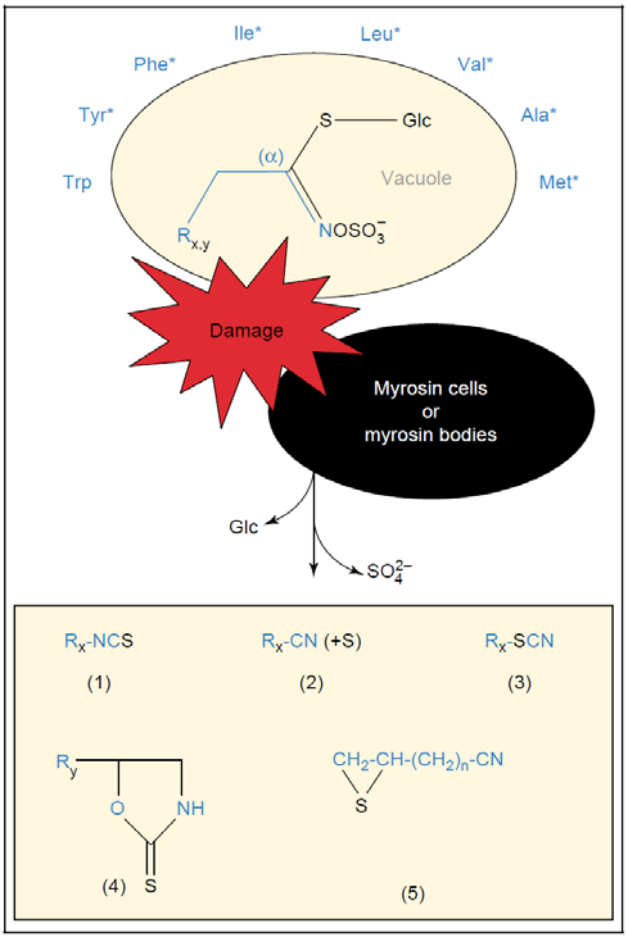

Fig. 1.3 The mustard oil bomb, a binary (glucosinolate-myrosinase) chemical defense system (from Grubb & Abel, 2006). 1: isothiocyanates; 2: nitriles and elemental sulfur; 3: thiocyanates; 4: oxazolidine-2-thiones; 5: epithionitriles.

1.1.4 GSLs and cancer

Cancer is a leading cause of death worldwide: it accounted for 7.4 million deaths (around 13% of all deaths) in 2004 (calculation from WHO). A review by Doll and Peto in 1981 summarized the available evidence for causes of cancer and suggested that diet is the primary causative factor in 35 % of all cancer deaths. Although diet may be a source of carcinogens, the authors concluded the most important role was as a source of cancer-inhibiting bioactive compounds, and diets that do not provide enough bioactive compounds may increase the risk of specific cancers. GSLs are not bioactive in the animal that consumes them until they have been enzymatically hydrolysed to an associated isothiocyanate by the endogenous myrosinase enzyme that is released by disruption of the plant cell through harvesting, processing, or mastication (Fig. 1.3). The breakdown products of GSLs are not only contributing to plant defense, but may also be benefic for human health, based on their cancer-preventing potential. *In vitro* and *in vivo* studies have reported that isothiocyanates affect many steps of cancer development including modulation of phase I and II detoxification enzymes (Rabot *et al*., 1993; Bogaards *et al*., 1994; Jiao *et al*., 1996; Talalay & Fahey, 2001), functioning as a direct antioxidant (Zhu *et al*., 2000; Zhu & Loft, 2001, 2003) or as an indirect antioxidant by phase II enzyme induction (Hayes & McLellan, 1999; Talalay & Fahey, 2001; McWalter *et al*., 2004), modulating cell signalling (Xu & Thornalley, 2001), induction of apoptosis (Yu *et al*., 1998; Chiao *et al*., 2002; Yang *et al*., 2002), control of the cell cycle (Yu *et al*., 1998; Zhang *et al*., 2003; Wang *et al*., 2004) and reduction of helicobacter infections (Fahey *et al*., 2002). Therefore, polymorphisms exist in the genes for the metabolizing enzymes, and the potential influence of these genetic alterations on risk for cancer, owing to diet-gene interactions, has become the focus of intense research interest.

In the biotransformation of a foreign compound or carcinogen, the first step typically involves the addition of one or more hydroxyl groups to a relatively nonpolar hydrocarbon, which transforms the compound into an electrophilic or more polar intermediate. These oxidation reactions are carried out by phase I or activating enzymes, the cytochromes P450, which are coded by CYP genes. The cytochrome P450 enzymes also catalyze the oxidation of several endogenous compounds, such as steroid hormones and vitamin D metabolites. Phase II or conjugating enzymes catalyze conjugation reactions to compounds such as glutathione, which facilitates

elimination. Phase II enzymes include the glutathione S-transferases (GSTs), N-acetyltransferases (NATs), microsomal epoxide hydrolase, sulfotransferases, and UDP-glucuronosyl-transferases. Whether a polymorphic variant of these enzymes increases or decreases risk for cancer depends on the specific enzymatic activity that is being stimulated and the substrate involved. Similarly, the induction of metabolizing enzyme activity by nutritional factors may result in either the activation of a carcinogen or in the detoxification of a reactive intermediate metabolite. Fig. 1.4 illustrates the interrelationships between the biotransformation enzyme systems.

In cruciferous vegetables, sulforaphane (SF), a breakdown product of glucoraphanin (GR) which is a major aliphatic glucosinolate, has been reported on induction of phase II detoxification enzymes (Munday & Munday, 2004; Paolini *et al.*, 2004; Cornblatt *et al.*, 2007).

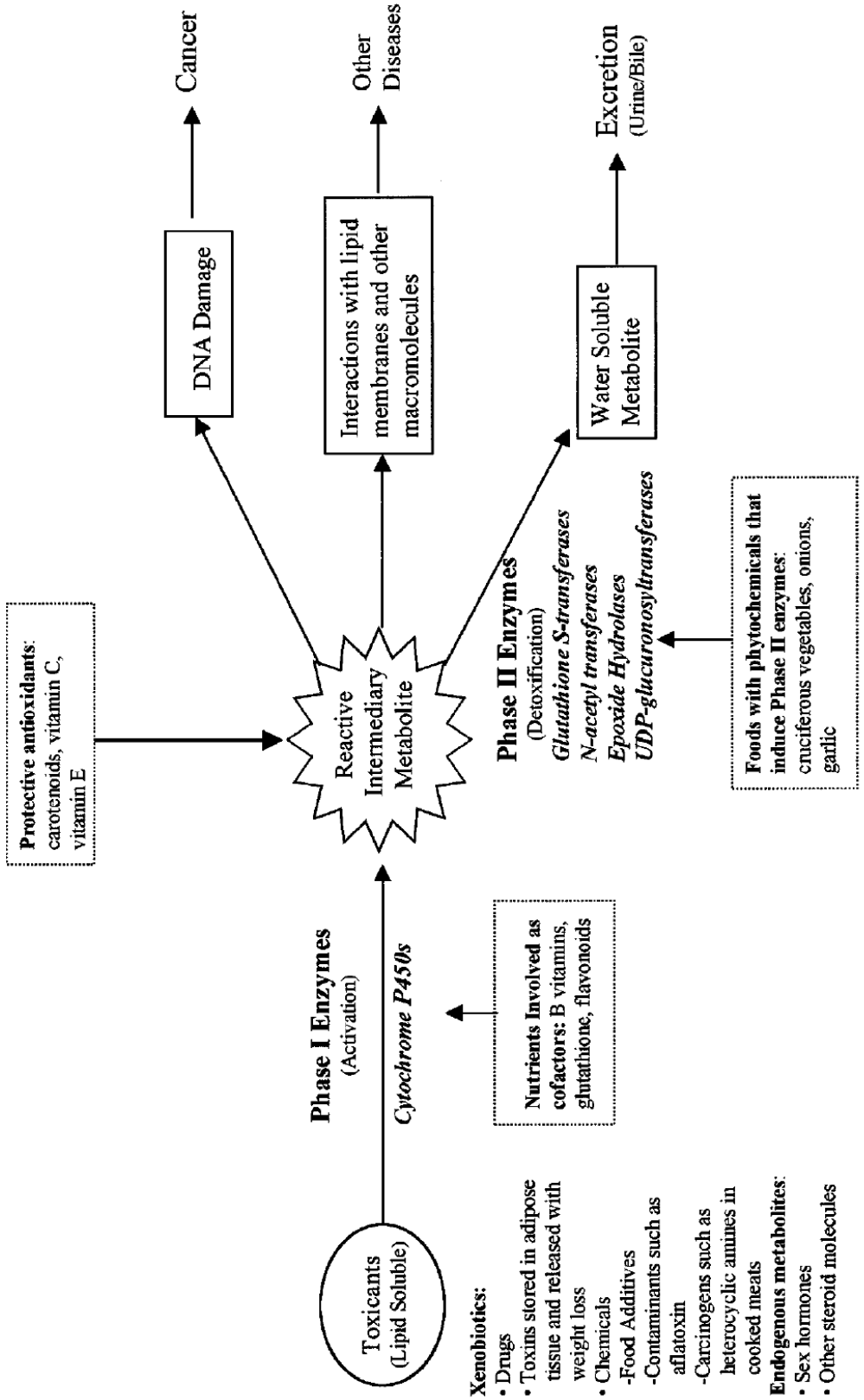

Fig. 1.4 Interrelationships between the biotransformation enzyme systems (from Rock *et al*., 2000).

1.2 Sulfur and Selenium metabolism in plants

In plants, sulfate and selenate share the initial pathway for uptake, assimilation and incorporation into *O*-acetylserine (OAS), resulting in the formation of cysteine (Cys) and seleno-cysteine, respectively (Fig. 1.5; Hell, 1997; Pilon-Smits *et al.*, 2002; Sors *et al.*, 2005a; Sors *et al.*, 2005b; Li *et al.*, 2008).

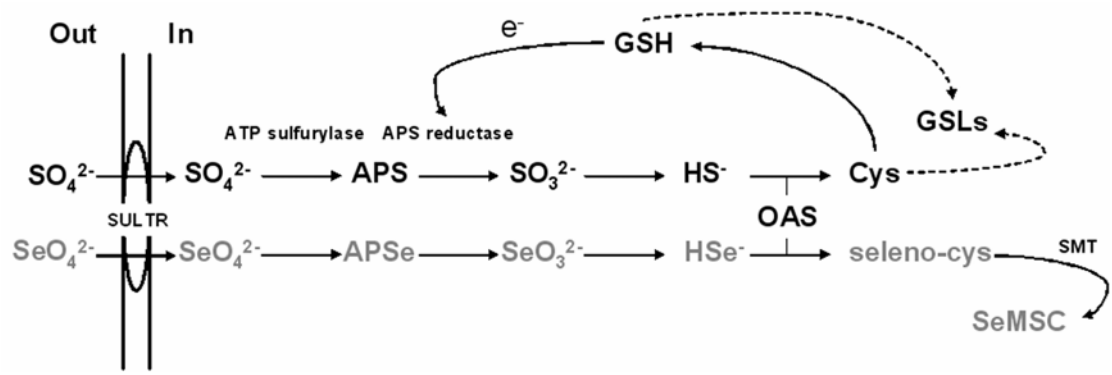

Fig. 1.5 Current model of sulfate and selenate uptake and assimilation pathways in plants. SULTR, sulfate transporter; APS, 5'-adenylylsulfate; APSe, 5'-adenylylselenate; OAS, *O*-acetylserine; Cys, cysteine; GSH, glutathione; GSLs, glucosinolates; SMT, selenocysteine methyltransferase; SeMSC, Se-methylselenocysteine. Note that a recent study demonstrated that the tripeptide glutathione (GSH) is the sulfur donor (Geu-Flores *et al*., 2009) for GSL biosynthesis.

1.2.1 S-metabolism

Sulfur (S) is an essential element for growth and physiological functioning of plants. Sulfate taken up by the roots is the major sulfur source for growth, though it has to be reduced to sulfide before it is further metabolized. Root plastids contain all sulfate reduction enzymes, however, the reduction of sulfate to sulfide and its subsequent incorporation into cysteine (Cys) takes predominantly place in the shoot in the chloroplast. Cysteine is the precursor or reduced sulfur donor of most other organic sulfur compounds in plants. The predominant proportion of the organic sulfur is present in the protein fraction (up to 70 % of total sulfur), as cysteine and methionine residues. Cysteine and methionine are highly significant in the structure, conformation and function of proteins. Plants contain a large variety of other organic sulfur compounds, as thiols (glutathione), sulfolipids and secondary sulfur compounds (alliins, glucosinolates, phytochelatins), which play an important role in physiology and protection against environmental stress and pests (Fig. 1.6; Rausch & Wachter,

2005; Mullineaux & Rausch, 2005). Sulfur compounds are also of great importance for food quality and for the production of phyto-pharmaceutics. Sulfur deficiency will result in the loss of plant production, fitness and resistance to environmental stress and pests.

1.2.2 Se-metabolism

The chemical and physical resemblance between selenium (Se) and sulfur (S) establishes that both these elements share common metabolic pathways in plants. The presence of isologous Se and S compounds indicates that these elements compete in biochemical processes that affect uptake, translocation and assimilation throughout plant development (Fig. 1.5). The main bioavailable form of Se in soils is selenate, which can be taken up by plants via sulfate transporters and assimilated into selenocysteine (SeCys) and selenomethionine (SeMet). Unlike selenate, there is no evidence that the uptake of selenite is mediated by membrane transporters.

Se is an essential micronutrient and has important benefits for animal and human nutrition. However, the question of the essentiality of Se as a micronutrient in higher plants is unresolved and remains controversial. From the ability for accumulating Se, plants can be divided into 3 groups: non-accumulator, Se-indicator and Se-accumulator. Non-accumulator plants are unable to grow on seleniferous soils and Se is toxic at tissue concentrations as low as 10-100 mg Se kg^{-1} dry weight, whereas Se-indicator (secondary accumulator) plants can colonize both non-seleniferous and seleniferous soils and tolerate tissue Se concentrations approaching 1000 mg Se kg^{-1} dry weight. Most of *Brassica* plants (e.g. broccoli) are secondary accumulators. Se-accumulator plants are able to hyperaccumulate Se in their shoots when they grow on seleniferous soils. They can accumulate from hundreds to several thousand mg Se kg^{-1} dry weight in their tissues. The largest group of Se-hyperaccumulating plants belongs to the genus *Astragalus* (*Fabaceae*). Twenty-five species of *Astragalus* have been characterized as Se hyperaccumulators (Shrift, 1969). While there is no proof of essentiality for Se in plants, there have been reports of beneficial effects of Se on plant growth. Among higher plants, the largest beneficial effects of Se on growth (up to 2.8-fold higher biomass with Se) have been observed in the Se hyperaccumulator plants, and Se has been suggested to be essential for these species (Shrift, 1969). While Se is generally metabolized by sulfur pathways, there is some evidence that

plants have evolved Se-specific enzymes that facilitate Se accumulation, perhaps to serve an ecological or physiological function. Hyperaccumulators such as two-grooved milkvetch (*Astragalus bisulcatus*) has a Se-specific selenocysteine methyltransferase (SMT), leading to accumulation of Se as relatively non-toxic methyl-selenocysteine (Fig. 1.7; Sors *et al*., 2005a; Sors *et al*., 2009). There is also a report of an *Arabidopsis thaliana* Se-binding protein that conferred Se tolerance when overexpressed (Agalou *et al*., 2005).

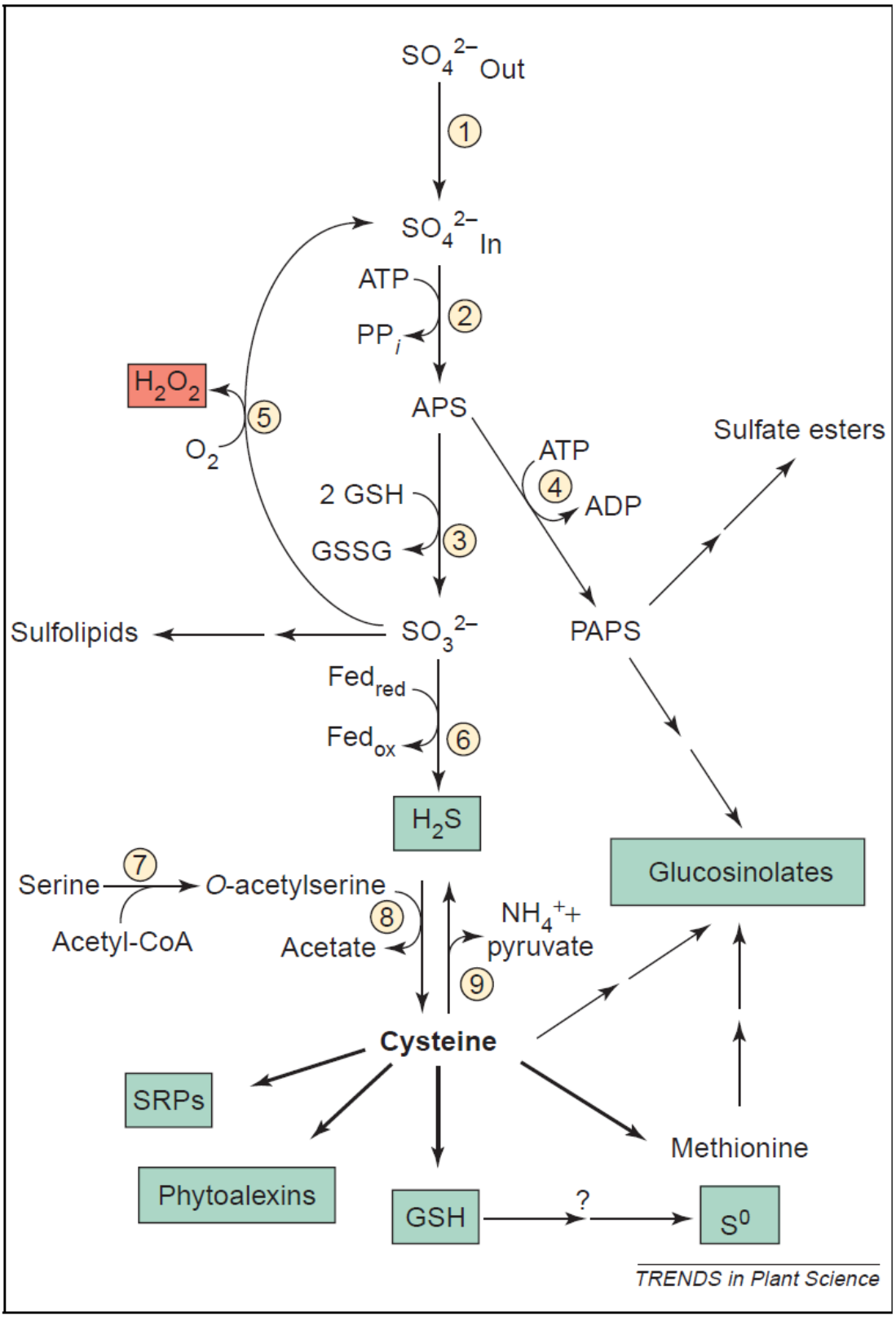

Fig. 1.6 Sulfur assimilation as a platform for the biosynthesis of sulfur-containing defence compounds (SDCs, depicted in green) (from Rausch & Wachter, 2005). After uptake of sulfate from the soil by high-affinity transporters (1), sulfate is largely transported to the shoot, where it becomes activated by ATP via ATP sulfurylase (2) in the leaves. The

product, APS (50-adenylylsulfate), is reduced by APS reductase (3), with the tripeptide glutathione (GSH) acting as an electron donor. Alternatively, APS is further activated by APS kinase (4) to form 30-phosphoadenylylsulfate (PAPS), which is required for various sulfatation reactions, including the biosynthesis of glucosinolates. Sulfite is reduced by sulfite reductase (6) to H_2S, which is incorporated into *O*-acetylserine via *O*-acetyl(thiol)lyase (8) to form cysteine. Cysteine, the primary product of S-assimilation is incorporated into sulfur-rich proteins (SRPs; including thionins) and GSH. Furthermore, cysteine is the donor of reduced sulfur for glucosinolate biosynthesis and for the synthesis of phytoalexins (including camalexin). Finally, H_2S can be released from cysteine via the action of desulfhydrases (9), whereas elemental sulfur, S^0, is possibly released from GSH. Note that via the activity of sulfite oxidase (5), excess sulfite is converted to sulfate, a reaction that uses O_2 as an electron acceptor, thereby releasing H_2O_2 (which could act as a defence signal). Sulfate assimilation [reactions (2), (3), (6), (8)] is localized in the plastids, whereas H_2S release occurs in plastids, mitochondria and cytosol. Sulfite oxidase is confined to peroxisomes.

1.2.3 Interaction between S and Se metabolism

While it was previously assumed that cysteine acts as donor of reduced sulfur for GSL biosynthesis, a recent report has demonstrated that the tripeptide glutathione is the sulfur donor (Geu-Flores *et al*., 2009). Thus, a negative impact of selenate on cysteine and/or GSH biosynthesis could impair GSL formation. Se-accumulating plant species are known to express a selenocysteine methyltransferase (SMT; Sors *et al*., 2009). While broccoli has the capacity to convert selenocysteine to Se-methylselenocysteine (SeMSC), it is not yet clear whether the cloned putative broccoli SMT (Lyi *et al.*, 2005) is indeed *a bona fide* SMT or rather a homocysteine methyltransferase with some SMT activity (Lyi *et al.*, 2005; Sors *et al*., 2009). An increased formation of SeMSC in broccoli would be desirable for human consumption, however, the interference with cysteine synthesis could compromise the plants ability to channel cysteine into sulfur-based defense compounds (SDC) (Rausch & Wachter, 2005), including not only GSLs but also the cellular antioxidant glutathione (Mullineaux & Rausch, 2005). Thus, manipulating the plant's cysteine metabolism via addition of selenate could cause side effects on plant stress tolerance which have to be considered.

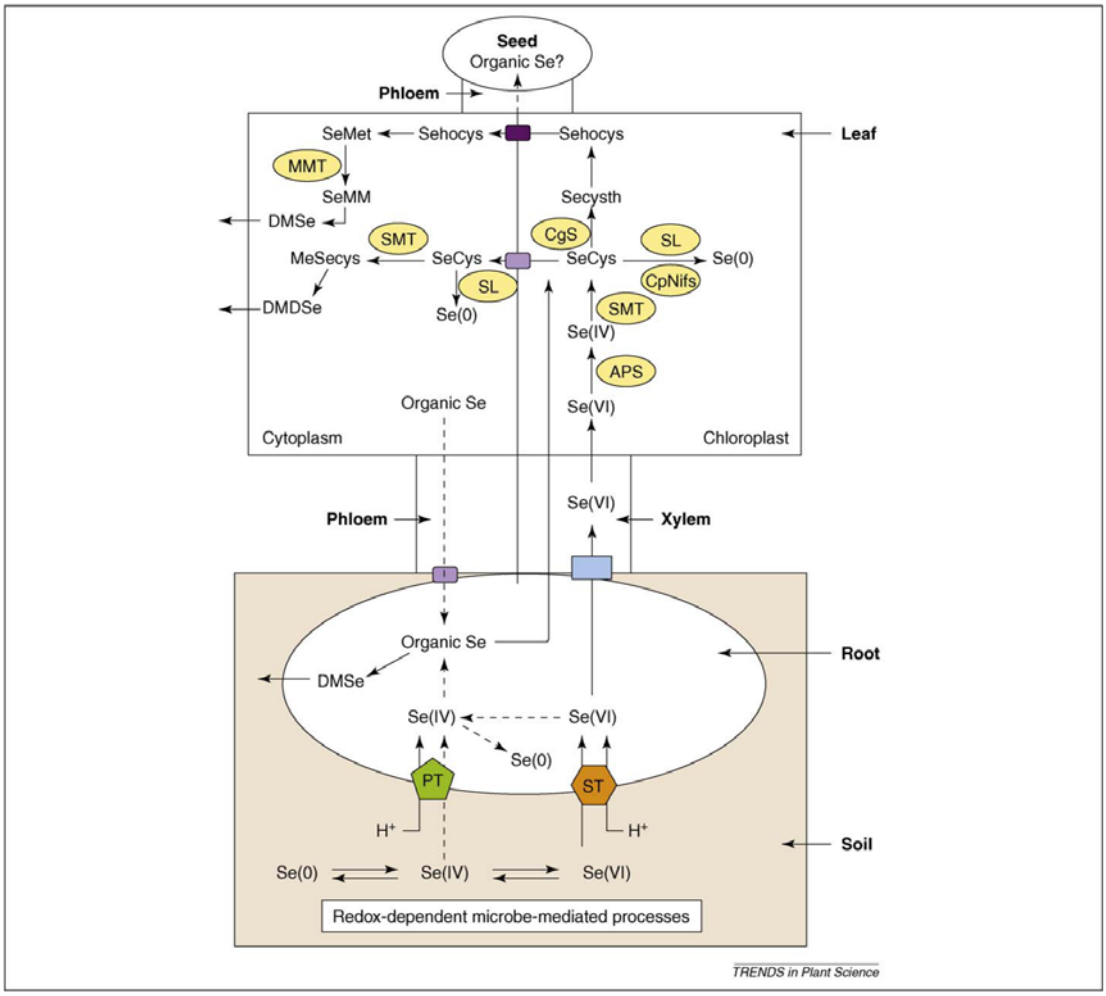

Fig. 1.7 Overview of Se metabolism and partitioning in plants, with an emphasis on genetic engineering approaches that have been shown to modify these processes (from Zhu *et al*., 2009). Enzymes in the yellow circles are those known to promote the conversion after overexpression (or at those points where Se uptake and metabolism can be manipulated); arrows leaving cells indicate the translocation of Se within and from the plant; dashed arrows indicate that the process is not yet confirmed. Abbreviations: PT, high-affinity phosphate transporters; Secysth, Se-systathionine; Sehocys, Se-homocysteine; ST, high-affinity sulfate transporters. Purple box = unknown transport for organic Se; blue box = selenate efflux pump.

While interference with cysteine biosynthesis could result in a negative impact of selenate-fertilization on GSL formation, selenate has also been shown to strongly induce a high affinity sulfate transporter in *Arabidopsis thaliana*, namely the root-expressed AtSULTR1;1 isoform, and, to a lesser extent also other *SULTR* isoforms (Takahashi *et al*., 2000; Yoshimoto *et al*., 2002). The analysis of a mutant in the isoform *AtSULTR1;2* has revealed its dominant role for selenate uptake via the root system (Shibagaki *et al*., 2002; El Kassis *et al*., 2007). Since in *Arabidopsis*

thaliana, the selenate-induced up-regulation of *SULTR* isoforms in the root also leads to increased sulfate accumulation, it may be speculated that low selenate exposure could even have beneficial effects on sulfur assimilation under certain conditions.

1.2.4 Se and human health

The reason for Se's essentiality for some organisms is that it is a structural component of specific selenoproteins and seleno-tRNAs. Selenoproteins contain SeCys in their active site and often have a redox function, such as the scavenging of free radicals that cause oxidative stress and cancer. The SeCys in selenoproteins is encoded by the opal stopcodon when present in the context of a specific secondary mRNA structure (SeCys insertion sequence). The importance of Se to human health has become a focus in recent years. Although Se-deficiency is rare, it does occur in several parts of the world, such as China, where concentrations of Se in the soil are low. Se-deficiency can lead to heart disease, hypothyroidism and a weakened immune system (Combs, 2000). The recommended intake of Se for a person is 50-70 μg day^{-1} (U.S. Department of Agriculture, 2003), with the maximum safe dietary intake being in the range of 600-800 μg day^{-1} (Whanger, 2004). Earlier studies concluded that a dietary Se-supplement of 100 to 200 μg day^{-1} can reduce the risk of cancer (Clark *et al*., 1996; Ip & Ganther, 1992), and an anti-carcinogenic function has been documented for different cancer types (Ganther, 1999; Whanger, 2004; Finley, 2005). Vegetables are major sources of Se-intake for human consumption. However, under field conditions, Se-compound formation in plants is limited by the low selenate-availability in most soils. Consequently, in most Western European countries human Se-intake is below the dietary recommendation (Combs, 2001).

1.3 Broccoli and cancer prevention

Broccoli (*Brassica oleracea* var. italica) is one of the most important vegetables because it is a major winter crop and a rich source of health promoting substances. It contains significant amounts of ascorbic acid, β-carotene, vitamin E, various flavonoids, fibers, and minerals such as magnesium (Mg) and calcium (Ca). In addition, like other *Brassica* species, broccoli contains significant quantities of glucosinolates (GSL), compounds rich in nitrogen (N) and sulfur (S), consisting of a thioglucose unit, a sulfonated oxime unit, and a variable side chain. The aliphatic GSL glucoraphanin (GR) is a major component in broccoli. In addition, broccoli can accumulate high amounts of selenium (Se) as other *Brassica* plants. Therefore, it has been advocated as a functional food for cancer prevention (Zhang *et al*., 1992; Fahey *et al*., 1997; Nestle, 1997; Nestle, 1998; Kristal & Lampe, 2002; Dinkova-Kostova *et al*., 2006; Munday *et al*., 2008; Traka *et al*., 2008).

2. AIM

Based on previous investigations, which predicted complex effects of selenate-application on broccoli (*B. oleracea* var. *italica*, a candidate for functional food), I have addressed in this study the following questions: First, does selenate-treatment affect sulfate uptake and, possibly, root-shoot transfer in broccoli in a way similar to previous reports for *A. thaliana*? Second, does selenate-fertilization negatively impact on glucoraphanin content (and, possibly, glutathione content) due to interference with primary sulfate assimilation? Third, does selenate-treatment modulate the expression of genes in GSLs biosynthesis and S-metabolism? Finally, I have also explored the potential of direct selenate leaf-spray to fully-grown broccoli plants, immediately before head expansion, as an alternative for soil-based selenate fertilization.

3. RESULTS

3.1 The effect of Se-fertilization on plant growth

3.1.1 Different broccoli cultivars show variation in glucoraphanin content and Se-tolerance between different Se-treatments (selenate vs. selenite).

In order to confirm whether Se-fertilization affects the plant-growth and any difference between various broccoli cultivars, first the glucoraphanin (GR) content was quantified in different broccoli cultivars (Fig. 3.1). Here we chose 4 commercial cultivars: Marathon, Monaco, Montop, and Ironman. 1-week-old seedlings after germination were harvested and measured the GR content by HPLC. Variation of GR content was observed between different cultivars and Monaco had higher GR content (avg. 1.44 mg GR / g DW) than other cultivars on average. Besides, Se-tolerance was assayed via root-length measurement (Fig. 3.2). Broccoli seeds were grown on medium with 50μM selenite or selenate. After 4 weeks, root-length was measured. From this experiment, selenate seems to have more effect on the root-growth than selenite (Fig. 3.2a, b). All of four cultivars with selenate-treatment had obviously shorter root-length than control plants. In addition, Montop and Ironman cultivars showed sensitive to selenite (Fig. 3.2c).

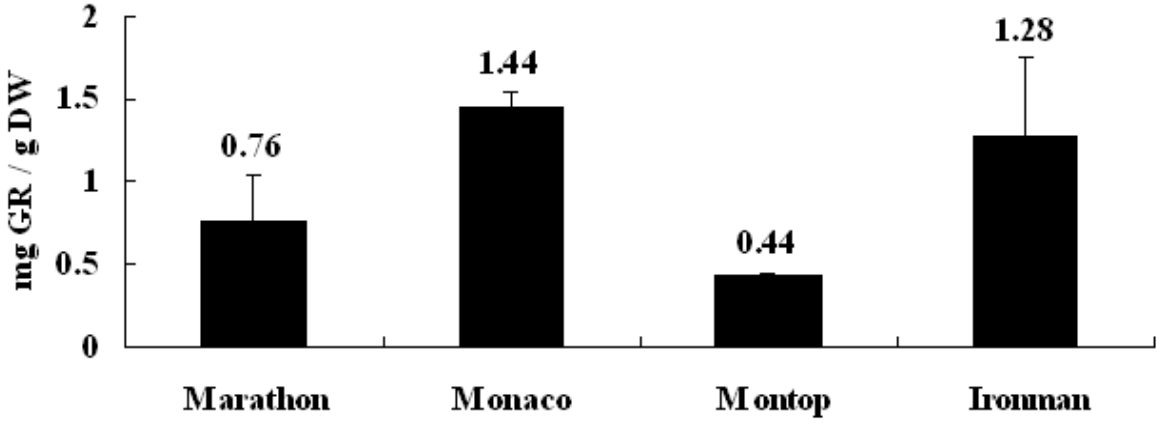

Fig. 3.1 Variation of GR concentration between different broccoli cultivars. Four broccoli cultivars were used for GR quantification (Marathon, Monaco, Montop and Ironman). 1-week-old seedlings were harvested after germination. The GR content was quantified by HPLC. Monaco is a cultivar which contains higher GR contents (avg. 1.44 mg GR / g DW). Bars represent arithmetic means; error bars represent SD from 3 independent experiments.

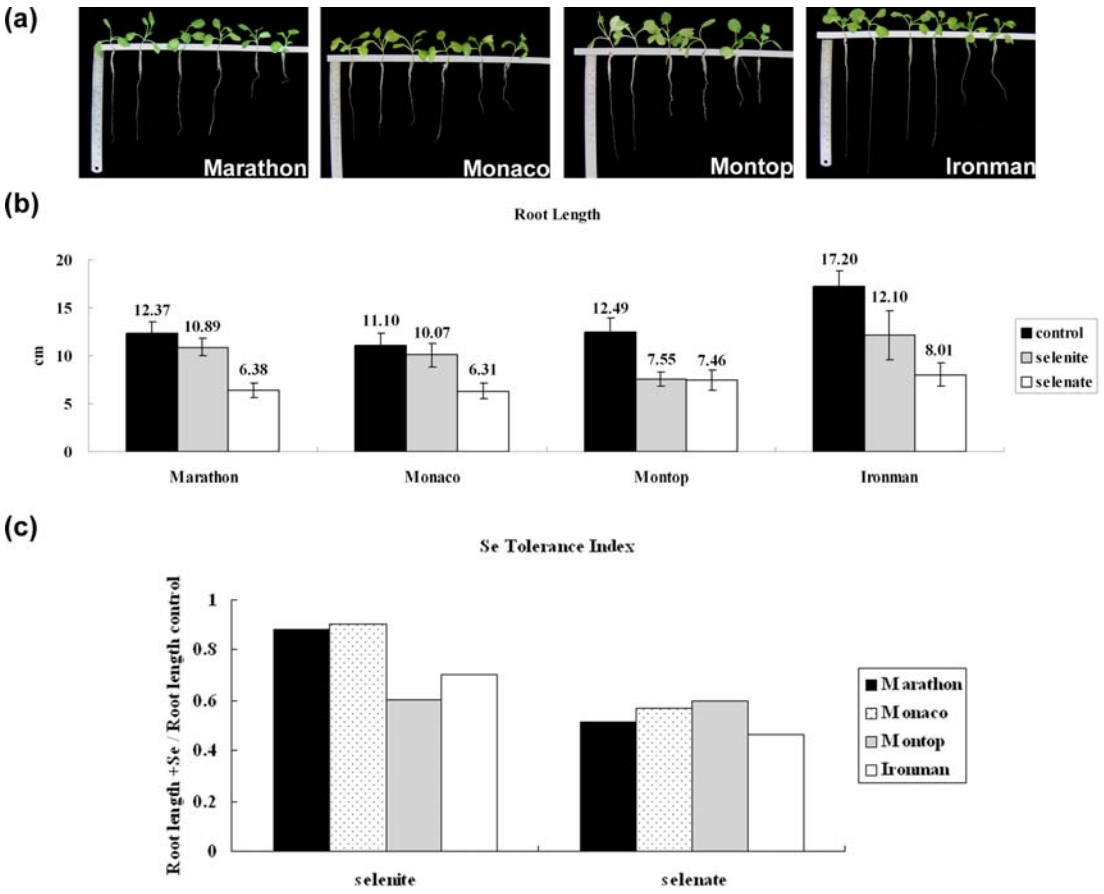

Fig. 3.2 The effect of Se-treatment on the root-growth of different broccoli cultivars. Four broccoli cultivars were used for Se-tolerance experiment (Marathon, Monaco, Montop and Ironman). Seeds were sterilized and grown on the agar medium with / without Se-treatment. The root-length of 4-week-old plants after Se-treatment was measured (a). The Se-treatment from left to right is: control, 50µM selenite, and 50µM selenate (two plants show one Se-treatment). (b) The root-length of 4-week-old broccoli plants. Ironman has longest root-length than others. Bars represent arithmetic means; error bars represent SD from 5 independent experiments. (c) The Se-tolerance index of different broccoli cultivars. The average root-length of selenate- / selenite-treated plants divided by the average root-length of control plants equals the Se-tolerance index. Selenate has stronger effect on the root-length than selenite. Montop and Ironman are more sensitive to selenite-treatment than Marathon and Monaco.

3.1.2 Selenate-fertilization did not affect the shoot-growth of young broccoli plants.

To address the effect of selenate on early plant development and concentrations of sulfur metabolites, broccoli plants were cultivated on sand in the greenhouse for 6 weeks with or without selenate fertilization. For the purpose of this study, I deliberately chose a selenate dosage comparable with previous studies (see Table 3.1), which have been shown to lead to substantial Se accumulation in broccoli shoots and significant formation of the Se-metabolite methylselenocysteine (Lyi *et al.*, 2005). Control plants (5 per pot) obtained nutrient solution (100 ml per pot) at weekly intervals. For the selenate treatment, the nutrient solution was supplemented with 40 µM sodium selenate, starting at the end of the second week. Thus, selenate dosage was about 0.8 µmol plant^{-1}, applied 4 times during the 6-week culture period, corresponding to a total amount of 3.2 µmol selenate or 253 µg Se per plant. At this selenate dosage, shoot growth and morphological development were not affected (Fig. 3.3a). A consistent ratio of fresh weight (FW)/dry weight (DW) (12.4 ± 0.4) over the entire growth period further supports our observation that selenate-fertilization did not affect young plant development.

Table 3.1

Selenium accumulation in *Brassica oleracea*.

Summary of previous studies on *B. oleracea* with selenate treatment, indicating Se contents achieved and impact on levels of aliphatic and indolic glucosinolates, respectively. In the study of Lyi *et al.* (2005), the accumulation of Se-methylselenocysteine was also determined, which corresponded to 273 µg g^{-1} DW or 13% of total Se accumulated.

Plant, cultivation method	**Applied Se concentration**	**Applied S Concentration**	**Se content**	**S content**	**reduction of GSL content**	**reference**
Broccoli, hydroponic culture	40 µM Na_2SeO_4	1 mM $MgSO_4*7H_2O$	900 µg g^{-1} DW (leaf)	n. d.	n. d.	Lyi *et al.*, 2005
Rapid-cycling *Brassica oleracea,* hydroponic culture	48 µM Na_2SeO_4	1 mM $MgSO_4*7H_2O$	731.6 µg g^{-1} DW (shoot)	21 mg g^{-1} DW (shoot)	aliphatic GSLs: - 67 % indolic GSLs: - 69 %	Charron *et al.*, 2001
Rapid-cycling *Brassica oleracea,* hydroponic culture	8 µM Na_2SeO_4	1 mM $MgSO_4*7H_2O$	373 µg g^{-1} DW (leaf)	18 mg g^{-1} DW (leaf)	aliphatic GSLs: - 36 % indolic GSLs: - 22 %	Toler *et al.*, 2007
Broccoli, soil fertilization	100 ppm Se 10.000 ppm Se	n. d. n. d.	99 µmol g^{-1} DW 879 µmol g^{-1} DW (broccoli head)	n. d. n. d.	aliphatic GSLs: - 30 % indolic GSLs: + 4 % aliphatic GSLs: - 43 % indolic GSLs: + 4 %	Robbins *et al.*, 2005

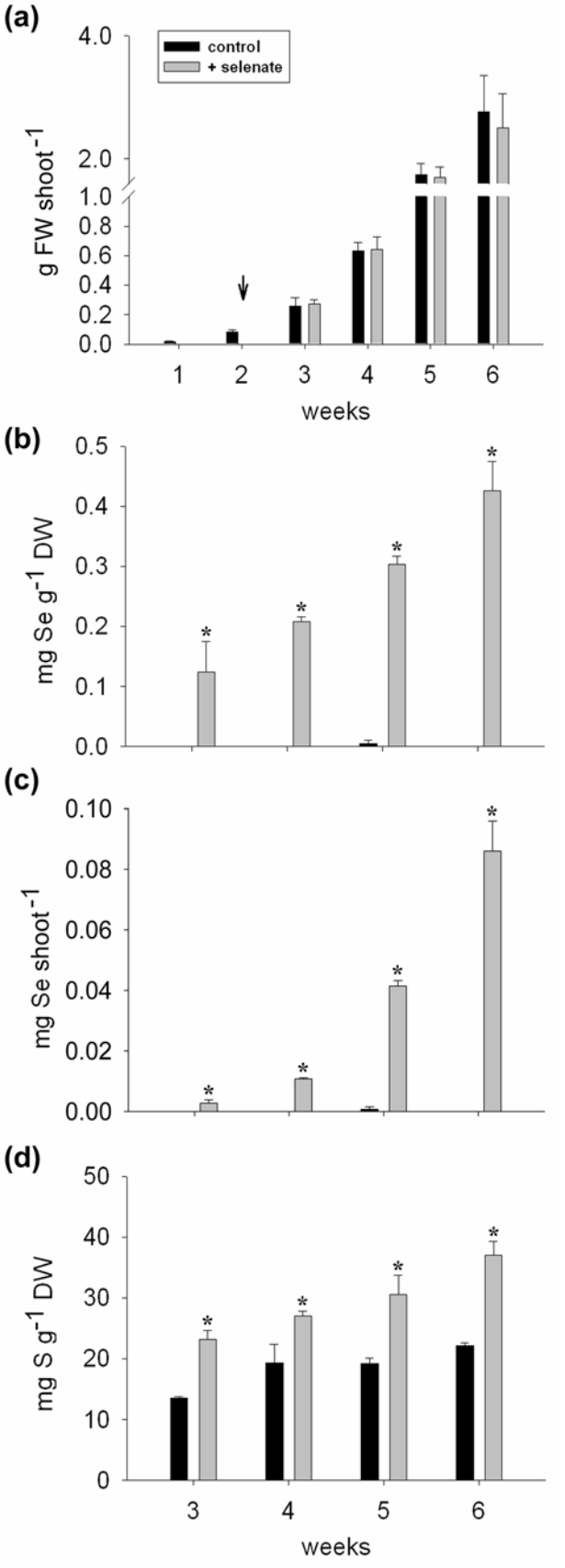

Fig. 3.3 Effect of selenate-fertilization on biomass and accumulation of selenium (Se) and sulfur (S) in shoots of young broccoli plants. Broccoli cultivar, Monaco, was grown by

sand-culture in greenhouse. 40 μM selenate was applied weekly, starting after the second week (arrow). (a), shoot biomass of 1 to 6 weeks old plants; (b) to (d), quantification of Se and S in the shoot of 3 to 6 weeks old plants after starting selenate-fertilization. (b), total selenium concentration; (c) shoot selenium content; (d) total sulfur concentration. Se-accumulation and increase of S was observed in the shoots of selenate-treated plants. Black bars, control plants; gray bars, selenate-fertilized plants. Bars represent arithmetic means; error bars represent SD from 3 independent experiments. Asterisks mark statistically significant differences between control and selenate-fertilized plants; *, $P < 0.001$.

3.2 The effect of Se-fertilization on S-metabolites

3.2.1 The Se-accumulation in the shoots of young broccoli plants.

Se accumulated in the shoot, reaching 130 µg g^{-1} DW after the 1st application and 420 µg g^{-1} DW after the 4th application, respectively (Fig. 3.3b), or, on a per shoot basis, increasing from 2.7 µg to 86 µg over a period of 4 weeks. Total Se content per shoot increased 4-fold from the 3rd to the 4th week, and again 4-fold from the 4th to the 5th week, and still 2-fold from the 5th to the 6th week (Fig. 3.3c), whereas shoot fresh weight increased only 2.4-fold, 2.6-fold and 1.5-fold, respectively (Fig. 3.3a), in these intervals. Based on the total amount of Se applied during the 4-week treatment, about 34% was accumulated in shoots. Note that this rather efficient accumulation of Se in the shoot was observed in the presence of a 25-fold higher sulfate concentration (1 mM) in the nutrient medium.

3.2.2 In the shoots of young broccoli plants, selenate-fertilization resulted in increased total sulfur and sulfate concentrations.

While in control plants, total sulfur content per DW remained unchanged after the 4th week, selenate-treated plants showed significantly higher total sulfur contents during the entire treatment (ranging from +40% to +72%) with a tendency for further increase (Fig. 3.3d). Total sulfur and sulfate concentrations, respectively, were already increased one week after the 1st selenate-treatment (Fig. 3.4a), finally reaching up to 2-fold higher levels compared to control plants at the end of the experiment. In 5-week-old control plants sulfate-S was 9.86 mg g^{-1} DW whereas in selenate-treated plants sulfate-S was 19.52 mg g^{-1} DW, accounting for 51.2% and 63.9% of total sulfur, respectively.

3.2.3 Selenate-fertilization did not affect the concentrations of cysteine, glutathione, total glucosinolates and glucoraphanin in the shoots of young broccoli plants.

In contrast to the Se-induced increases in sulfate and total sulfur, the concentrations of the sulfur metabolites cysteine and glutathione were unchanged upon selenate-treatment during the entire growth period (Fig. 3.4b,c). Cysteine concentrations strongly decreased from the 1st to the 3rd week (-78%), but remained

rather stable thereafter (Fig. 3.4b). Also, glutathione concentrations were significantly higher in the 1st and 2nd week than in the following growth period after the onset of selenate treatment (Fig. 3.4c).

Se-fertilization was reported on decreasing the GSLs content in broccoli (Table 1), however, it is controversial since Se-fertilization can increase total sulfur and sulfate concentrations in the shoots of broccoli (Fig. 3.3d; Fig. 3.4a) and GSLs are S-containing metabolites. In order to confirm whether Se-fertilization does affect the GSLs content, the concentration of total GSLs in the shoots of young broccoli plants was quantified (Fig. 3.5). However, Se-fertilization did not affect the total GSLs content in those plants and the concentration of total GSLs went down from 3 to 6 weeks.

Furthermore, GR, the precursor of sulforaphane and major aliphatic GSLs in broccoli, was quantified by HPLC. For the GR concentration (expressed per g DW), a pronounced decrease by about 80% was observed between the 1st and the 2nd week, which was obviously linked to early plant development (Fig. 3.4d). As during the same growth interval the dry weight per plant increased 5-fold, this apparent decrease is likely the consequence of a growth-related "dilution effect" (i.e. no net *de novo* synthesis). Following this initial lag period, the content of GR per plant increased 4-fold from the 2nd (7.5 µg) to the 3rd (32.0 µg) week, indicating that *de novo* synthesis of GR largely follows vegetative growth after the lag period. Over the entire growth period, selenate-fertilization did not have any significant effect on total GSLs and GR concentrations.

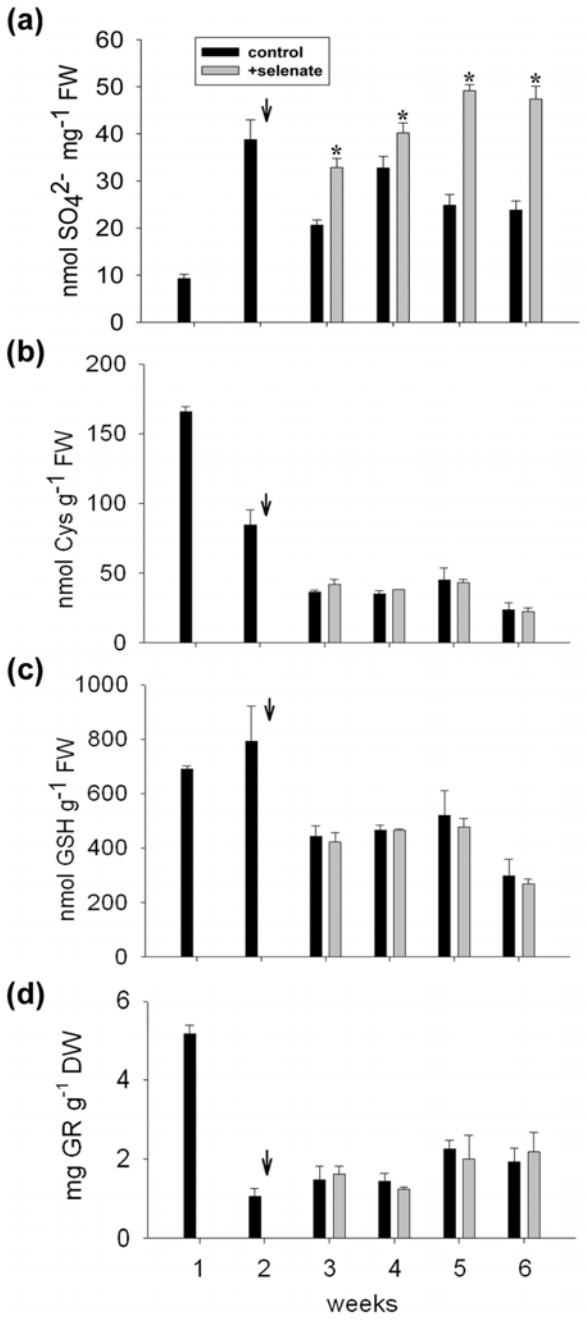

Fig. 3.4 Effect of selenate-fertilization on sulfate, cysteine, glutathione and glucoraphanin concentrations in shoots of young broccoli plants. Broccoli cultivar, Monaco, was grown by sand-culture in greenhouse. 40 μM selenate was applied weekly, starting after the second week (arrow). The concentrations of different S-containing metabolites in shoots were quantified from 1 to 6 weeks old plants by HPLC. (a), sulfate; (b), cysteine; (c), glutathione; (d), glucoraphanin. The increase of sulfate was observed in the shoots of selenate-treated plants. However, selenate-treatment did not significantly affect the concentrations of Cys, GSH and GR. A dramatic drop of Cys-, GSH- and GR-concentration showed in first 2 weeks. Black bars, control plants; gray bars, selenate-fertilized plants. Bars

represent arithmetic means; error bars represent SD from 3 independent experiments. Asterisks mark statistically significant differences between control and selenate-fertilized plants; *, $P < 0.001$.

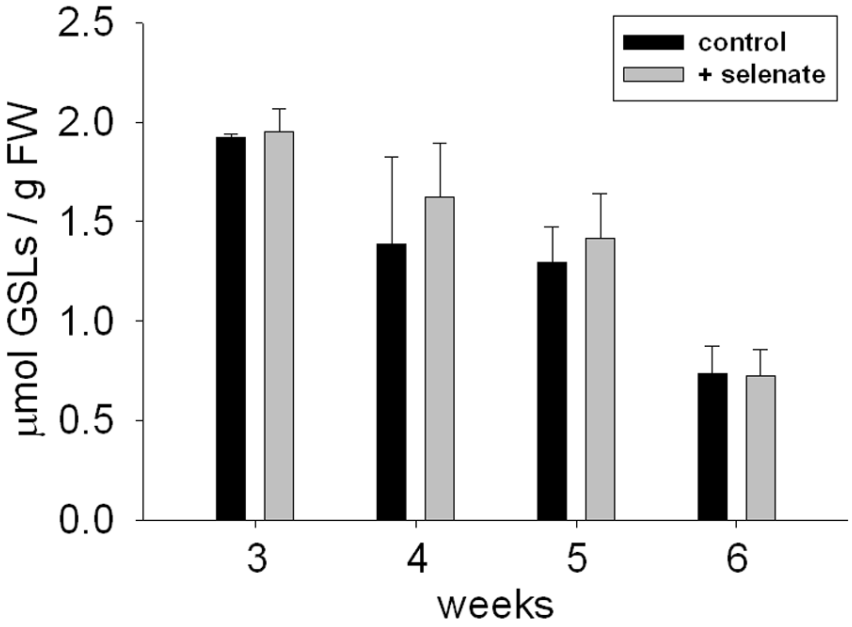

Fig. 3.5 Effect of selenate-fertilization on total GSLs concentration in shoots of young broccoli plants. Broccoli cultivar, Monaco, was grown by sand-culture in greenhouse. 40 μM selenate was applied weekly, starting after the second week. Total GSLs concentration in shoots was quantified from 3 to 6 weeks old plants by glucose assay. Selenate-fertilization did not have obvious impact on total GSLs concentration in the sand-cultured broocoli plants. Black bars, control plants; gray bars, selenate-fertilized plants. Bars represent arithmetic means; error bars represent SD from 3 independent experiments.

3.3 The effect of Se-fertilization on gene expression

3.3.1 The master regulator of aliphatic GSLs biosynthesis, *BoMYB28* transcription factor, was isolated from broccoli.

In *Arabidopsis thaliana, AtMYB28*, the major positive regulator of genes encoding the enzymes of aliphatic glucosinolate biosynthesis, has been reported (Gigolashvili *et al.*, 2007; Hirai *et al.*, 2007). Therefore, to address the question whether selenate-treatment affected the expression of the closest *MYB28* homologue in broccoli plants, I have cloned a full-length cDNA for an orthologous gene *BoMYB28* (GenBank accession: GQ478992; Appendix 8.1) and monitored its expression (Fig. 3.6). The amino acid sequence of *BoMYB28* was aligned with the entire subclade of *Arabidopsis thaliana* R2R3-MYB transcription factors known to be involved in the regulation of aliphatic and indolic glucosinolate biosynthesis, using ClustalW2 (EBI database). The derived phylogram confirmed that *BoMYB28* has the highest sequence similarity with *AtMYB28*, showing 77% identity (note that this high similarity extends beyond the N-terminal DNA-binding motif which is highly conserved among all R2R3-MYB factors!), while sharing only 57% identity with *AtMYB29* (Fig. 3.6a,b); *AtMYB*29 has recently been reported as an accessory factor in response to methyl jasmonate signaling and a positive regulator of aliphatic glucosinolate biosynthesis (Hirai *et al.*, 2007; Gigolashvili *et al.*, 2008).

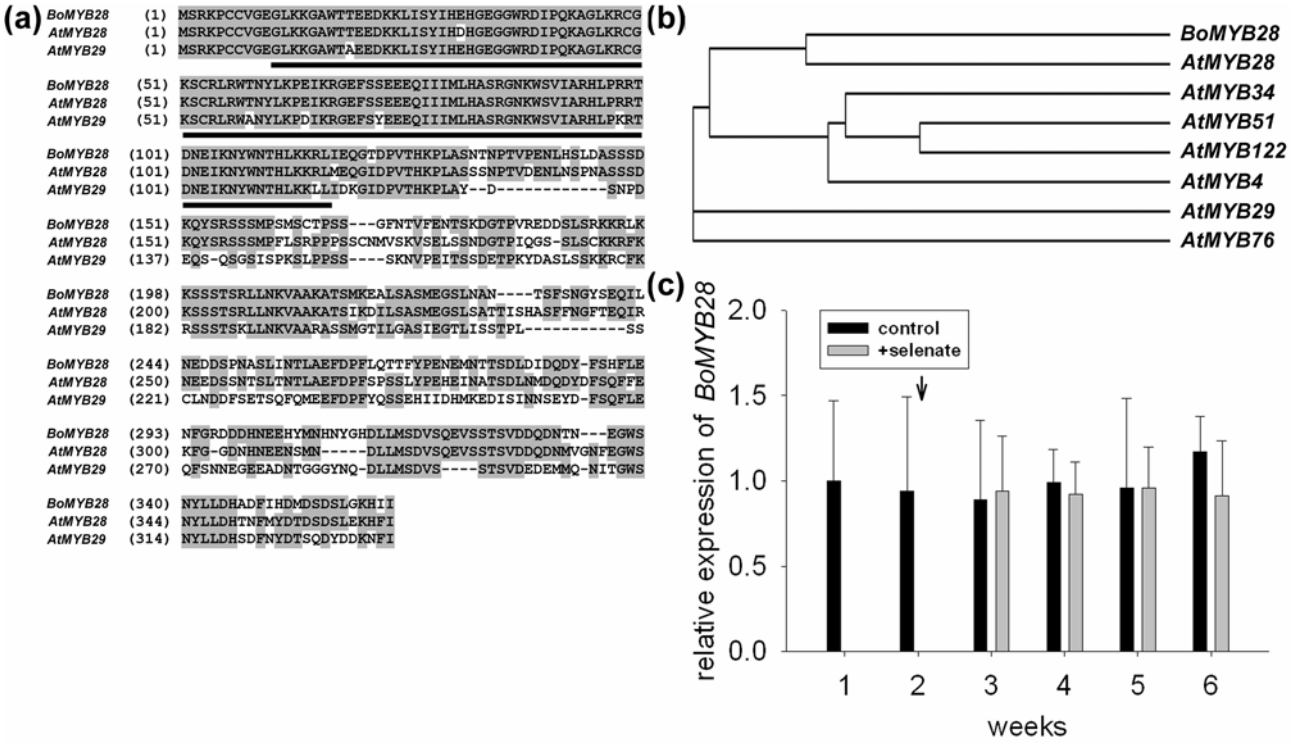

Fig. 3.6 cDNA cloning and expression analysis of *BoMYB28*. (a), alignment of *BoMYB28* amino acid sequence with *AtMYB28* and *AtMYB29* from *Arabidopsis*; conserved residues are shaded in gray. The R2R3 DNA-binding motif is underlined in black. (b), amino acid sequence-based phylogram, depicting the relationship of *BoMYB28* with all R2R3-MYB transcription factors of Arabidopsis thaliana known to be involved in the control of aliphatic (*MYB28/29/76*) or aromatic (*MYB51/34/122*) GSL biosynthesis. (c), effect of selenate-fertilization on the expression of *BoMYB28* in shoots of young broccoli plants. Broccoli cultivar, Monaco, was grown by sand-culture in greenhouse. 40 μM selenate was applied weekly, starting after the second week. The relative expression of *BoMYB28* was determined by qPCR using actin as reference gene; subsequently, all relative transcript levels were normalized to the average relative transcript level in shoots of 1-week-old plants. The expression of *BoMYB28* in shoots of young broccoli plants was not affected by selenate-treatment. Black bars, control plants; gray bars, selenate-treated plants. The arrow indicates the onset of selenate-treatment. Error bars indicate SD from 3 independent experiments. GenBank accession numbers: BoMYB28 (GQ478992); AtMYB28 (NP_851241); AtMYB29 (NP_196386); AtMYB34 (NP_200897); AtMYB51 (NP_173292); AtMYB76 (NP_196387); AtMYB122 (NP_177548); AtMYB4 (NP_195574).

3.3.2 The expression of the transcription factor gene *BoMYB28* was not affected in selenate-fertilized broccoli plants.

Expression analysis for *BoMYB28* in shoots of 1- to 6-week-old plants by qPCR (Fig. 3.6c) did not reveal any change in response to selenate-fertilization during the entire growth period. Interestingly, steady-state *BoMYB28* transcript levels were constant in 1- and 2-week-old plants, respectively, a growth interval during which GSLs content did not show a net increase (see above).

3.3.3 The expression of *BoMYB28* was increased by glucose and decreased by NAA.

According to the microarray analysis for *MYB28*-stimulus treatment in *Arabidopsis* (Appendix 8.2; from GENEVESTIGATOR microarray database, https://www.genevestigator.com/gv/index.jsp), glucose- and NAA-treatment can modulate the expression of *AtMYB28*. To confirm whether *BoMYB28* does work on the GSLs biosynthesis, like *AtMYB28* in *Arabidopsis*, glucose- and NAA-treatment were done with 2-week-old broccoli seedlings (Fig. 3.7). The expression of *BoMYB28* was increased more than 2-fold by glucose and decreased about 34% by NAA. Glucose-treatment can increase the expression of *BoMYB28*, since glucose is released during myrosinase acts on GSLs, thus possibly generating a signal for *MYB28* induction. Some of genes in GSL biosynthesis are also involved in auxin biosynthesis, like *CYP83A1* and *CYP83B1* (Bak & Feyereisen, 2001). Therefore, auxin could be a signal to repress the expression of those genes in GSL biosynthesis.

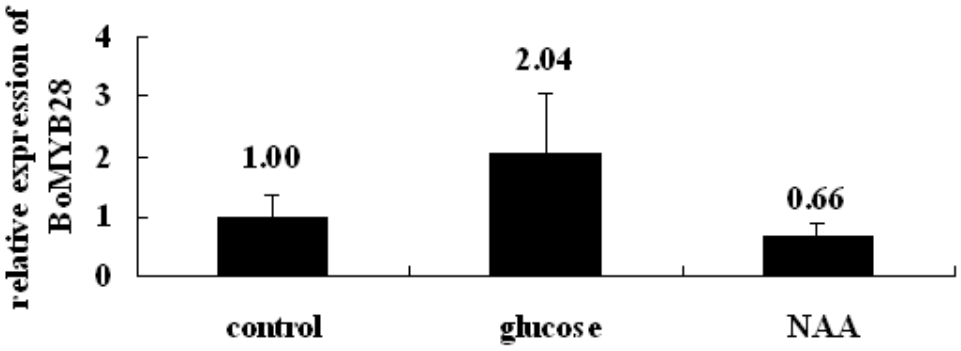

Fig. 3.7 The expression of *BoMYB28* in the shoots of 2-week-old broccoli plants after glucose or NAA treatment. Seeds of broccoli cultivar, Monaco, were sterilized and grown on agar medium. Two weeks after germination, uniform seedlings were transferred to the medium contains 1% glucose or 1 μM NAA. After 24 hrs, the shoots of 2-week-old plants were harvested and measured the expression of *BoMYB28* by qPCR. The relative expression of *BoMYB28* was determined by qPCR using actin as reference gene; subsequently, all relative transcript levels were normalized to the average relative transcript level in control plants. Glucose-treatment could increase the expression of *BoMYB28*. On the other hand, the expression of *BoMYB28* was decreased by NAA-treatment. Error bars indicate SD from 6 independent experiments.

3.3.4 *BoMYB28* regulated genes of the aliphatic GSL biosynthetic pathway

To further confirm that the putative *BoMYB28* is indeed functionally equivalent to its *AtMYB28* homolog, its potential to specifically regulate genes of the aliphatic GSL biosynthetic pathway was explored in a transient promoter activation assay. This technique has recently been used to functionally characterize transcription factors and their target specificities in the flavonoid pathway (Bogs *et al*., 2007; Czemmel *et al*., 2009). In *Arabidopsis thaliana*, *AtMYB28* has been shown to activate the expression of genes for aliphatic GSL biosynthesis (see above; Gigolashvili *et al*., 2007b; Hirai *et al*., 2007). Therefore, promoter regions of the assumed target genes *MAM1*, *CYP83A1* and *AOP2* were isolated from *Arabidopsis* genomic DNA (Appendix 8.3; 8.4; 8.5) and cloned into a luciferase reporter vector. The chosen genes are involved in different stages of aliphatic GSL biosynthesis and are upregulated in an *AtMYB28*-overexpressing mutant (Hirai *et al*., 2007). For transient expression in Arabidopsis protoplasts, the full-length cDNA of *BoMYB28* was cloned into the expression vector pART7, allowing its expression under control of the 35S promoter. For comparison, the *AtMYB114* transcription factor involved in anthocyanin biosynthesis and a promoter-luciferase reporter construct for its target gene *UFGT* (UDP-Glc:flavonoid-3-O-glucosyltransferase) were included in the analysis. As *AtMYB114* is dependent on group IIIf bHLH and the WD40 type transcription factor TTG1 (Gonzalez *et al*.2008), expression vectors for the bHLH factor EGL3 and for TTG1 were included in all transfection assays.

Co-transfection of *Arabidopsis* leaf protoplasts with *AtMYB114* and the *UFGT*-reporter construct resulted in a normalized luciferase activity of about 10 (positive control), whereas co-transfection with the empty vector pART7 yielded a background luciferase activity of < 0.1 (negative control; Fig. 3.8). Likewise, co-transfection of *BoMYB28* with the *UFGT*-reporter construct did not cause promoter activation. As expected, *AtMYB114* did not induce the promoter activities of *CYP83A1* (average 0.08: 0.01-0.15) and *AOP2* (average 0.05: 0.01-0.1). Surprisingly, *MYB114* apparently induced the promoter activity of *MAM1* (average 2.2: 0.2-4.7), albeit with high variation. Conversely, *BoMYB28* consistently induced the promoter activities of *MAM1*, *CYP83A1* and *AOP2*. The average normalized luciferase activities were 3.1 for *MAM1* (1.5-5.2), 1.0 for *CYP83A1* (0.8-1.6), and 4.4 for *AOP2* (1.0-9.7). Thus, *BoMYB28* may be regarded as a functional homolog to the previously

characterized *AtMYB28*.

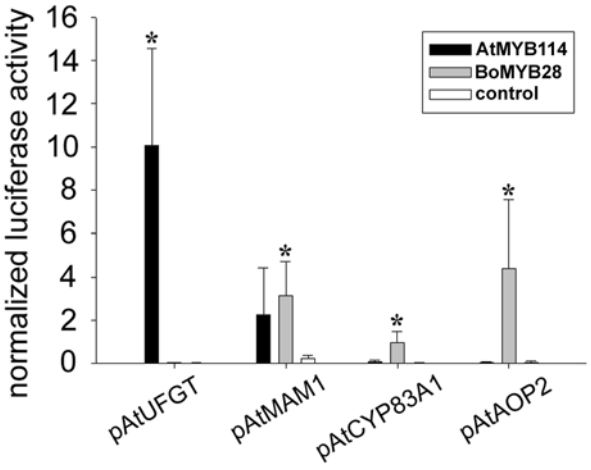

Fig. 3.8 Confirmation of *BoMYB28* function as a regulator of aliphatic glucosinolate biosynthesis in a transient target promoter activation assay.

Transcription factors and promoter fragments used for transfection of *Arabidopsis* protoplast are indicated. *AtMYB114* (black bars) and *BoMYB28* (gray bars) were cloned into the transient-expression vector, pART7, and empty vector was used as the control (white bars). Promoter activation was assessed by determining activities of the reporter luciferase (for further details see Materials and Methods). *AtMYB114* activates its target gene, *UFGT*, which is involved in anthocyanin biosynthesis. *MAM1*, *CYP83A1* and *AOP2* are genes of aliphatic GSL biosynthesis in *Arabidopsis*, and their promoters are activated by *BoMYB28*. Each transfection contained the *Renilla* luciferase plasmid pRluc (Horstmann et al., 2004) as internal control and the transcription factors EGL3 and TTG1 in pART7 as co-factors. Normalized luciferase activities were calculated as ratio between the *Firefly* and the *Renilla* luciferase activity. Error bars indicate SD from 6 independent experiments. Asterisks mark statistically significant differences between control and cells transfected with transcription factors; *, $P < 0.001$.

3.3.5 The expression of sulfate transporter genes was altered in response to selenate-fertilization.

High-affinity sulfate transporters (SULTRs) play the major role for the initial uptake of sulfate and selenate and are primarily expressed in roots. Yoshimoto *et al.* (2002) reported that in *Arabidopsis thaliana* the expression of two distinct high-affinity *SULTR* genes (*AtSULTR1;1* and *AtSULTR1;2*) was increased to different degrees in response to selenate-treatment, correlating with an increased sulfate concentration in leaves. The observation of selenate-induced expression of *AtSULTR1;1* was recently confirmed by microarray-based expression profiling, together with an increased sulfate content in the shoot (Van Hoewyk *et al.*, 2008). Therefore, based on our observations of increased total sulfur and sulfate contents in young broccoli plants, I monitored the expression of several sulfate transporters by qPCR, using primers based on conserved regions of *AtSULTR1;1, AtSULTR1;2, AtSULTR2;1* and *AtSULTR2;2* cDNAs. The latter two transporters were included as they are thought to play a role in long distance transport, *AtSULTR2;1* is primarily expressed in the xylem, whereas *AtSULTR2;2* expression appears to be localized to the phloem (Takahashi *et al.* 2000; Kataoka *et al.* 2004). All *SULTR* PCR products from broccoli were sequenced and their sequence similarities to the corresponding *Arabidopsis thaliana* genes confirmed (Fig. 3.9). As a cautious note, it has to be emphasized that it cannot be excluded that in *B. oleracea* more than one closely homologous gene exists for each of the corresponding *AtSULTR* gene (and this holds true also for *BoMYB28*). However, the primers used in our qPCR analysis always amplified only a single product.

In agreement with previous reports on *Arabidopsis*, I observed a dramatically increased *BoSULTR1;1* expression in the roots of selenate-treated plants by more than 60-fold when compared to control plants (Fig. 3.10b). Furthermore, transcript levels for *BoSULTR1;2* and *BoSULTR2;1* in the roots increased 2-fold in response to selenate-treatment, whereas transcripts for *BoSULTR2;2* decreased. Conversely, in shoots transcripts levels of *BoSULTR1;2, BoSULTR2;1* and *BoSULTR2;2* were all moderately lower in selenate-treated plants than in controls, and, as expected because of the root-specific expression of *AtSULTR1;1, BoSULTR1;1* transcripts were undetectable (Fig. 3.10). Assuming that the observed changes in *BoSULTR* gene transcript levels in response to selenate fertilization result in corresponding changes of

transporter activities, increased uptake rates and increased root-to-shoot transport of sulfate and selenate would be expected. To test this, I determined the root-shoot distribution of total sulfur and selenium (Fig. 3.11). In fact, the increased total S concentration in shoots of selenate-treated plants (see also Fig. 3.3d) was accompanied by a significant reduction in roots, and the total Se-concentration in shoots was about 60-fold higher than in roots.

In summary, monitoring plant development and concentrations of major sulfur metabolites in young broccoli plants did not reveal any negative effect of selenate fertilization via the root system. The increase in total sulfur (and sulfate), together with the efficient Se-accumulation in the shoot rather reflect transport-specific effects of selenate on sulfate uptake and root-shoot transfer.

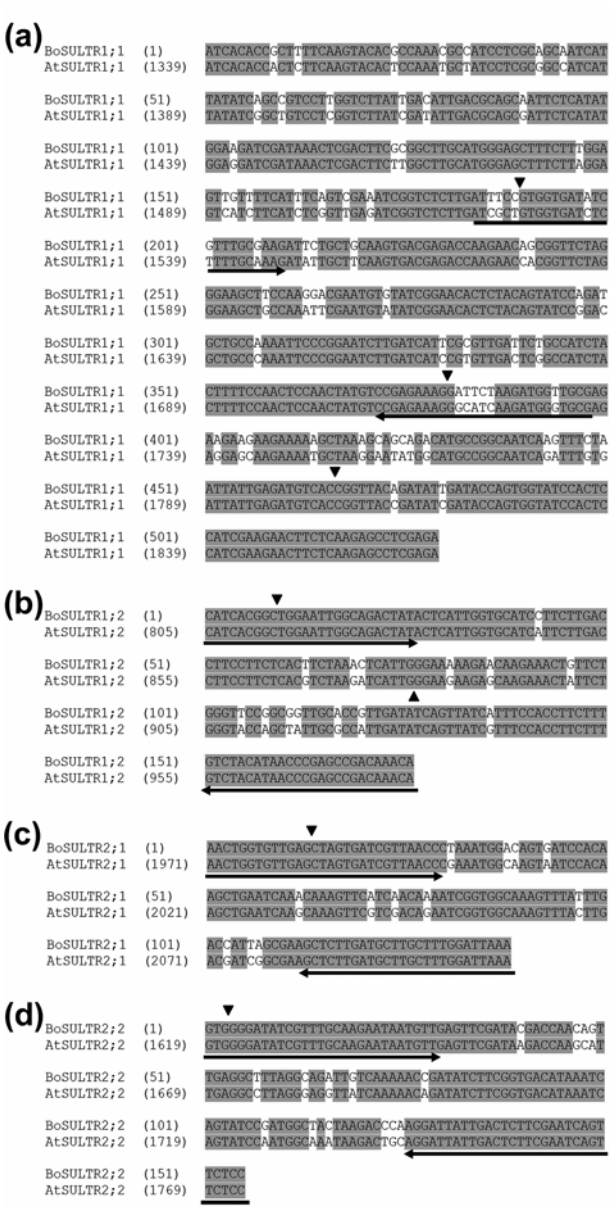

Fig. 3.9 Alignment of partial cDNAs of *BoSULTRs* and *AtSULTRs*. Alignment of partial cDNA sequences of *BoSULTR*s with *AtSULTR1;1* (NM_116931), *AtSULTR1;2* (NM_106449), *AtSULTR2;1* (NM_121056), and *AtSULTR2;2* (NM_106448). Conserved nucleotides are shaded in gray. Arrows mark the annealing sites of the primers used for RT-PCR and qPCR. Solid triangles indicate the positions of introns.

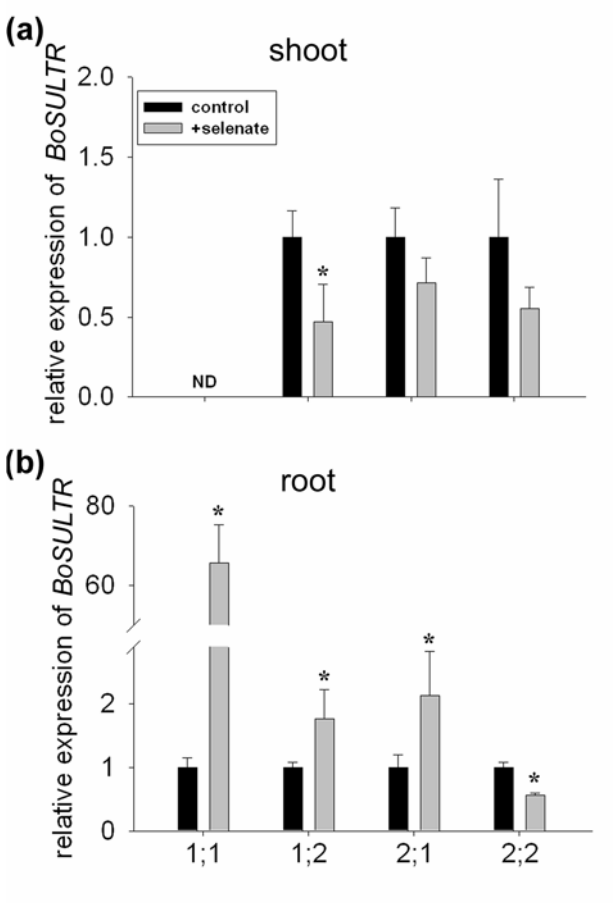

Fig. 3.10 Effect of selenate-fertilization on the expression of sulfate transporter (*SULTR*) genes in shoots and roots of 6-week-old broccoli plants. Partial cDNAs of broccoli *SULTR*s (*BoSULTR1;1, 1;2, 2;1,* and *2;2*) were cloned by RT-PCR, using primers based on the sequences of the closest homologous genes from *Arabidopsis thaliana* (see Materials and Methods, and Fig. 3.9). The relative expression of *BoSULTR* genes was determined by qPCR using actin as reference gene; subsequently, relative transcript levels were normalized to the average relative transcript level of the respective control plants. Black bars, control plants; gray bars, selenate-treated plants. Error bars indicate SD from 3 independent experiments. Asterisks mark statistically significant differences between control and selenate-fertilized plants; *, $P < 0.001$.

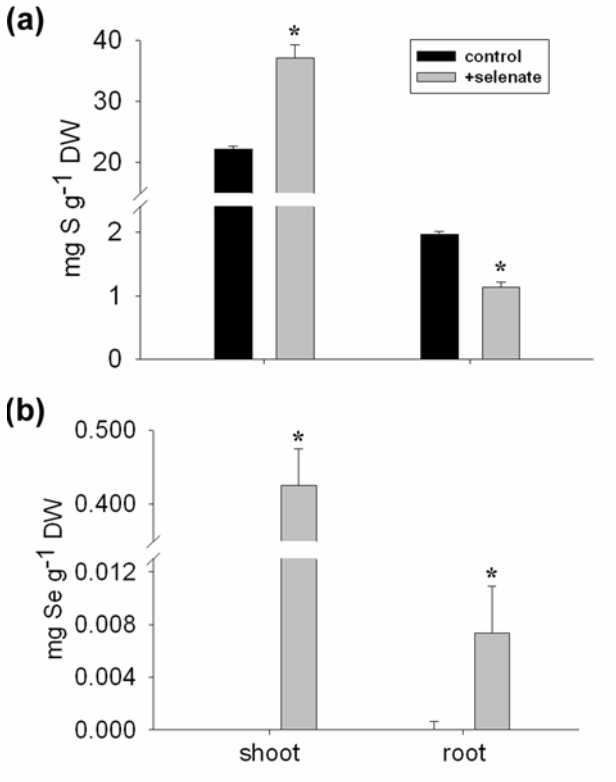

Fig. 3.11 Effect of selenate-fertilization on the accumulation of total sulfur (S) and selenium (Se) in shoots and roots of 6-week-old broccoli plants. (a), total sulfur concentrations in shoots and roots, respectively. (b), total selenium concentrations in shoots and roots, respectively. Note that selenate-application was initiated at a plant age of 2 weeks (see also Fig. 3.3, 3.4). Black bars, control plants; gray bars, selenate-fertilized plants. Bars represent arithmetic means; error bars represent SD from 3 independent experiments. Asterisks mark statistically significant differences between control and selenate-fertilized plants; *, $P < 0.001$.

3.4 Leaf-fertilization of mature broccoli plants with selenate: Evidence for efficient leaf-to-head transfer under field conditions.

Under field conditions, Se-accumulation in crops and vegetables is limited by low selenate concentrations and availability in most soils. While application of selenate to the root system represents a valid option to fortify broccoli with selenium without impairing glucoraphanin biosynthesis (see above), I also explored the efficiency of spraying selenate-solution directly to leaves of 3-month-old field-grown broccoli plants at an early stage of head development. Selenate application via leaf spray resulted in efficient Se-accumulation in the broccoli head (about 25% of the applied selenium was recovered in the broccoli head), indicative of efficient leaf-to-head transfer (Fig. 3.12f), however, again the concentrations of the different sulfur metabolites, including GR, were not significantly affected (Fig. 3.12a-e). For the 20 mg Se-application, the distribution of selenium between floret and upper stem did not reveal a significant difference, in agreement with the distribution of sulfate (Fig. 3.12d,f; for the 2 mg Se application the data showed a higher variability). While sulfate concentrations were similar in florets and upper stem of control and selenate-treated plants, cysteine, glutathione and total sulfur concentrations were about 2-fold higher in florets as compared with the upper stem (Fig. 3.12b-e). Conversely, GR concentrations were 3- to 4-fold higher in upper stem than in florets (Fig. 3.12a).

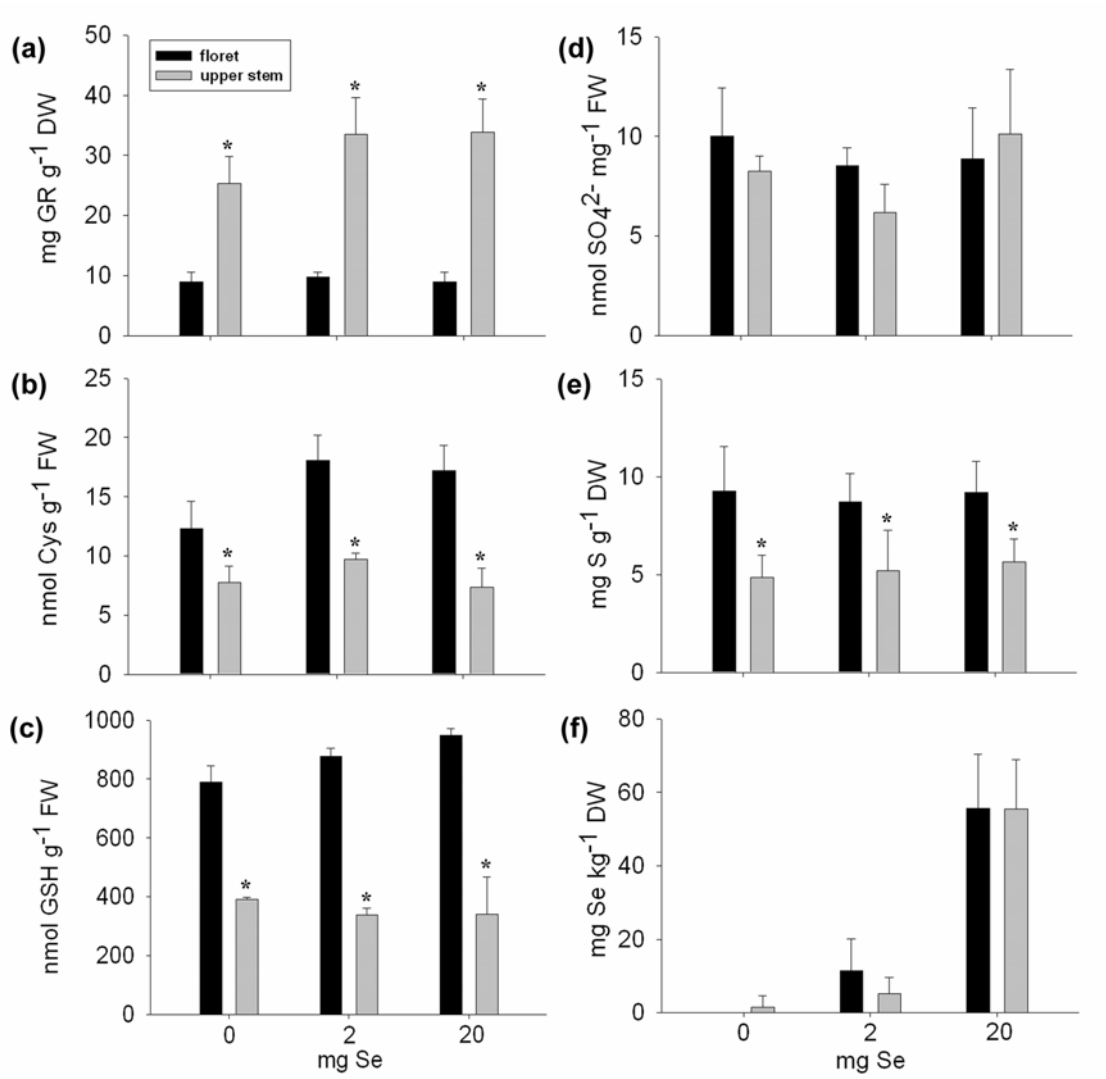

Fig. 3.12 Effect of selenate-application to leaves through spraying on 3-month-old field-grown broccoli plants: Se-accumulation and S-metabolite concentrations in broccoli heads. Selenate corresponding to 0, 2, or 20 mg Se plant^{-1} (0, 25, and 250 µmol plant^{-1}) was applied as a single spray to the leaves of plants with a head diameter of 2 cm (head was covered during spray). After 1 week, head diameter had reached 10 cm and terminal florets and upper stem were harvested for total Se, total S, and S-metabolite analysis, respectively. Bars represent arithmetic means of concentrations of GR (a), Cys (b), GSH (c), sulfate (d), total S (e), and total Se (f); error bars indicate SD from 3 independent experiments. Asterisks mark statistically significant differences between floret and upperstem; *, $P <$ 0.001.

4. DISCUSSION

4.1 Does Se-treatment affect the growth of broccoli?

The essentiality of Se for the growth is still unclear in plants. Se accumulator, *Astragalus pectinatus*, was observed an increase in biomass production after treating 0.38 mM Se (Trelease & Trealease, 1939). These results were challenged subsequently by Broyer *et al*. (1972), who attributed the growth stimulation in the *Astragalus* plants to the ability of Se in the nutrient solution to counteract phosphate toxicity; at low phosphate concentrations, growth was not stimulated by Se-treatment. There is no evidence for a Se requirement in nonaccumulators (Shrift, 1969). Broccoli is secondary accumulator and a putative SMT was found for Se-metabolism in broccoli (Lyi *et al*., 2005). Therefore, the effect of Se-treatment on the growth of broccoli is interesting to be investigated. From the root-length experiment (Fig. 3.2), the root-growth of broccoli young plants was affected by Se-treatment. Selenate has a stronger effect on the root-growth than selenite. Perhaps, this is due to that the uptake of selenate via roots can trigger the S-starvation effect in roots and then influences the root-growth. However, the growth of shoots was not significant influenced by Se-treatment, whatever selenite or selenate. The fresh weight of shoots from sand-culture broccoli plants was not affected by selenate-fertilization, too (Fig. 3.3a). According to our observation, Se-treatment has impact on the root-growth, however, no significant effect on the shoot-growth of broccoli.

Besides, variations of GSL-content (Fig. 3.1; Kliebenstein *et al*., 2001a) and Se-tolerance (Fig. 3.2; Zhang *et al*., 2007) have been observed between different broccoli cultivars and *Arabidopsis* ecotypes. However, according to those studies, GSL-content in plant seems to have no connection with Se-tolerance.

4.2 In broccoli, selenate-treatment increases sulfate-uptake and sulfate-transfer from root to shoot

4.2.1 S-content is increased in the shoots and decreased in the roots by selenate-fertilization

Previous studies had demonstrated that in *Arabidopsis thaliana* selenate-treatment can increase sulfate uptake (Takahashi *et al*. 2000; Yoshimoto *et al*. 2002; White *et al.*, 2004; Van Hoewyk *et al.*, 2008). However, for broccoli a significant competition between sulfate and selenate for uptake and assimilation has also been demonstrated, selenate negatively affecting the biosynthesis of the sulfur-containing secondary metaboite glucoraphanin (Lyi *et al.*, 2005; Finley *et al.*, 2005). Regarding the relevance of such a possible "antagonism" for the production of Se-enriched broccoli as an improved functional food for cancer prevention, I have readdressed this apparent contradiction and monitored the contents of sulfate and total sulfur in shoots of young broccoli plants, treated with a moderate dose of selenate. In fact, I observed a substantial increase in total sulfur in the shoots which was largely due to an increased sulfate accumulation in the shoot. (Fig. 3.3d; Fig. 3.4a; Fig. 3.11a). Conversely, the content of total sulfur in the root revealed a pronounced decrease in response to selenate-fertilization (Fig. 3.11a). These results are in agreement with previous observations of Yoshimoto *et al.* (2002) and El Kassis *et al.* (2007), who after short-term selenate-treatment of *Arabidopsis* plants observed increased sulfate contents in shoots but reduced sulfate amounts in roots.

4.2.2 Selenate-fertilization stimulates the expression of *SULTR1;1*, *1;2* and *2;1* in the roots for the initial sulfate-uptake and long-distance transport from root to shoot

In addition, I have demonstrated that in young broccoli plants selenate-treatment significantly affected the expression of different *SULTR* isoforms. A similar effect was earlier observed for *Arabidopsis thaliana* (Takahashi *et al*. 2000; Yoshimoto *et al*. 2002; Van Hoewyk *et al*. 2008). A detailed analysis of the effects of selenate-treatment on *BoSULTR* expression in roots of young broccoli plants (Fig. 3.10b) revealed an interesting pattern. First, the expression of the *BoSULTR1;1* and *1;2* was increased, these two transporter isoforms being responsible for the primary

uptake of sulfate and selenate at the rhizodermis, and, second, the ratio of transcripts for the *BoSULTR2;1* isoform to the *BoSULT2;2* isoform showed a pronounced increase. Assuming that the latter two transporter isoforms are, like their *Arabidopsis* orthologs, localized in the xylem and phloem, respectively (Takahashi *et al*., 2000; Kataoka *et al*., 2004), this shift might be responsible for an increased root-shoot transfer of sulfate, in agreement with the observed shift in total sulfur contents (Fig. 3.11a). I therefore conclude that in young broccoli plants selenate-treatment modulates the expression of different *BoSULTR* isoforms such as to trigger the selenate flux from root to shoot, and, as a direct consequence causes an increased root-shoot transfer of sulfate (Fig. 4.1).

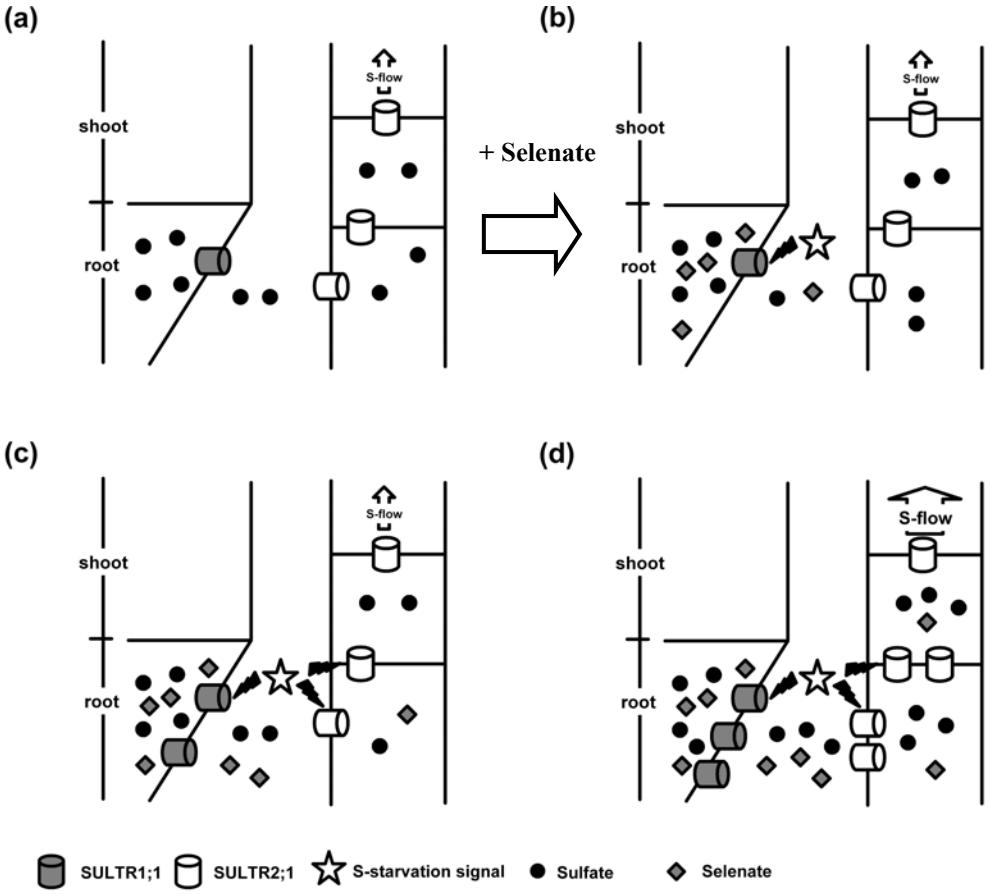

Fig. 4.1 Selenate-fertilization triggers the local S-starvation signal in the root to increase the expression of *SULTR1;1* and *2;1* and the S-flux from root to shoot. (a) *SULTR1;1* is a high-affinity SULTR for the initial uptake of sulfate and selenate from soil to root. The expression of *SULTR1;1* is major in roots and is stress-inducible during S-starvation. *SULTR2;1* is a low-affinity SULTR for long distance transport in xylem. After selenate-treatment (b), S-starvation signal is induced and triggers to increase the expression of *SULTR1;1* and *2;1* in roots (c), since sulfate and selenate are competitors for the initial uptake. Therefore, the S- and Se-flux from root to shoot is enhanced, due to the increased expression of *SULTR1;1* and *2;1* (d).

4.2.3 The Se-induced increase of sulfate-content in the shoots is not subject to further metabolism

Interestingly, selenate-treatment did not affect the levels of several S-metabolites, i.e. cysteine, GSH and glucoraphanin, the increased sulfate being most likely compartmentalized in the vacuole. Under the chosen growth conditions (1 mM sulfate in the nutrient solution), the tissue sulfate availability was apparently high enough to saturate the corresponding metabolic routes. It remains to be shown whether under sulfate limitation, selenate treatment might even stimulate glucosinolate biosynthesis by increasing sulfate uptake and root to shoot transfer. It is noteworthy that our transcript analysis of BoSULTR isoform expression was done at a growth stage where the system was already adapted to the selenate-treatment (i.e. 4 weeks after the first application), indicating that the observed changes were part of a long-term switch in transporter expression.

S-starvation was previously shown to repress the expression of *AtMYB28* (Hirai *et al.*, 2007) and to increase the expression of *AtSULTR1;1* (Yoshimoto *et al.*, 2002; El Kassis *et al.*, 2007; Yoshimoto *et al.*, 2007; Van Hoewyk *et al.*, 2008). Thus, it may be speculated that the observed drastic increase of *BoSULTR1;1* expression by selenate-treatment may be due to a local sulfur starvation signal limited to the root. In fact, as a result of an increased root-shoot transfer of sulfate (see above), the total sulfur content in roots decreases in response to selenate-treatment (Fig. 3.11a).

4.3 Selenate-application did not affect the concentration (and content) of glucoraphanin and total GSL in broccoli

In *Arabidopsis*, several genes of GSL biosynthesis are repressed in response to sulfate-limitation and Se-stress (Grubb & Abel, 2006; Hirai *et al.*, 2007; Van Hoewyk *et al.*, 2008). In addition, several studies have demonstrated that selenate-fertilization may significantly decrease the contents of GSLs in broccoli when plants were treated with a high selenate dosage (>800 μg Se g^{-1} FW; Finley *et al.*, 2005; Robbins *et al.*, 2005). However, in our study, selenate did not affect glucoraphanin content, even after a 4-week-long exposure (Fig. 3.4d). Also, when using a leaf spray application mode, the substantial Se accumulation in the broccoli head did not result in any interference with glucoraphanin accumulation during the period of head expansion (Fig. 3.12a). Interestingly, at the chosen developmental stage of head formation, the glucoraphanin content was 2-3 folds higher in the upper stem as compared with the terminal floret (Fig. 3.12a). Whether this discrepancy reflects a transient phenomenon during head development remains to be shown. Possibly, upper stems may contain high glucosinolate content in transit to the developing flowers where they accumulate later during seed formation.

It is noteworthy, that the direct application of selenate to the leaves of adult broccoli plants did not result in changes of total sulfur or sulfate in the broccoli head, corroborating the interpretation of root-localized changes in *BoSULTR* isoform expression (Fig. 3.10) being responsible for the effects. As the illustration in Fig. 4.1, selenate-treatment via root increases the expression of *SULTR1;1* and *2;1* and then enhances the S- and Se-flux from root to shoot. However, leaf-fertilization can not trigger this process.

4.4 *BoMYB28*: A regulator of aliphatic GSL biosynthesis functionally homologous to *AtMYB28*

Although the sequence of *BoMYB28* has highest homologous with *AtMYB28* (Fig. 3.6a, b), besides, the expression of *BoMYB28* is increased by glucose-treatment and is decreased by NAA-treatment (Fig. 3.7). However, I still need more direct evidence to confirm whether *BoMYB28* does work on GSL biosynthesis in broccoli. Here, I confirmed the target genes of *BoMYB28* by luciferase assay (Fig. 3.8). The promoter region of three genes in the aliphatic GSL biosynthesis was isolated from *Arabidopsis*. *MAM1* is a member of a gene family sharing approximately 60% amino acid sequence similarity with 2-isopropylmalate synthase, an enzyme of leucine biosynthesis that condenses 2-oxo-3-methylbutanoate with acetylcoenzyme A (Kroymann *et al.*, 2001). *CYP83A1* catalyzes the initial conversion of aldoximes to thiohydroximates in the synthesis of glucosinolates not derived from tryptophan (Bak & Feyereisen, 2001; Naur *et al*., 2003). *AOP2* catalyzes the conversion of methylsulfinylalkyl glucosinolates to alkenyl glucosinolates (Kliebenstein *et al.*, 2001b). They have been reported on different stages of aliphatic GSL biosynthesis and upregulated in *AtMYB28*-overexpressed mutant (Fig. 1.1; Hirai *et al.*, 2007). In our luciferase assay, *BoMYB28* significantly induced the promoter activity of *MAM1*, *CYP83A1* and *AOP2* (Fig. 3.8). Since that, the ability of *BoMYB28* to regulate genes of aliphatic GSL biosynthetic pathway as *AtMYB28* is directly confirmed.

4.5 Conclusions and perspectives

In this study, the effect of selenate-treatment on young broccoli plants was examined, with a focus on growth effects and possible interference with sulfur metabolism, including the biosynthesis of glucoraphanin, which is a major aliphatic glucosinolate in broccoli and the precursor of the anti-cancer compound sulforaphane. Furthermore, the transcription factor *BoMYB28*, the closest homolog to *AtMYB28*, which regulates aliphatic glucosinolate biosynthesis, and partial cDNAs of putative sulfate transporters (*BoSULTR*) were cloned from broccoli. Our study has revealed that a possible competition between selenate and sulfate, which was postulated based on the identical primary uptake and assimilation routes for both oxoanions, does not impair glucosinolate biosynthesis. Instead, selenate fertilization stimulates the expression of several sulfate transporters, which results in increased total sulfate levels in the shoot, as a consequence of both increased root uptake and, most likely, increased root-shoot transfer. In addition, selenate application did not negatively affect plant growth or the level of glutathione, a central component of cellular redox control and an important metabolite for plant defence against biotic and abiotic stress (Rausch & Wachter, 2005; Mullineaux & Rausch, 2005).

I conclude that broccoli may be fortified with Se without negative trade-offs towards glucosinolate accumulation and plant growth, provided an appropriate selenate-fertilization scheme is adopted. In fact, as a major result of selenate uptake the total sulfur status of the plant changes towards an increased sulfur-content in the shoot. However, this latter effect is limited to selenate application via the soil. Under these conditions, the major "detoxification" pathway for selenate appears to be the efficient transfer from root to shoot, which requires upgrading the directed sulfate transport.

Some questions remain to be solved in the future. First, does Se-fertilization affect glucosinolate biosynthesis in broccoli? It is really controversial, since selenate-fertilization can enhance S-accumulation in the shoots and also can compete with sulfate for the initial uptake. In our experiments, appropriate dosage of selenate-application, which is at a level suitable for human consumption, did not have obvious impact on the concentration of GSLs and GR in broccoli (Fig. 3.4d; Fig. 3.5).

Besides, the expression of *BoMYB28* was not influenced by selenate-fertilization, too (Fig. 3.6c). However, several studies have shown the decreasing of GSLs in broccoli by Se-fertilization (Table 3.1). In order to clear this conflict, the detailed ratio of selenate to sulfate for fertilization should be examined. In addition, which developmental stage of broccoli treated with selenate is also an important point.

Second, how do GSLs and their precursors transport in the whole plant? This is a long-standing question. Recently, the plastidic bile acid transporter 5 (*BAT5*) was identified as the first transporter component of the aliphatic glucosinolate biosynthetic pathway (Gigolashvili *et al*., 2009). *BAT5* has been identified as the target gene of *MYB28*, the major positive regulator of aliphatic GSL biosynthesis and annotated as a bile acid transporter is involved in the transport of 2-keto acids between chloroplasts and the cytosol (Fig. 4.2). This is the first evidence for the subcellular transport of GSLs biosynthesis. However, the long-distance transport of GSLs is still unknown and whether a specific transporter works for this. Furthermore, an interesting observation in our field experiment is that stems of broccoli head had higher GR content than floret (Fig. 3.12a). That perhaps means GSLs is delivered from leaves (source) to flower (sink), since seeds have highest GSLs contents in whole developmental-stage of plant.

Third, what is the S-starvation signal? S-deficiency and selenate-treatment can trigger S-starvation signal to increase the expression of *SULTR1;1, 1;2,* and *2;1* for the initial S-uptake and transport. However, the S-starvation signal is still unclear. The possible candidate is like Cys, GSH, or *O*-acetyl-L-serine (OAS). Cys and GSH are considered the major S-donor for most S-containing metabolites and GSLs (Rausch & Wachter 2005; Geu-Flores *et al*., 2009). Especially, what role GSH play in GSLs biosynthesis still needs to be investigated. In addition, OAS is the most possible candidate, since OAS has been reported as a general regulator for global gene expression under sulfur-nutrition stress (Hirai *et al*., 2003). The increasing of OAS content in the plant was also observed under S-starvation treatment.

Last, the function of *BoMYB28* has to be confirmed, although its sequence and target genes have been proved as *AtMYB28*. To compensate *Arabidopsis myb28* mutant probably can provide the direct evidence.

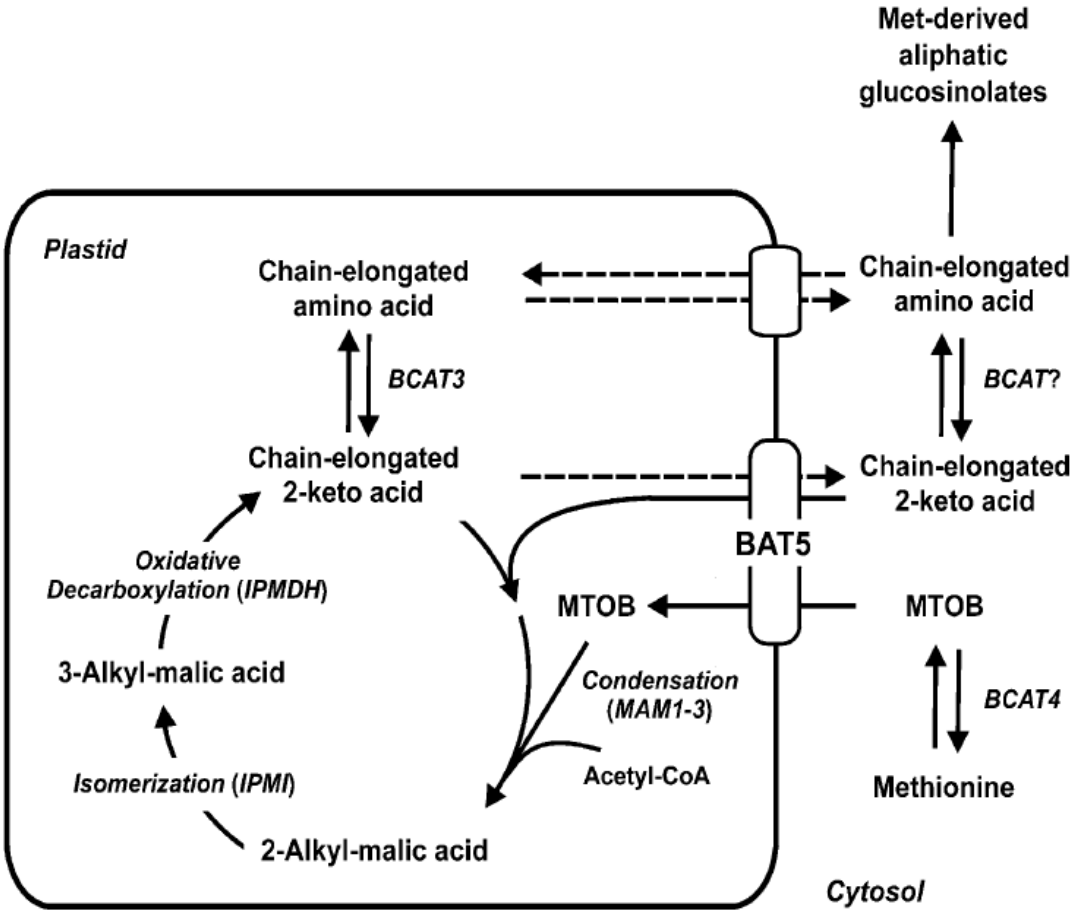

Fig. 4.2 Schematic representation of the role of BAT5 in the transport of 2-keto acids, side chain elongation of 2-keto acids, and biosynthesis of met-derived GSLs. (from Gigolashvili *et al*., 2009)

5. MATERIALS AND METHODS

5.1 Plant material and cultivation

5.1.1 Plant material and sterilization of seeds

For Se-tolerance experiment, 4 commercial cultivars of Broccoli (*Brassica oleracea* var. *italica*) were chosen: Marathon, Monaco, Montop, and Ironman. 1-week-old seedlings were harvested for glucoraphanin quantification. In addition, Monaco was used to study the effects of selenate-, glucose- and NAA-treatment at the young plant and the adult plant stage (leaf application), respectively.

In order to allow seeds to germinate on the medium with or without different treatment, broccoli seeds were sterilized through 3 short incubations in a bleaching solution containing 4% NaOCl in water supplemented with 0.02% of Triton X-100. During the last step, the samples were transferred to a sterile bench and thereafter the seeds were washed three times with sterile ddH_2O before being transferred on medium. Plates were sealed with Micropore 3M surgical tape and incubated at 4 °C for two to three days before being transferred to a growth chamber or the greenhouse.

5.1.2 Medium for root-length experiment, glucose-treatment and NAA-treatment.

The medium contains half-strength Murashige and Skoog (MS) medium (Murashige & Skoog, 1962), 1% sucrose and 0.75% plant agar (pH 5.8). For Se-tolerance experiment, 50 μM sodium selenite or 50 μM sodium selenate was added in the medium. 1% glucose and 1μM NAA was added to confirm the function of *BoMYB28*.

5.1.3 Sand-culture

Nutrient solution:

(a) Macronutrients:

KNO_3:	808.88 mg l^{-1}	$Ca(NO_3)_2*4H_2O$:	944.64 mg l^{-1}
KH_2PO_4:	136.09 mg l^{-1}	$MgSO_4*7H_2O$:	246.47 mg l^{-1}

(b) Micronutrients:

Fe-EDTA:	7.34 mg l^{-1}	H_3BO_3:	1.55 mg l^{-1}
$MnSO_4$*H2O:	0.236 mg l^{-1}	$CuSO_4*5H_2O$:	0.150 mg l^{-1}
$Na_2MoO_4*2H_2O$:	0.072 mg l^{-1}	$CoCl_2*6H_2O$:	0.002 mg l^{-1}
$ZnSO_4*H_2O$:	0.179 mg l^{-1}		

After germinating broccoli seeds on moist filter paper in a petri dish, 4-day-old seedlings were selected for uniformity and transferred to Mitscherlich pots for sand culture in the greenhouse (10 kg of washed sand and 5 plants per pot, respectively).

The nutrient solution was supplied once per week at 100 ml pot^{-1}. For the selenate-treatment, the nutrient solution was supplemented with 4 µmol sodium selenate pot^{-1} (selenate concentration: 40 µM), starting after 2 weeks. At the time points indicated in the Results section, entire shoots (except cotyledons) were harvested and their fresh and dry weights determined. For metabolite analysis and RNA extraction, fresh shoots were directly immersed in liquid nitrogen. For 6-week-old plants, roots were also harvested for determination of total S and Se contents by ICP-AES (Inductively-Coupled Plasma Atomic Emission Spectrometry), and for expression analysis of *SULTR* genes by qPCR.

5.1.4 Selenate-application in the field by leaf-fertilization

For direct selenate-application to leaves, three-month-old adult broccoli plants cultivated under field conditions were chosen for uniformity at a stage of 2.0±0.5 cm head diameter. Sodium selenate solution was sprayed once onto the leaves (40 ml $plant^{-1}$, corresponding to 0, 2, or 20 mg Se $plant^{-1}$). Control plants were sprayed with the corresponding amount of water. During selenate-application, underlying soil and young broccoli heads were covered with aluminium foil to prevent direct contact of broccoli head with selenate solution and to exclude selenate transfer to the soil. After 1 week, when broccoli head diameter had reached 10 cm, heads were harvested, and their contents of total S, S-metabolites and Se were determined in floret (0-1 cm from head surface) and upper stem (1-3 cm from head surface), respectively.

5.2 Microbiological techniques

5.2.1 *Escherichia coli* strains

For cloning procedures *E. coli* strain XL1-Blue (Stratagene) or DH5α (Invitrogen) were used.

Genotypes:

XL1-Blue: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *pro*AB *lacIq ZΔM15* Tn*10* (Tetr)].

DH5α: *supE44, Δ lacU169 (phi 80 lacZ Δ M15), hsdR17, recA1, endA1, gyrA96, thi-1, relA1*.

5.2.2 Media and antibiotics

E. coli bacteria were grown in low salt LB-medium (5 g/L NaCl, 5 g/L Yeast Extract, 10 g/L Tryptone/Peptone) for cloning purposes. Selection was carried out with the following concentrations of antibiotics: Ampicillin 100 µg/ml, Kanamycin 50 µg/ml, Spectinomycin 100 µg/ml, Tetracyclin 12.5 µg/ml.

5.2.3 Preparation of electrocompetent *E. coli* cells and transformation

One liter of low salt LB containing the appropriate antibiotics was inoculated with 20 ml of an over night bacterial culture and incubated until OD600nm reached 0.7 – 1.0. The culture was chilled to 4 °C and cells were collected by centrifugation. The pellet was washed twice with 500 ml of ddH2O, then with 40 ml 10% glycerol and finally resuspended in 4 ml 10% glycerol, frozen in 50 µl aliquots in liquid nitrogen and stored at -80°C.

The electrocompetent cells were transformed by electroporation with a GenePulserII (Bio-Rad) set to 200 W, 1.8 kV, 25 µF and incubated in 1 ml SOC-medium for 1 h at 37 °C before plating variable volumes on selective LB-plates.
SOC-medium: 20 g/l tryptone; 0.5 g/l yeast extract; 0.5 g/l NaCl; 0.186 g/l KCl; 2.03 g/l $MgCl_2$; 3.96 g/l glucose-monohydrate; pH 7.0

5.3 Nucleic acid techniques

5.3.1 Genomic DNA extraction

100 mg of homogenized material were mixed with 500 µl of extraction buffer (200 mM Tris-HCl, pH 9, 400 mM LiCl, 25 mM EDTA, 1 % SDS). After centrifugation at 13.000 rpm for 10 min, supernatant was transferred to a new tube and mixed with the same volume of PCI (Phenol/Chloroform/Isoamylalcohol 25:24:1). In order to precipitate the DNA, the upper phase was transferred to a new eppendorf and mixed carefully with 1/10 volumes of a 3 M Na-Acetat solution (pH 7.2) and 1 volume of Isopropanol. After incubation for 15 minutes at room temperature, the DNA was precipitated by centrifugation at 13,000 rpm for 15 minutes.

The pellet was washed with 70% EtOH followed by centrifugation for 5 minutes at 13,000 rpm. The dried pellet was dissolved in TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8) and stored at -20 °C.

5.3.2 RNA extraction

Total RNA was extracted from 100 mg of frozen and homogenized tissue with the GeneMATRIX Universial RNA purification Kit from EURx according to the manufactures's instructions. RNA quantity was estimated by measuring the optical density of a 1:300 dilution at 260 nm and the appearance of rRNA on 1.4% agarose gel.

5.3.3 Determination of nucleic acid concentrations

Nucleic acid concentrations were determined spectrophotometrically. The concentration of DNA samples (µg/ml) can be calculated as A260nm x 50 x dilution factor, the concentration of RNA samples as A260nm x 40 x dilution factor. The A260/A280 ratio was used to determine the purity of DNA and RNA samples (protein contamination), in addition the A260/A230 ratio was used to determine the purity of RNA samples (polysaccharide or polyphenolic contamination).

5.3.4 Separation of DNA by agarose gel electrophoresis

For separation of purified DNA, 0.75 to 1.5% agarose gels were prepared in 1xTAE-buffer (Sambrook et al., 1989). DNA samples were prepared by adding a suitable volume of 5x loading buffer (50% glycerol, 5x TAE-buffer, 1% Orange G (w/v)). As molecular weight markers, either SmartLadder (Eurogentec) or the 2-log ladder (NEB) were used. After the gel run, DNA was stained using a solution of 0.1 µg/ml ethidium bromide in water for few minutes and was visualized under UV light.

5.3.5 Separation of RNA by agarose gel electrophoresis

20x MOPS buffer:	400 mM MOPS
	100 mM NaOAc
	20 mM EDTA
	pH 7
10x RNA loading buffer:	50% (v/v) glycerol
	1x MOPS
	1% (w/v) bromphenol blue
1.4% RNA agarose gel:	1.4% (w/v) agarose
	1x MOPS
	2% (v/v) formaldehyde (37%)

For RNA separation, samples were brought to 12.7 µl with formamide and upplemented with 2 µl 10x RNA loading buffer, 3.3 µl formaldehyde (37%), 1 µl 20x MOPS and 1 µl EtBr (0.5 mg/ml) and denatured for 10 min at 65°C. Samples were separated on a 1.4% RNA agarose gel in 1x MOPS buffer at constant voltage (70 V).

5.3.6 Reverse transcription

Total RNA was treated with RQ1 RNase-Free DNase (Promega, Mannheim, Germany) to remove genomic DNA contamination at 37 ℃ for 30 min. To terminate the reaction, 2 µl Stop-solution was added and RNA samples were transfer to 65 °C for 10 minutes and then immediately placed on ice to destroy secondary structures. Synthesis of cDNA was performed with AMV-Reverse Transcriptase from Roboklon. Typically 2.9 µl of RNA (irrespective of its concentration) were incubated with 1 µl of AMV buffer, 0.5 µl of oligo-dT Primer (40 µM) 0.5 µl of dNTP solution (40 mM) and 0.125 µl of AMV-RT for 1 hour at 42 °C followed by denaturation of the enzyme at 85

°C for 5 minutes. The cDNA was stored at – 20 °C.

5.3.7 Polymerase chain reaction

For DNA amplification via polymerase chain reaction (PCR) Taq polymerase (Sigma-Aldrich) was used. A typical PCR mix contained the components shown in Table 5.1. PCRs were carried out in a Biometra personal cycler with the program shown in Table 5.2.

Component	Volume (µl)
10x reaction buffer	5
dNTPs (10 mM each)[a]	1
Primer 1 (10 µM)[b]	1
Primer 2 (10 µM)[b]	1
DNA template (10 pg-1µg)	1
Polymerase	1
H2O	to 50 µl

Table 5.1 Components of a typical PCR mixture.

[a] 10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM dTTP

[b] Primers are shown in Table 5.3

Step	Temperature	Duration	Cycle number
Denaturation	95℃	5 min	1x
Denaturation	95℃	30 sec	
Annealing	49-60℃[a]	30 sec	35x
Extension	72℃	1-2 min[b]	
Extension	72℃	5 min	1x
	4℃	∞	

Table 5.2 Typical PCR program.

[a] depending on primer annealing temperatures

[b] depending on fragment length, 1 min per kb

Primer name	Comment	Primer sequence (5'→3')
cloning cDNA of *BoMYB28* from broccoli		
bMYB-1	full-length cDNA of *BoMYB28* (forward)	gaaaatcacagttcacgcctcttactcc
bMYB-2	full-length cDNA of *BoMYB28* (reverse)	tgattctcaatatcagagaaaccctcgttt
AtMyb28-91F	internal primer for 3'RACE (forward)	gaccacggcgagggaggctgg
AtMyb28-640F	internal primer for 3'RACE (forward)	catcaaggctcttgaacaaagttgcggcta
AtMyb28-330R	internal primer for 5'RACE (reverse)	cgtgttccagtagttcttgatctcgttgtc
AtMyb28-640R	internal primer for 5'RACE (reverse)	tagccgcaactttgttcaagagccttgatg
qPCR		
BoMYB28_3UTR-F	*BoMYB28* (forward)	cccaagcagaaaggtttcaa
BoMYB28_3UTR-R	*BoMYB28* (reverse)	ccctaaacttgggactaacaacc
BoSULTR1;1-Q1	*BoSULTR1;1* (forward)	atttccgtggtgatatcgtttgcgaag
BoSULTR1;1-Q2	*BoSULTR1;1* (reverse)	cgcaaccatcttagaatcctttctcgg
BoSULTR1;2-Q1	*BoSULTR1;2* (forward)	catcacggctggaattggcagactat
BoSULTR1;2-Q2	*BoSULTR1;2* (reverse)	tgtttgtcggctcgggttatgtagac
BoSULTR2;1-Q1	*BoSULTR2;1* (forward)	aactggtgttgagctagtgatcgttaacc
BoSULTR2;1-Q2	*BoSULTR2;1* (reverse)	tttaatccaaagcaagcatcaagagct
BoSULTR2;2-Q1	*BoSULTR2;2* (forward)	gtggggatatcgtttgcaagaataatgtt
BoSULTR2;2-Q2	*BoSULTR2;2* (reverse)	ggagaactgattcgaagagtcaataatcct
Phactin_L	Actin (forward)	ggtaacattgtgctcagtggtgg
Phactin_R	Actin (reverse)	ctcggccttggagatccacatc
cloning promoter sequences of genes in aliphatic GSLs biosynthesis		
MAM1_5UTR_B	promoter of MAM1 (*Bam*HI, forward)	ttacttttaaattaggatccaatcccaatcccacagcactg
MAM1_5UTR_X	promoter of MAM1 (*Xho*I, reverse)	tggagtacgctcgagaaaaaagagagagatactt
CYB83A1_5UTR_B	promoter of CYB83A1 (*Bam*HI, forward)	tatgggatccgcttttggttgatctaaacacaaa
CYB83A1_5UTR_X	promoter of CYB83A1 (*Xho*I, reverse)	ccgacctcgagtttttaatcaaatggttactccc
AOP2_5UTR_B	promoter of AOP2 (forward)	agtcaaaaacttgatcgatcgtctcgtattt
AOP2_5UTR_X	promoter of AOP2 (*Xho*I, reverse)	cgtctcgagatgttagcagatagtagcaataa
sequencing constructs		
M13 fw	In pCR2.1 vector (forward)	gtaaaacgacggccagt
M13 rev	In pCR2.1 vector (reverse)	ggaaacagctatgaccatg
35S	In pART7 vector (forward)	caatcccactatccttcgcaa
ocs rev	In pART7 vector (reverse)	ggcggtaaggatctgagcta
LUCF	In pLUC vector (forward)	ctaacatacgctctccatca
LUCR	In pLUC vector (reverse)	ggatagaatggcgccgg

Table 5.3 Oligonucleotides used for PCR, cloning, and sequencing.

5.3.8 Quantitative real time PCR

To confirm the expression of *BoMyb28* and *BoSULTR* genes, two µg DNase-treated RNA was reverse-transcribed using AMV reverse transcriptase (EURx, Gdansk, Poland) and an oligo-dT primer at 42°C for 1 h. qPCR was performed using the JumpStart Taq DNA Polymerase (Sigma-Aldrich, Taufkirchen, Germany) and SYBR-Green (Invitrogen, Karlsruhe, Germany) as fluorescent reporter in the iCycler™ (BIO-RAD, Munich, Germany). Primers were listed in Table 5.3 and actin primers were used for reference gene validation. qPCR included 45 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 20 sec. Each reaction was performed in triplicates, and specificity of amplification products was confirmed by melting curve and gel electrophoresis analysis. Relative expressions of *BoMYB28* and *BoSULTR* genes transcripts were calculated and normalized with respect to actin transcripts. All qPCR transcript quantifications were done with three independent biological replicates.

5.3.9 Gel extraction and PCR purification

For the purification of DNA fragments from agarose gels or the clean-up of PCR products the NucleoSpin Extract II Kit (Macherey-Nagel) was used according to the manufacturers instructions.

5.3.10 Plasmid minipreparation

Plasmid DNA was extracted from 3 ml bacterial cultures with the Nucleospin Plasmid (Macherey-Nagel) miniprep kit according to the manufacturer's instructions.

5.3.11 Plasmid maxipreparation

LB medium:	10g/l peptone
	5g/l yeast extract
	5g/l NaCl
	pH 7.2
1x TE buffer:	10 mM Tris HCl
	0.1 mM EDTA
	pH 8.0
TE 50/1 buffer:	50 mM Tris HCl
	1 mM EDTA

	pH 8.0
RNase A mixture:	100 µl RNase A solution (20 mg/ml)
	150 µl 10% Triton X-100
	750 µl TE 50/1 buffer
Lysozyme:	10 mg lysozyme
	1 ml TE 50/1 buffer
0.5M EDTA:	pH 8.0

For maxipreparation, a pre-culture was made with 2,5 ml LB medium and cultured at 37℃ for 3 hours. The pre-culture was transferred to pre-warmed 500 ml LB and incubated over night at 37℃. Cells were precipitated by centrifugation at 5,500 rpm for 15 min. The pellets were resuspended with 4 ml pre-cold TE 50/1 buffer and transferred to 10 ml tube on ice. 2.5 ml fresh-prepared lysozyme solution was added quickly and mixed by inverting several times. After incubation on ice for 5 min, 2 ml 0.5 M EDTA was added to stop the reaction and gently mixed by inverting several times. After incubation on ice for 5 min, 1 ml RNase A solution was added to remove RNA contamination and then put on ice for 60 min. After centrifugation at 13.000 rpm for 30 min, supernatant was transferred to a new 50 ml falcon tube and added equal-volume phenol (pH 8) for purification. After shaking vigorously for 1 min, the solution was separated by centrifugation at 13.000 rpm for 20 min. The water phase (upper layer) was transferred to a new tube and added equal-volume chloroform. The further steps were repeated as after adding phenol. For precipitating DNA, 1/10 (v/v) 5 M $NaClO_4$ and 0.6 to 1-time volume of isopropanol were added and the solution was centrifuged at 13,000 rpm for 15 min. DNA pellet was resuspended in 500 µl TE buffer after air dry. The concentration and quality of plasmid DNA were confirmed by spectrophotometer.

5.4 Cloning techniques

5.4.1 T/A cloning of PCR products

Cloning of PCR fragments via PCR derived poly-A overhangs was carried out with the Invitrogen Original TA cloning kit (pCR2.1 vector) according to the respective manufacturer's instructions. PCR products were purified in advance with the Nucleospin Extract II kit (Macherey-Nagel) according to the manufacturer's instructions.

5.4.2 Cloning via restriction enzyme digestion

Restriction enzymes were purchased from New England Biolabs and used according to the manufacturer's instructions. Usually 4 U per µg plasmid DNA were used, for control digestions for 1 h in a 10 µl volume, for cloning purposes in accordingly upscaled reactions.

5.4.3 Cloning of *BoMYB28*

Total RNA was isolated from shoots of 4-week-old broccoli plants. Primers for amplification of a partial cDNA of *BoMYB28* were designed according to cDNA sequence of *AtMYB28* (NM_180910). For obtaining the full length *BoMYB28* cDNA, 5' and 3' cDNA ends were amplified by GeneRacer kit (Invitrogen, Karlsruhe, Germany). The full-length cDNA of *BoMYB28* was then amplified using the gene-specific primer (Table 5.3). The amplified fragments were ligated into the pCR2.1 TA cloning vector (Invitrogen, Karlsruhe, Germany) and sequenced. Sequence-analysis was performed using the Vector NTI software (Invitrogen, Karlsruhe, Germany) and ClustalW2 program (EMBL-EBI: http://www.ebi.ac.uk/Tools/clustalw2/index.html).

5.4.4 Cloning of constructs for luciferase-assay

For transient expression of *AtMYB114* (AT1G66380), *BoMYB28*, EGL3 (AT1G63650) and TTG1 (AT5G24520), theirs full-length cDNA were amplified and cloned into the vector pART7 (Gleave, 1992), allowing constitutive gene expression by the CaMV 35S promoter. Promoter fragments of *AtUFGT* (AT4G14090), *AtMAM1* (AT5G23010), *AtCYP83A1* (AT4G13770), and *AtAOP2* (At4g03060) were amplified

from *Arabidopsis* genomic DNA and cloned into the luciferase reporter vector pLuc (Horstmann *et al*., 2004). The constructs for *AtMYB114* and *AtUFGT* were gifts from Dr. Jochen Bogs. Full-length cDNA of *BoMYB28* was first cloned into the pCR2.1 TA cloning vector. The construct of pCR2.1-*BoMYB28* was digested with *Eco*RI and then cloned into pART7 vector. The promoter fragments of *AtMAM1*, *AtCYP83A*, and *AtAOP2* were amplified by specific primers containing restriction sites (Table 5.3) for cloning into pLuc vector as a *Bam*HI/*Xho*I fragment.

5.5 Elemental analysis of total sulfur and total selenium

Tissues samples were dried at 65°C in an oven for 3 days. Dried samples were weighed, and 20 mg of dried tissue was incubated in 2 ml HNO_3 at room temperature for 3 days. Samples were then transferred to 95°C for 3 hours, followed by addition of 1 ml H_2O_2 (30 %) and further incubation at 95°C for 1 hour. Thereafter, samples were diluted with double-distilled water to 10 ml. Total element contents were determined by inductively coupled plasma atomic emission spectrometry (ICP-AES, Thermo Elemental, Dreieich, Germany) using an IRIS Advantage Duo ER/S. S and Se were determined using the 182-nm line and 196-nm line respectively.

5.6 Quantification of sulfate and S-metabolites

5.6.1 High-Performance Liquid Chromatography (HPLC)

For quantitative determination of sulfate, Cys and GSH, frozen plant material was ground in liquid nitrogen and extracted with 1 ml 0.1 M HCl per 0.1 g fresh weight. The extract was 10-fold diluted in water and used for sulfate determination by ion chromatography according to Wirtz and Hell (2007), using a Dionex HPLC system. Cys and GSH were quantified after derivatization with monobromobimane (Calbiochem, Bad Soden, Germany). The derivatization procedure and separation of thiol derivatives were performed as previously described by Wirtz *et al.* (2004). Depending on cysteine formation in the assay, 10 µl or 50 µl of assay supernatant was reduced at room temperature for 60 min in a total volume of 0.27 ml containing 134 mM TRIS-HCl pH 8.3, 1 mM DTT. Afterwards thiols were derivatized for 15 min by adding 0.03 ml monobromobimane to a final concentration of 3 mM, representing more than 2.5-fold excess above the total thiol concentration. The resulting monobromobimane derivatives were stabilized by the addition of 0.7 ml 5% acetic acid and detected by fluorescence (Fluorometer RF 551, Shimadzu) at 480 nm after excitation of the adduct at 380 nm after separation.

Extraction and quantitative analysis of glucoraphanin were performed according to Rangkadilok *et al.* (2004) and Rochfort *et al.* (2006), with some modifications. Briefly, young plant shoot samples (8-10 g for 6-week-old plants and 2-4 g for all other samples) were boiled in water for 5 min to inactivate myrosinase. Samples were then transferred to a mortar with 5 ml double distilled water (10 ml for 6-week-old plants) and thoroughly homogenized. Extracts were placed on a shaker for 10 min and then centrifuged at 5,000 rpm for 10 min at room temperature. Supernatants were vacuum-filtered through filter paper (Whatman No. 1, Dassel, Germany) and then centrifuged again at 5,000 rpm for 10 min. Finally, the cleared extracts were filtered through 0.22 µm Millipore filter (Millipore, Jaffrey, NH, USA) and stored at -20°C for analysis. For glucoraphanin quantification, a 4 µm 4.6 x 250 mm C18 column (Nova-Pak, Waters, Milford, MA, USA) was used, the mobile phase consisting of 0.1% v/v formic acid/water and used with a flow rate of 1ml per min. After each analysis, the column was washed with 20% v/v acetonitrile/water, containing 0.1%

v/v formic acid. The elution profile was monitored at 234 nm. The peak corresponding to glucoraphanin was identified by co-chromatography with authentic standard (a gift from Dr. Jonathan Gershenzon). Peak purity was confirmed by comparative scanning of the UV spectrum using a photodiode array detector (Waters, Milford, MA, USA).

5.6.3 Glucose assay

Extraction and quantitative analysis of total GSLs were modified from Smith and Dacombe (1987). 100 mg frozen sample was ground in liquid nitrogen and added 280 µl acidified methanol (40% methanol and 0.5% acetic acid) as the sample-blank or added 280 µl ddH_2O at 37℃, 10 min for myrosinase hydrolysis. To stop the reaction, 210 µl 100% methanol was added and a bit of activated carbon also was added to precipitate polyphenolic substances. Samples were centrifuged twice at 13,000 rpm, 4℃ for 10 min and supernatant was transferred to a new tube. 100 µl extract was mixed with 400 µl glucose-assay reagent (Sigma-Aldrich, Taufkirchen, Germany) and incubated at 37℃ for 30 min. 400 µl 12N H_2SO_4 was added to stop the reaction and the colour of solution turned to violet. Colorimetric assay proceeded with HITACHI U-2000 spectrophotometer at 540 nm. According to glucose standard, the amount of released glucose was calculated by subtracting the value of sample-blank from the value of sample. The amount of released glucose indicates the amount of total GSLs hydrolyzed by myrosinase.

5.7 Transient expression of *BoMYB28* and its functional assay

5.7.1 Protoplast isolation

Enzyme solution: 1% cellulose 'Onozuka' R10 (Yakult, Tokyo, Japan)
0.25% macerozyme 'Onozuka' R10 (Yakult, Tokyo, Japan)
0.4 M mannitol
10 mM $CaCl_2$
20 mM KCl
0.1% BSA
20 mM MES
pH 5.7
autoclave stock solution without enzymes and BSA

W5 solution: 154 mM NaCl
125 mM $CaCl_2$
5 mM KCl
5 mM glucose
2 mM MES
pH 5.7
sterile filtration

MMg solution: 0.4 M mannitol
15 mM $MgCl_2$
4 mM MES
pH 5.7
autoclave

For *Arabidopsis* protoplast isolation and transfection, leaves were collected from 3 to 5-week-old plants. The generation of protoplasts was performed as previously described by Wu *et al.* (2009). Washing and transfection of protplasts was performed as described by Yoo *et al.* (2007). The upper epidermal surface was stabilized by affixing a strip of Time tape (Time Med, Burr Ridge, IL) while the lower epidermal surface was affixed to a strip of Magic tape (3 M, St. Paul, MN). The Magic tape was then carefully pulled away from the Time tape, peeling away the lower epidermal surface cell layer. The peeled leaves still adhering to the Time tape, were transferred

to a Petri dish containing 10 ml of enzyme solution. The leaves were gently shaken (40 rpm on a platform shaker) in light for 20 to 60 min until the protoplasts were released into the solution. The protoplasts were centrifuged at 100 x G for 2 min and then washed twice with 50 ml of pre-chilled W5 solution and incubated on ice for 30 min. During the incubation period, protoplasts were counted using a hemocytometer under a light microscope. The protoplasts were then centrifuged and resuspended in MMg solution to a final concentration of 2 to 5 x 10^5 cells / ml.

5.7.2 PEG-transfection

PEG solution: 30% (w/v) PEG (MW 4000, Roth)
0.1 M $CaCl_2$
0.2 M mannitol

WI solution: 4 mM MES (pH 5.7)
0.5 M mannitol
20 mM KCl

Approximately 2 x 10^4 protoplasts were mixed with 10 µg transcription factor construct, 10 µg promoter construct and 1µg the *Renilla* luciferase plasmid pRluc (Horstmann *et al*., 2004). An equal volume of a fresh-prepared PEG solution was added and the mixture was incubated at room temperature for 5 min. After incubation, 400-440 µl of W5 solution was added slowly and mixed gently by inverting the tube. Protoplasts were precipitated by centrifugation at 100 x G for 2 min. The protoplasts were resuspended gently in 1 ml WI solution and were incubated in 24-well plates coated with 1% BSA at room temperature for 16 hr in light.

5.7.3 Luciferase assay

For the measurement, protoplasts were harvested by centrifugation at 100 x G for 2 min and resuspended with 40 µl 2-fold passive lysis buffer (Promega). 20 µl of the supernatant was used to measure *Firefly* and *Renilla* luciferase activity with the dual-luciferase reporter assay kit (PJK, Kleinblittersdorf, Germany), by sequential addition of 50 µl Beetle Juice and Renilla Juice pH5. Light emission was measured with a Lumat LB9507 Luminometer (Berthold Technologies) and the relative luciferase activity was calculated as the ratio between the *Firefly* and the *Renilla*

luciferase activity. All transfection experiments were performed in triplicates and each set of promoter experiments was repeated with similar relative ratios to the respective control.

5.8 Statistical analysis

All experiments were done at least three times, each with three independent biological replicates. The variation was calculated as standard deviation (SD). Comparison of means from different data sets was analyzed for statistical significance with the student's t-test by Excel (Microsoft). Significance was considered if P value < 0.001.

6. ABBREVIATION INDEX

AMV	alfalfa mosaic virus
A. thaliana	*Arabidopsis thaliana*
B. oleracea	*Brassica oleracea*
BLAST	basic local alignment search tool
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
CaMV	cauliflower mosaic virus
$Ca(NO_3)_2$	calcium nitrate
cDNA	complementary DNA
cm	centimeter
$CoCl_2$	cobalt chloride
Col-0	Columbia zero ecotype of *Arabidopsis thaliana*
$CuSO_4$	copper sulfate
Cys	cysteine
ddH_2O	double distilled water
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
DW	dry weight
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
e.g.	for example (lat. exempli gratia)
EtBr	ethidium bromide
EtOH	ethanol
F	farad
Fig.	figure
FW	fresh weight
g	gram
G	acceleration of gravity
GFP	green fluorescent protein
GR	glucoraphanin

GSL	glucosinolate
GSH	glutathione
GST	glutathione S-transferase
h	hour
H_2O	water
H_2O_2	hydrogen peroxide
H_2SO_4	sulfuric acid
H_3BO_3	boric acid
HCl	hydrogen chloride
HNO_3	nitric acid
HPLC	high-performance liquid chromatography
ICP-AES	Inductively-Coupled Plasma Atomic Emission Spectrometry
i.e.	that is (lat. id est)
IPTG	isopropyl ß-D-1-thiogalactopyranoside
kb	kilo base pairs
KCl	potassium chloride
KH_2PO_4	potassium dihydrogen phosphate
KNO_3	potassium nitrate
l	liter
LB	Luria-Bertani (medium)
LiCl	lithium chloride
µ	micro (10^{-6})
M	molar (1 M = 1 mol/l)
MES	2-(*N*-morpholino)ethanesulfonic acid
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulfate
min	minutes
$MnSO_4$	manganese sulfate
mol	mole
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger RNA
MS	Murashigge-Skoog (medium)
MW	molecular weight
n	number of replicates

N	Normality
NAA	1-naphthaleneacetic acid
NAT	N-acetyltransferase
NaCl	sodium chloride
NaH_2PO_4	sodium phosphate
NaOAc	sodium acetate
NaOCl	sodium hypochlorite
$NaOCl_4$	sodium perchlorate
NaOH	sodium hydroxide
Na2MoO4	sodium molybdate
Na_2SeO_3	sodium selenite
Na_2SeO_4	sodium selenate
$(NH_4)_2SO_4$	ammonium sulfate
nm	nanometer
nt	nucleotide
OAS	*O*-acetylserine
ODx nm	optical density at x nm wavelength
PCI	phenol/chloroform/isoamylalcohol
PCR	polymerase chain reaction
PEG	polyethylene glycol
pH	negative decadic logarithm of [H+]
qRT-PCR	quantititative real-time polymerase chain reaction
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
RT	reverse transcription
S	sulfur
SD	standard deviation
SDS	sodium dodecyl sulfate
Se	selenium
SeCys	selenocysteine
SeMet	selenomethionine
SeMSC	Se-methylselenocysteine

SF	sulforaphane
SMT	selenocysteine methyltransferase
SOC	super optimal broth with catabolite repression (SOC) medium
SULTR	sulfate transporter
U	unit
UTR	untranslated region
UV	ultraviolet
V	volt
v/v	volume/volume
var.	variety
W	watt
w/v	weight/volume
WHO	World Health Organization
WT	wildtype
$ZnSO_4$	zinc sulfate

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8. APPENDIX

8.1 Full-length cDNA sequence of *BoMYB28*

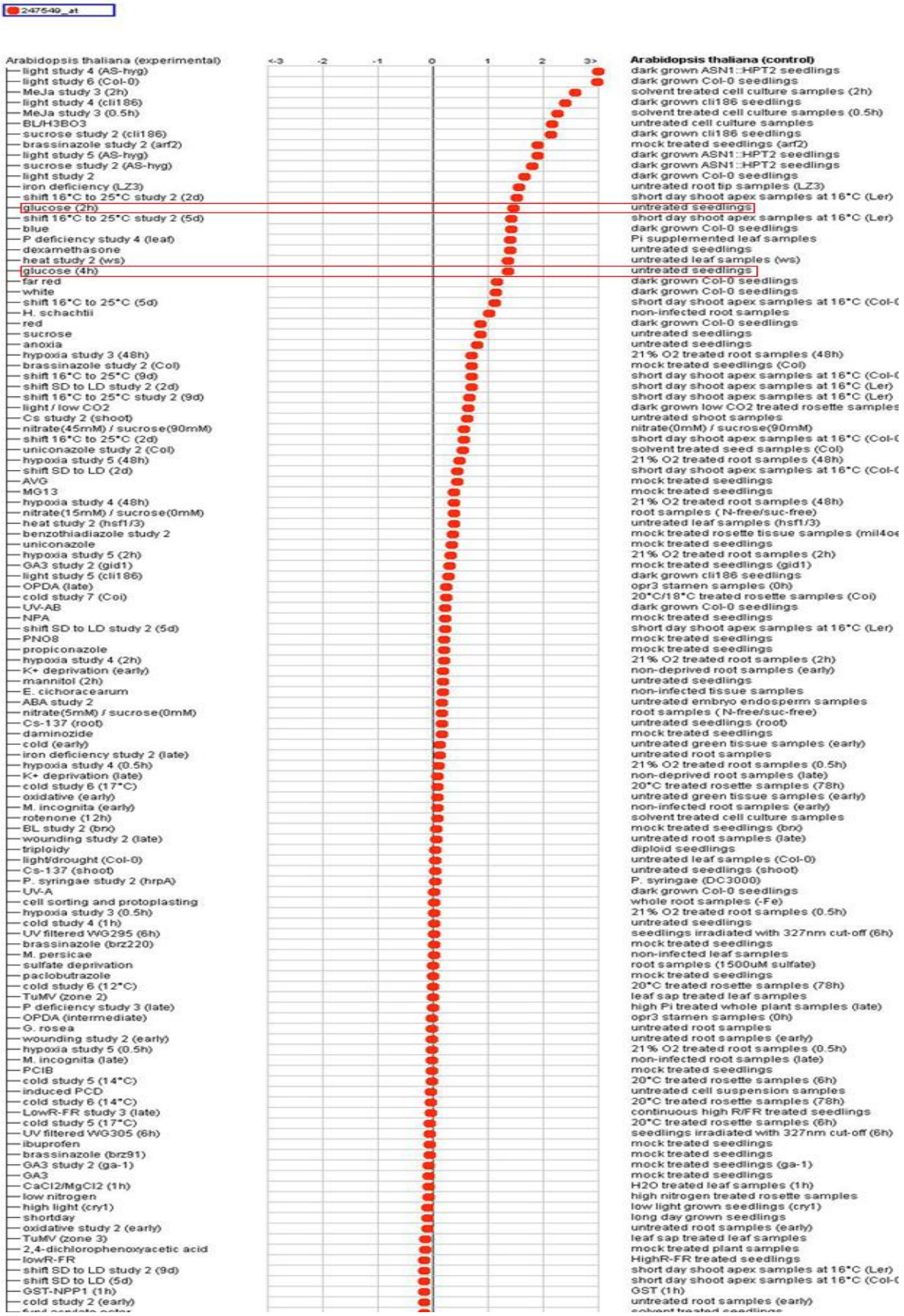
GAATTCGGCTT*gaaaatcacagttcacgcctcttactccatgagcttctctattctcatcct*
agtgttataatcttgcaaacacatatagaaagcaagatttggagtgtacgagaaaaacatga
aaacacctagaagctctgtgggtaagacccaagagcgtttctcgattagtttcatatacaga
tgcatcagagttctcatcaaccgatctacttctttcttatcttattagaaaaaaaatcctat
caaaatttactttcctgcaagtatatttttctttacattttcattttcttgagtgttatttg
*agtgaagttatattaaaatattgtaatagagttcatatatatcgaaa*ATGTCAAGAAAGCCA
TGTTGTGTCGGAGAAGGGCTGAAGAAAGGGGCATGGACCACCGAGGAAGATAAGAAACTCAT
CTCTTACATCCATGAACATGGAGAAGGAGGCTGGCGTGACATTCCTCAAAAAGCTGGATTGA
AAAGGTGTGGAAAGAGTTGTAGACTGCGATGGACTAACTACCTAAAACCTGAAATCAAAAGA
GGCGAGTTTAGTTCAGAGGAGGAACAGATTATCATCATGCTTCATGCTTCTCGTGGAAACAA
GTGGTCGGTCATAGCGAGACATTTACCTAGAAGAACAGACAATGAGATCAAGAACTACTGGA
ACACACATCTCAAGAAACGTTTGATCGAACAGGGTACTGATCCCGTGACTCACAAGCCACTA
GCTTCTAATACAAACCCTACTGTACCTGAGAATTTGCATTCCCTAGATGCATCTAGTTCCGA
CAAGCAATACTCCCGGTCAAGCTCAATGCCTTCCATGTCTTGTACTCCTTCCTCCGGTTTCA
ACACGGTTTTCGAGAATACCAGCAAAGATGGGACACCAGTTCGTGAGGACGATTCCTTGAGT
CGCAAGAAACGTTTGAAGAAATCAAGTTCTACATCAAGGCTTTTGAACAAAGTTGCGGCTAA
GGCCACTTCCATGAAAGAAGCTTTGTCTGCTTCCATGGAAGGTAGCTTGAATGCTAATACAA
GCTTTTCCAATGGCTACTCTGAGCAGATTCTCAATGAAGATGATAGTCCTAATGCATCCCTC
ATAAACACTCTCGCCGAGTTCGATCCCTTCCTCCAAACAACGTTTTACCCTGAGAATGAAAT
GAATACTACTTCTGATCTCGATATAGATCAGGACTACTTCTCACATTTTCTCGAAAATTTCG
GCAGAGATGATGACCACAATGAGGAGCACTACATGAATCATAACTATGGTCATGATCTTCTT
ATGTCCGATGTGTCCCAAGAAGTCTCATCAACTAGCGTTGATGATCAAGACAATACTAATGA
GGGTTGGTCAAATTATCTTCTTGACCATGCTGATTTTATACATGACATGGATTCTGATTCCC
TCGGAAAGCATATCATATGA*atcttcatgcccaagcagaaaggtttcaaacttttgaaactt*
gtcagaacaagaagttatgtatgtattctattatatggattgtttagtacatgtccaagatc
atggttgttagtcccaagtttagggtttgtataatatacaataagggacgttatcttataaa
*acgagggtttctctgatattgagaatca*AAGCCGAATTC

Underlined: *Eco*RI site in pCR2.1 vector

Italic and small: 5' and 3' UTR

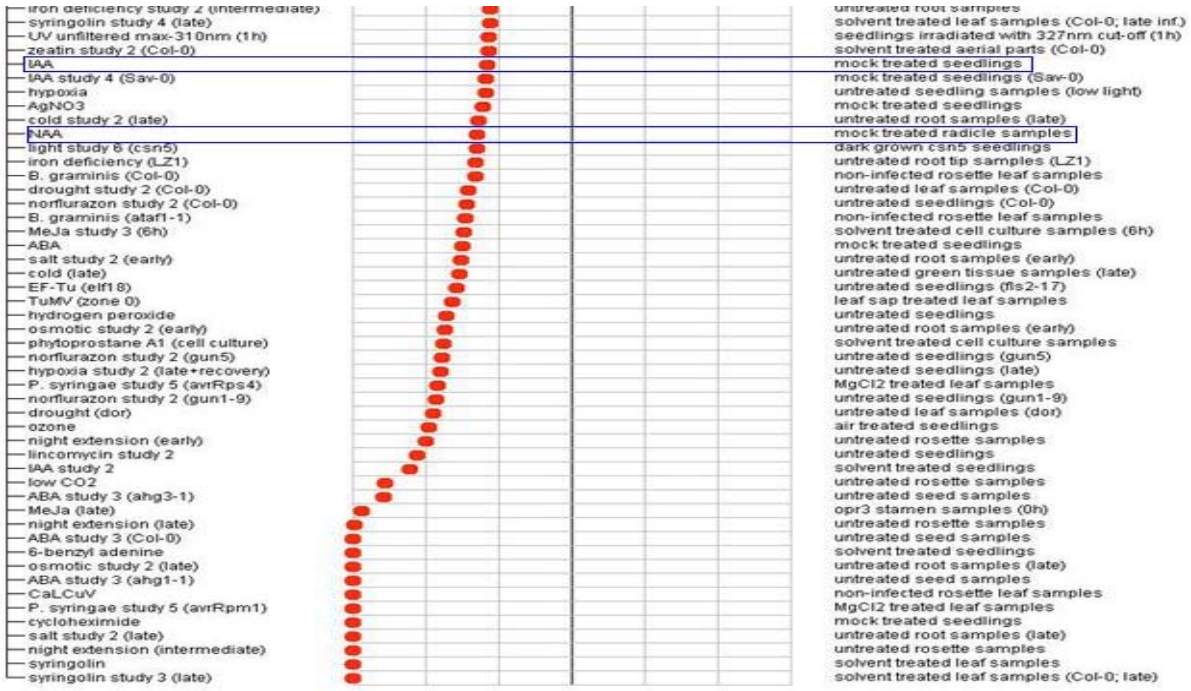
Boxed: start and stop codon

8.2 *AtMYB28*-stimulus microarray data



8.2 *AtMYB28*-stimulus microarray data (continued)

8.2 *AtMYB28*-stimulus microarray data (continued)



analysis from GENEVESTIGATOR microarray database

https://www.genevestigator.com/gv/index.jsp

8.3 sequence of *AtMAM1* promoter region

GGATCCAATCCCACAGCACTGTATGGCTGAAGTTATCCACAACTCACCTACCTATACAAGCA
TACACGTGATGATATCATGCTAATCACTTCTGAAGGAACAATGCCGAAAATTAAACCTTATT
ATGCATTCATTAACATCCGAATCCATTTTTCTACTTTGACAAAATGAACAATATCCACATGC
CAGCATCTAGCTAGATGCAGACCTGGTAAGGCACCAATGGGTTTGAAACCTTCCTCCATATT
GTTCCAATTTGTATATATTGCATTTCAAAAAACCAGAAATATTTACTGTACCATTCACGATC
GACCCAAAGGATTTGAACCCAATCTCATAGCCTAATGAATCGTATTCAAAATTGATATGCAC
CATGAGAAACTGGTATCATAAACAAAAACTGATATGCAAATTAATTAATTAAATCACACATA
TGCTATATTTTCTCTATGGGCTGGACTGGTGTTTTGTTTTAATTATTATGATAAGAAGAATT
GCACCAAAAAAAAAAAAGAAAAAAAAAAAGAAGAAAATCGTATCTTTTTGTTCTACCTATAC
AGACACATAATGCATCATTTTAGTTTTTGGGACAAAGACATAATGCATCAACGTGTCAGCAA
TATATGAAACGTGAAGAATGTTTTGTCTGAGTCTGAGTAAAATTACTAGAAATTAAAATTAG
TAAGACTGACTAATTACAAATATCCCAAGTCTGTGTTTATTCTAAGACAACTACTAGAAAAC
TTAACTATATTAGACTACCAACTAGGCAACAAATATCACAAAGAATATCGTATGTCACCTAC
CTGGAGGTGCATACCACGTGATTTTATCCCCATTTTAGATATGGTCATATCGATTAGTTATT
GTATATAAAAAAAAAATTCTTACAGGCTATAAACTATTATGCTACAAATTTTGGTAAAAACC
TATTACTTGTTATTCCGTTTCCAAAACATATTATGGCTATATTAAAGTGTGTATAAATGAGT
TAAAACATTTTTAACAACAAATAAATGTAAAAAAAATGAGTTTAACATCGTTGTAAGTAAAC
TTAGGATTTGTTTGTTACCTCAAACTTAAATATTATTCCCTCTGTTTCTAACTAAGTGTAGT
TTAAAGGTTTTTTATTTTTTTCAGTATAAGTATTGTTTTCACTTTTCGATGCAAACATTAAA
TGTATTTAATAGTTTTTAACCAATTATATTTTACATCATATTTTTTATTGGTTGGATTAGTT
GTAATTGGTGATATTTTTTTTAAAAAAAGATAAATCAAATGAGATTTATATATTTTCTTAAT
TTGCGTGCAAAAACTTTAAATTAAAAATATTAAGAAACAGAGAGAGTATCTTTTCTATACAT
AGGTATATCACTTATATATATATATATGTATACAGCTAAATATTTATGTAAAAATGTAAACA
TACGAAACTGTTTATAGAAAGTATAATATTCTAAAATAAGATATCAAACACAGTATAATATT
TAATTTTAAAGAAGATACTATTTTGCGTTTAATGTTTTCATCGAATATAATTTCTTATTCCG
CTAACTCAAATGTTTATTATTTTTAACATCAAAATGTTTCTAATACTAAAAAGTTTAATAAA
TAAAAAAATCTCTCTATAAATAGATAAATTATATCGTATAATGTTCAAAACAATTCCCACAC
TATCTTTCCTCCACATTAAAGTAAAGTATCTCTCTCTTTTCTCGAG

AtMAM1 (AT5G23010; -1730 to -23 nt)

Underlined: *Bam*HI and *Xho*I sites in pLuc vector

8.4 sequence of *AtCYP83A1* promoter region

GGATCCGCTTTTGGTTGATCTAAACACAAAAGAGAATCTAGTTGCTTGCTACAAAGATGTGC
CCTTCACTACATCTGCAGGTGTATGCAAAGTTTCATCCATCAGCAATGGCACGATCCGGTAA
AAGCTTACTTGAGCCATAAGCAAACTAATATTCTCCATTTCTATCTTTCTCTGTAAGAGAAA
CGAAATTCGGAGATTTTCATTTGAAAATTTGATTCTAATTACAAACATGTTCTTGTAAACTT
AAAGCTTGGTACAATTGTTGTTATTGATTGATGTTGTGAGAAACATTCAGCTTTTTAAAATT
GGGCCCAGACGCAAAGCAACCACACAACTTACGATTTCAAATCTTTTATGGTACTAGTTGAG
GTTTTGTGAAGGTTGATGTTTTTCTTAATTTTTTAATATTCTAGTTCTCGTGTTTAAAAATA
GTCGCATCATTCTTTTTTTCTGGCGGTTCGTAATTGTGTTCCCTTGTAATACTGTATAGGGT
TGTATACAATCGAACGTTTGAAGTGATCATGTAATTCAATCATAAATTTAAATCTTTGGTTA
TTGAAAACATTTTAAGCATTCTATTTTTATTGACCTTTAAGAGATTTTGGCATTTTATTGGC
TTGAGTTCACTGAGCGTTTCCAAGTTACCAACTACACCAACCCTTCGGATTCAAATCTGTGT
TGATGTTTTGCGATTCCATATCATTACTATATCAACGAGTTGATTTTTTTTTTTGGTCAACC
AGCAAGTTGATTTTTGAAAAACTCTTACTCTACAATTTGCGACTGACGACTGTTTAGAATTT
TGGGTACGTACCAATAATCCAATATGTTATATTTTCGATATAGAAATGGATTGTGATTGATA
TAAGTCTTGGGTTCGTCGAACAAAGTTAAACAAAGATTTGTGTAAATAACAAATAGATAAAA
ACAAGAATGAGTGTGACTGCATGAACCACTAAAACATATACGACGTTAGAGGGATGTTGGGT
AGGTGAAGGTGTTTATTAATTCTAACACGTGAGGTTTCGTAAGTAGGTATAAGGGAGTAACC
ATTTGATTAAAACTCGAG

AtCYP83A1 (AT4G13770; -1201 to -142 nt)

Underlined: *Bam*HI and *Xho*I sites in pLuc vector

8.5 sequence of *AtAOP2* promoter region

GGATCCGGATCCAACCGAGATAAGTAAAAAAAGATTCTATCTTTAAAATGGGACCTCTCTCC
TTTTTCTCTTCATTATTGCTATGGTTCAAAACGTACAATCATCGGCCTCGCTCGGAATTTAA
ATCATATACAATATGCTTACTAAGTATTATTCACGTATAAGTAACAATATGCAACTAAAGGT
TTTGGGCCACGAATTACTTACAATAAAAAACTTTTGTAAACTCAAGTTCCAAATGTTTAGTA
ATATAAATTCACATTTTATCTCAAAAAAAAAGAGAAAAAGAGAAAACTTTTTATCAAAAGAG
AAAAGTTAACTTAAGTTATAACTCGTTGAGTTGTTCTTCATCTTGTACAACAATAATGCCCA
CACATCGTATGGATTGATATTTTCTATGATGAAAATAAACGATTCACCAACTTTCCTTTTTT
TGTATATAAAATGTGAACTTTTAATAGTATATACCAACTTTCCTTCCTCTGTCTATTATTAT
TAAGCATGTGTTTGCTTAAATTAAGCAAAGCGACAAAAAAAAAAACTTAATACAATCACTTGT
GAATAATTTCTCTATAAAATGGGGACCCTTCACTATTCTTACTCACACAGAAGAGAAAAATC
TCTAGAGCTAGCAAAGTAAAAACAATTAATATAACAGAAAGTCCAAAGGTAATTTTCTTATG
CGTTTCGAATGTTTTTTTTTCTTATTAATTATTGCTACTATCTGCTAACATCTCGAG

AtAOP2 (AT4G03060; -761 to -41 nt)

Underlined: *Bam*HI and *Xho*I sites in pLuc vector