SILVIO JÚNIO RAMOS

BIOFORTIFICAÇÃO, VARIAÇÃO GENOTÍPICA E METABOLISMO ENVOLVENDO Se EM PLANTAS DE ALFACE E BRÓCOLIS

LAVRAS – MG
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Orientador
Dr. Valdemar Faquin

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APROVADA em 24 de Fevereiro de 2011.

Dr. Luiz Roberto Guimarães Guilherme	UFLA
Dr. Evaristo Mauro de Castro	UFLA
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RESUMO

O selênio (Se) tem sido estudado extensivamente por causa da sua essencialidade em animais e seres humanos. Entretanto, a diferença entre os teores benéficos e tóxicos do Se é bastante estreita, fazendo com que tanto a deficiência quanto a toxidez se tornem comuns em várias regiões do planeta. Nesse contexto, as plantas demonstram ser uma solução promissora para ambos os problemas. Assim, no presente trabalho foram conduzidos três experimentos, em que avaliou-se: a) a biofortificação com Se e a atividade de enzimas antioxidantes em plantas de alface tratadas com selenato e selenito; b) a variação genotípica em germoplasmas de alface, o efeito das formas de Se sobre o teor total de Se, S e aminoácidos, além da expressão de genes envolvidos no transporte e assimilação de Se; c) a variação genotípica em germoplasmas de brócolis ao tratamento com selenato, o efeito no crescimento vegetal, no teor total de Se, macro e micronutrientes, na síntese de glucosinolatos (GLS), nas formas orgânicas de Se, na atividade de enzimas antioxidantes e na expressão de genes envolvidos no transporte e assimilação de Se. Pelo resultado do primeiro experimento conclui-se que a aplicação do Se na forma de selenato, em baixas concentrações, foi mais apropriada para a biofortificação de alface, pois favoreceu o maior crescimento vegetal, a translocação de Se e o teor de Se na parte aérea. No segundo experimento, o selenato foi superior ao selenito no teor de Se na parte aérea e, essa forma de Se apresentou relação sinérgica entre o Se e o S em quase todos os acessos utilizados. Além disso, o maior estímulo no crescimento vegetal quando aplicou-se o selenato e, a inibição do crescimento quando aplicou-se selenito, foram correlacionadas com a atividade de enzimas antioxidantes na maioria dos acessos avaliados. Também, destaca-se que a diferente capacidade dos acessos em acumular Se pode estar associada a alterações nos genes envolvidos na absorção e assimilação do Se. No terceiro experimento, a aplicação de selenato proporcionou diferentes respostas no teor de micronutrientes, na síntese de GLS, na atividade antioxidante e, o teor de Se variou em mais de duas vezes entre as plantas utilizadas, mostrando assim uma grande variação genotípica entre os acessos de brócolis. Além disso, a regulação transcricional da adenosine 5`phosphosulfate sulfyrulase (*APS*) e a expressão gênica da selenocysteine Se-methyltransferase *(SMT*) contribuiu no aumento do teor de Se em plantas de brócolis.

Palavras-chave: *Lactuca sativa*. *Brassica oleracea* var. *italica*. Selênio. Bioforticifação. Expressão de genes.

ABSTRACT

Selenium (Se) has been studied extensively because of its essentiality for animals and humans. However, the difference between beneficial and toxic levels of Se is quite narrow, making both Se deficiency and Se toxicity common problems in different regions of the world, and plants appear to be a promising solution for both sides of the Se problem. Thus, the aim of this study was to evaluate: a) selenium biofortification and antioxidant activity in lettuce plants fed with selenate and selenite; b) the genotypic variation in lettuce germplasm, and the effect of forms of Se on total levels of Se, S and amino acids, and the expression of genes involved in transport and assimilation of Se; c) the genotypic variation in broccoli germplasm in response to treatment with selenate and selenite, and the effect of Se on plant growth, total levels of Se, macronutrients and micronutrients, synthesis of glucosinolates (GLS), on organic forms of Se, activity of antioxidant enzymes and expression of genes involved in transport and assimilation of Se. The results of first experiment shows that Se application as selenate at low concentration was more appropriate for lettuce biofortification, because it favors shoot biomass growth, Se translocation, and Se levels in shoot biomass. In the second experiment, the selenate was superior to selenite in increase total Se levels in shoot, and synergistic relationship between Se and sulfur accumulation was observed in nearly all accessions at the selenate dosage applied. Moreover, the growth-stimulated effect by selenate and the growth-inhibited effect by selenite were found to be correlated with the alteration of antioxidant enzyme activities. Besides this, the different ability of lettuce germplasm to accumulate Se following selenate treatment appeared to be associated to an altered expression of genes involved in Se uptake and assimilation. In third experiment, the application of selenate provided various responses in organic Se species production, micronutrient accumulation, GLS synthesis, and total antioxidant contents, and we found that total selenium content varied with over 2-fold difference among broccoli germplasm. Moreover, transcriptional regulation of adenosine 5′-phosphosulfate sulfurylase and selenocysteine *Se*-methyltransferase gene expression might contribute to the different levels of selenium accumulation in broccoli.

Keywords: *Lactuca sativa*. *Brassica oleracea* var. *italic*. Selenium. Biofortification. Gene expression.

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO

Aparentemente a nutrição mineral de plantas e a saúde humana são áreas muito distintas, mas, quando analisadas na essência, percebe-se que estão interligadas. A maioria dos nutrientes comprovadamente essenciais ao homem e aos animais também desempenha funções importantes no desenvolvimento vegetal e, em muitos casos, seus mecanismos de ação são similares. Nesse sentido, cita-se, por exemplo, a função antioxidante do selênio (Se) na eliminação de radicais superóxido, que ocorre tanto na planta quanto no organismo humano.

O Se é um elemento essencial para o metabolismo humano e animal e, a prevenção de certas doenças como câncer, controle no metabolismo de hormônios, algumas anomalias morfológicas, doenças cardiovasculares, entre outras, muitas vezes está relacionada com a concentração de Se presente nos alimentos consumidos e, consequentemente, no plasma sanguíneo.

Os seres humanos necessitam de mais de 22 minerais essenciais, os quais podem ser fornecidos por uma adequada dieta. Contudo, deficiências nutricionais são relatadas em vários países (GRAHAM et al., 2007; WHITE; BROADLEY, 2009). Com relação ao Se, os teores desse elemento nas plantas, animais e seres humanos, numa determinada região, estão diretamente relacionados com o conteúdo desse elemento presente no solo, que por sua vez está relacionado com a pedologia, gênese e localização das áreas de cultivo (ANDERSON; SCARF, 1983). Yläranta (1985) relatou que as plantas desempenham um papel único na introdução e reciclagem do Se a partir do solo

para a cadeia alimentar e, segundo Combs (2001), o nível de Se em uma população é altamente correlacionado com o teor de Se em culturas agrícolas. Nesse sentido, a biofortificação das plantas com Se, por meio da sua introdução juntamente com a adubação nas culturas, aliada ao melhoramento genético, seja por meio da seleção de genótipos ou na transferência de genes, tem-se mostrado ser uma técnica útil para a ingestão desse elemento em humanos e animais.

De acordo com Hawkesford e Zhao (2007), nas plantas, o Se apresenta propriedades químicas semelhantes às do enxofre (S), estando presente em aminoácidos sulfurados como selenometionina e selenocisteína, e essas substituições apresentam consequências deletérias, uma vez que o funcionamento desses não é o mesmo da metionina e cisteína. Além disso, as plantas apresentam capacidades diferenciadas na absorção e acumulação de Se do solo, onde esse elemento é encontrado principalmente como selenato e selenito, embora possam existir, também, Se elementar, seleneto e formas orgânicas de selênio (KABATA-PENDIAS; PENDIAS, 2001).

Apesar do Se não ser classificado como um micronutriente para plantas superiores, vários estudos têm mostrado que em baixas concentrações, esse elemento exerce efeito benéfico sobre o crescimento e a tolerância a estresses, por meio do aumento da sua capacidade antioxidante, tanto por diminuir a peroxidação de lipídios, quanto por aumentar a atividade da superóxido dismutase e glutationa peroxidase, tal como ocorre em humanos (DJANAGUIRAMAN et al., 2005; XUE; HARTIKAINEN; PIIRONEN, 2001). No entanto, as culturas agrícolas são sensíveis às altas concentrações de Se no tecido, e a sensibilidade varia entre espécies vegetais (LYONS et al., 2005).

Combs (2001) estimou que há cerca de 0,5 a 1 bilhão de pessoas no mundo com ingestão abaixo da adequada para Se. Estratégias para a ingestão

desse elemento estão sendo estudadas e desenvolvidas em muitos países. Na Finlândia, o aumento da concentração de selênio nas culturas, através da aplicação juntamente com adubação e melhoramento genético das culturas tem sido praticado desde meados da década de 1980 (EUROLA et al., 1991). Essa estratégia tem mostrado ser eficiente em aumentar a ingestão de Se na população (BROADLEY et al., 2006). Entretanto, no Brasil, trabalhos dessa natureza são raros, embora haja um indicativo de baixo consumo de Se pela população brasileira (FERREIRA et al., 2002). Assim, são necessários estudos mais específicos no Brasil, para a introdução desse elemento nos alimentos, na tentativa de definir melhor estratégia, como doses, cultivares, formas de aplicação, bem como começar a determinar o teor desse elemento nos alimentos, a fim de conhecer e proporcionar o consumo adequado de Se para a população e animais.

Nesse contexto, pelo presente trabalho objetivou-se avaliar a biofortificação com Se e a atividade de enzimas antioxidantes em plantas de alface e brócolis tratadas com Se, além da variação genotípica e a expressão de genes relacionados ao seu transporte e assimilação.

2 REFERECIAL TEÓRICO

2.1 Selênio e a saúde humana e animal

O Se é um elemento essencial para animais e seres humanos, embora tenha sido conhecido apenas pela sua toxicidade (DRAIZE; BEATH, 1935; SCHWARZ; FOLTZ, 1957). Doenças causadas pela deficiência de Se têm sido evidentes em várias partes do mundo, principalmente onde o teor de Se é baixo no solo. Nesse sentido, a doença de Keshan-Beck, que é uma cardiomiopatia frequentemente fatal e, relatada como sintoma clássico de deficiência de Se, é encontrada principalmente em áreas rurais da China, Tibete e na Sibéria, regiões essas que apresentam severas deficiências desse elemento no solo (COPPINGER; DIAMOND, 2001).

Em mamíferos, o Se é um componente de muitas enzimas e proteínas (KRYUKOV et al., 2003). A função nutricional de Se é exercida principalmente por selenoenzimas e selenoproteínas como a glutationa peroxidase, que está envolvida na proteção antioxidante (ROTRUCK et al., 1973), a tiorredoxina redutase que tem regulação redox (TAMURA; STADTMAN, 1996) e a iodotironina 5`deiodinase, que está envolvida na regulação hormonal e metabolismo (LARSEN; BERRY, 1995). Além disso, o Se faz parte de um dos sítios ativos da selenocisteína, que é crucial para as funções biológicas no corpo humano (STADTMAN, 1996; DRISCOLL; COPELAND, 2003).

Além de sua essencialidade nutricional, vários estudos mostram importantes benefícios do Se na saúde, como redução na incidência de câncer de próstata, cólon e pulmão (CLARK et al., 1996). Nesse sentido, por meio de um ensaio clínico com 1.312 pessoas mostrou-se que a suplementação de Se reduziu

a incidência de riscos de câncer de próstata em 63%, 58% para câncer de cólon e 46% para câncer de pulmão (CLARK et al., 1996) e, há evidências crescentes de que um maior consumo de Se está associado com a redução de incidência de câncer (RAYMAN, 2002).

Estudos mostram que uma baixa concentração de Se no plasma sanguíneo apresenta elevada correlação com infecções virais (BECK; LEVANDER; HANDY, 2003). Outros pesquisadores constatam com clareza que a deficiência de Se apresenta um profundo impacto na replicação do RNA viral, contribuindo para o surgimento de novas cepas virais, resultando no aumento da virulência e com o agravamento de doenças como resfriado comum, ebola e aids (BECK et al., 2001). Com relação ao vírus da aids, Sappey et al. (1994), em estudos *in vitro*, mostraram que o Se foi um potente inibidor da replicação do vírus e concluíram que o Se é um importante nutriente para indivíduos infectados com esse vírus.

Há muito tempo se sabe que a administração de Se em rações animais tem contribuído para o sucesso reprodutivo de bovinos, equinos, caprinos e ovinos (SANDERS, 1984). Nesse sentido, Hidiroglou (1979) mostrou que a suplementação com Se foi eficaz em evitar o aborto em ovinos. Da mesma forma para animais, pelas pesquisas observou-se que mulheres com baixo teor de Se no plasma sanguíneo apresentou significativa correlação com abortos ocorridos no primeiro trimestre de gravidez (BARRINGTON et al., 1997). Esses autores sugerem que a perda de gravidez precoce pode estar associada à reduzida proteção antioxidante de membranas biológicas e DNA, ocasionada por níveis relativamente baixos de atividade da glutationa peroxidase (GSH-Px), que apresenta correlação direta com a concentração de Se no plasma sanguíneo.

Também, foi verificado que animais alimentados com dietas deficientes em Se mostraram anormalidades estruturais em espermatozóides, além de baixa mobilidade e perda da cauda, diminuindo assim as chances de fertilização (WU et al., 1973) e, por meio de estudos infere-se que tal fato também ocorre com grande frequência em espermatozóides humanos, tornando homens subférteis (IWANIER; ZANCHARA, 1995).

2.2 Selênio no solo

O Se entra na cadeia alimentar por meio das plantas, mas a disponibilidade depende do teor de Se no solo, material de origem, bem como condições redox, pH do solo, presença e competição entre ânios, como fosfatos e sulfatos, além da atividade microbiana (BLAYLOCK; JAMES, 1994; DHILLON; DHILLON, 2003). Nesse sentido, algumas bactérias podem converter formas insolúveis de Se a formas solúveis que podem ser absorvidas pelas plantas (DIPLOCK, 1993).

A disponibilidade de Se para as populações em diferentes partes do mundo é altamente variável e especialmente baixa em certas regiões da China, Tibete, Sibéria, Nova Zelândia, parte leste da Europa, Rússia e África (COMBS, 2001). O teor de Se na maioria dos solos varia entre 0.1 e 2.0 mg kg^{-1} (DHILLON; DHILLON, 2003) e, o Se pode ser encontrado em quatro diferentes estados de oxidação: selenato (+6), selenito (+4), Se elementar (0), e como selenetos orgânicos e inorgânicos (-2), do quais o selenato e selenito as formas de Se predominantes em solos cultivados. Selenato é mais solúvel e disponível para as plantas em condições de solo oxidantes e alcalinas (MASSCHELEYN; DELAUNE; PATRICK, 1990) e o selenito é menos disponível para as plantas,

uma vez que é adsorvido mais fortemente por óxidos de ferro e argilas do solo (MIKKELSEN; PAGE; BINGHAM, 1989; YLÄRANTA, 1985). Entretanto, alguns autores mostram que a calagem reduziu a adsorção de selenito nas superfícies de óxidos de ferro, aumentando, assim, a sua disponibilidade para as plantas (CARLSTON; ADRIANO; DIXON, 1991; MIKKELSEN; PAGE; BINGHAM, 1989). Além de sulfato, a interação entre o Se e outros nutrientes, como fosfato, diminui a disponibilidade de Se para as plantas (HOPPER; PARKER, 1999; YLÄRANTA, 1985).

Por causa dos teores baixos de Se no solo, em alguns países como Finlândia, o Se é aplicado anualmente junto com fertilizantes a fim de manter uma concentração suficiente de Se em produtos agrícolas. Estudos demonstraram que, após o início do uso de Se em fertilizantes, que começou no início da década de 80, a concentração no plasma sanguíneo na população desse país apresentou aumentos significativos (EUROLA et al., 2003). Entretanto, há poucos relatos desse tipo de estudo no Brasil e, algumas pesquisam mostram que muitos solos estão numa faixa de deficiência de Se (FICHTNER et al., 1990; FARIA, 2009). Em um estudo realizado por Sillanpää e Jansson (1992), estes autores classificaram como baixo o teor de Se em solos brasileiros.

2.3 O selênio nas plantas

Estudos mostram que a absorção do Se na forma de selenato e sua distribuição nas plantas ocorre de forma mais rápida que o selenito (CARTES; GIANFERA; MORA, 2005; PILON-SMITS et al., 1999). Souza et al. (1998) observaram que o acúmulo total de Se em uma planta pode ser até dez vezes maior quando aplicado selenato, do que na forma de selenito. O selenato, por

apresentar semelhanças ao íon sulfato, é ativamente absorvido para as raízes por transportadores desse último (ASHER; BUTLER; PETERSON, 1977; TERRY et al., 2000). O mecanismo de absorção do Se na forma de selenito ainda não é totalmente compreendido, embora alguns autores assumam que ocorre de forma passiva (TERRY et al., 2000; WHITE et al., 2004). Além disso, as plantas podem absorver ativamente formas orgânicas de Se, como selenometionina (TERRY et al., 2000; ZAYED; LYTLE; TERRY, 1998).

Plantas superiores apresentam diferentes capacidades em acumular e tolerar Se. Elas são classificadas em não-acumuladoras, indicadoras e acumuladoras (DHILLON; DHILLON, 2003; TERRY et al., 2000; WHITE et al., 2004) e, algumas espécies de plantas são classificadas como hiperacumuladores de Se, sendo um dos maiores grupos de hiperacumuladoras pertencente ao gênero Astragalus (TERRY et al., 2000). Há indícios que o Se para esse gênero de plantas mostra ser essencial (TRELEASE; TRELEASE, 1939). Mas, de fato, a essencialidade de Se foi demonstrada para o crescimento de uma alga verde (*Chlamydomonas reinhardtii*), em que a atividade da GSH-Px foi identificada como dependente da presença de Se, tal como em humanos (FU et al., 2002). Em contraste, a maioria das espécies de culturas agrícolas, não parecem exigir Se para o seu crescimento (TERRY et al., 2000).

Em relação a plantas hiperacumuladoras de Se, essas são divididas em dois grupos: acumuladoras primárias de Se, que são capazes de acumular milhares de miligramas de Se (> 2000 mg kg^{-1}); e acumuladoras secundárias, que acumulam centenas de miligramas Se. Um grupo conhecido das brássicas, como a mostarda indiana, brócolis e canola são classificadas como acumuladoras primárias. Segundo White et al. (2004), a maioria das culturas agrícolas acumula e apresenta baixa tolerância a altos níveis de Se no meio de

cultivo, apresentando geralmente menos de 25 mg kg^{-1} de Se na massa seca e, sendo classificadas como não acumuladoras. Rani, Dhillon e Dhillon (2005) mostraram que plantas não acumuladoras podem tolerar e acumular altas concentrações de Se em solos enriquecidos com esse elemento. Entretanto, a maioria dos trabalhos publicados mostra o contrário (DJANAGUIRAMAN et al., 2005; HARTIKAINEN; XUE; PIIRONEN, 2000; RIOS et al., 2008; XUE; HARTIKAINEN; PIIRONEN, 2001) e, estudos revelaram que o Se aplicado em baixa concentração favoreceu o crescimento e a atividade antioxidante em mono e dicotiledôneas (HARTIKAINEN et al., 1997; HARTIKAINEN; XUE, 1999). Tal como em humanos, vários trabalhos também mostram a função protetora do Se contra estresse oxidativo em plantas, aumentando a atividade da GSH-Px e diminuindo a peroxidação lipídica (CARTES; GIANFERA; MORA, 2005; DJANAGUIRAMAN et al., 2005; HARTIKAINEN; XUE; PIIRONEN, 2000).

Dadas as características bioquímicas semelhantes entre o Se e S, é conhecido que o Se usa os mesmos transportadores do S e, a mesma via de assimilação (PILON-SMITS; QUINN, 2010). Nas raízes das plantas, o selenato (SeO_4^{-2}) é absorvido ativamente por transportadores de sulfato e permanece nessa forma até que a ATP sulfurilase (*APS*) entre em ação, transformando o SeO_4^{-2} em adenosina fosforoselenato (*APSe*) (Figura 1). Em seguida a *APSe* é convertida em selenito (SeO_3^{-2}) pela ação da enzima adenosina fosforoselenato redutase (*APR*). Na sequência, o SeO_3^{2-} é transformado em Se^{2-} pela intervenção da sulfito redutase e, finalmente o Se^{2-} é convertido em selenocisteina (SeCys) pela ação da O-acetilserina (tiol)liase (*OAS*). Por ser muito instável, a SeCys é logo transformada em outros compostos, como metilselenocisteina (MeSeCys), proteínas, etc. Todas as etapas citadas anteriormente ocorrem nos plastídios celulares. No citosol, ocorre a formação da selenometionina (SeMet), que é

transformada a partir da SehomoCys pela ação da metionina sintase. Ainda no citosol a SeMet pode se transformar em compostos voláteis de Se, proteínas ou, entrar no plastídios e se ligar a proteínas.

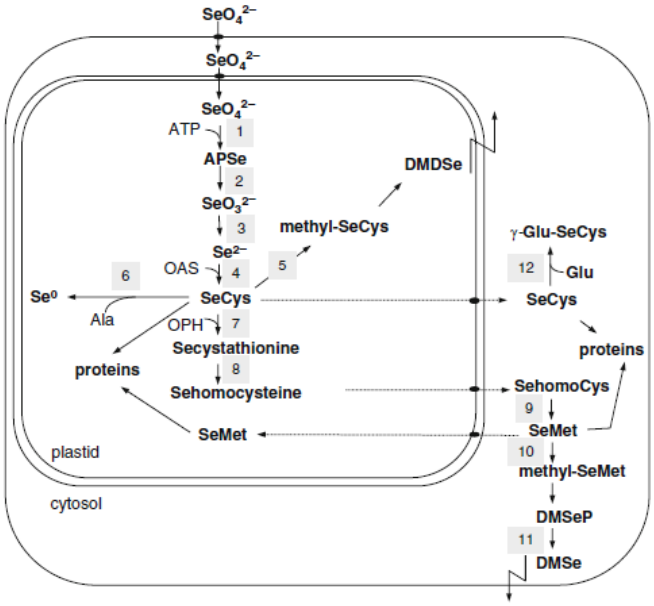

Figura 1 Esquema do metabolismo de Se nas plantas

APSe = adenosina fosfatoselenato; OAS = O-acetilserina (tiol)liase; OPH = O-fosforohomoserina; SeCys = selenocisteina; SeMet = selenometionina; DMSeP = di-metil selenoproprionato; DMSe = di-metil seleneto; DMDSe = di-metil di-seleneto. Os números indicam enzimas conhecidas. (1) ATP sulfirulase; (2) adenosina fosforoselenato redutase; (3) sulfito redutase; (4) O-acetilserina (tiol)liase; (5) SeCys metil transferase; (6) SeCys liase; (7) cistationa-*y*-sintase; (8) cystationa-β-liase; (9) metionina sintase; (10) metionina metil transferase; (11) DMSP liase; (12) *y*-glutamil-cisteina sintase

Fonte: Adaptado de Pilon-Smits e Quinn (2010)

Para as etapas envolvendo a volatilização do Se, essas ocorrem por duas vias. A primeira ocorre no citosol pela quebra da SeMet, que é transformada em metilselenometionina (*metil-SeMet*) pela ação da metionina metiltransferase, que produz um produto intermediário chamado de dimetilselenopropionato

(*DMSeP*), que, em seguida, é transformado em di-metil seleneto (*DMSe*), que é considerado, por alguns autores, como a principal forma de compostos voláteis produzidos em plantas não acumuladoras (LEWIS; JOHNSON; DELWICHE, 1966). A segunda etapa na volatilização de compostos de Se ocorre nos plastídios, onde a enzima SeCys-metiltransferase transforma a SeCys em methil-SeCys, que na sequência é transformado em di-metil di-seleneto (*DMDSe*). Plantas que são consideradas acumuladoras de Se, como brássicas, sintetizam DMDSe e, esse composto é responsável pelo odor e sabor característico nessas plantas (KUBEC; DRHOVA; VELISEK, 1998; WHITAKER, 1976).

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SEGUNDA PARTE

ARTIGO 1

Selenium biofortification and antioxidant activity in lettuce plants fed with selenate and selenite

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Selenium biofortification and antioxidant activity in lettuce plants fed with selenate and selenite

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ABSTRACT

Selenium is an important element associated with enhancing antioxidant activity in plants, microorganisms, animal, and humans. In Brazil, the information on Se in agricultural crops is lacking, though there are indications that low levels of Se are consumed by the population. The experiment was conducted under greenhouse conditions with pots containing 3 L of nutritive solution in a completely randomized factorial design, with seven Se concentrations (0, 2, 4, 8, 16, 32, and 64 μmol L^{-1}) and two forms of Se (sodium selenate – Na_2SeO_4 and sodium selenite – Na_2SeO_3), with six replicates. The application of Se as selenate at low concentrations is more appropriate for lettuce biofortification because it favors shoot biomass growth and Se levels in the shoot biomass. Selenium in both forms had two effects on lettuce plant metabolism: at low doses it acted as an antioxidant and enhanced plant growth, whereas at higher levels it reducing yield.

Keywords: selenate, selenite, antioxidant enzymes, biofortification

INTRODUCTION

Selenium (Se) is an important element for human and animal nutrition, due to its roles on a series of biochemical reactions enhancing antioxidant activity (Rayman, 2002). In contrast, Se has not yet been considered essential for plants (Terry et al. 2000, Kápolna et al. 2009). Nevertheless, several studies reported the beneficial effects of Se, because it increases the antioxidant activity in plants, leading to better plant yield (Hartikainen et al. 2000, Lyons et al. 2009).

Plants recycle Se within the food chain. Thus, biofortification of agricultural crops with Se, by means of adding Se along with fertilizers, is an useful technique to increase the consumption of Se by animals and man (Chen et al. 2002, Ríos et al. 2008, White and Broadley 2009, Broadley et al. 2010). Inorganic Se forms differ in terms of absorption and mobility within plants, being selenate being more easily transported to shoots, while selenite tends to accumulate in plant roots (Zhang et al. 2003). For this reason, in some Se biofortification programs, use of selenate is recommended over selenite (Ríos et al. 2008).

The antioxidative effect of Se has been related to an improved GSH-Px and SOD activity and a decreased lipid peroxidation in Se treated plants, like ryegrass (Hartikainen et al., 2000), lettuce (Xue et al., 2001) and soybean (Djanaguiraman et al., 2005). Moreover, Cartes et al. (2005) determined that selenite was more efficient that selenate in promoting enzymatic activity.

Even though there are reports of low Se consumption by the Brazilian population, studies on Se fortification of crop plants is meagre (Ferreira et al. 2002, Maihara et al. 2004). Since lettuce is the most-consumed leafy plant in Brazil and many parts of the world (Luz et al. 2008, Li et al. 2010), this crop

can be preferred in Se biofortification programs, as an efficient way of increasing intake of this element by the population. So in the present work the effect of the biofortification of different Se forms on the yield and antioxidant systems in lettuce plants is studied.

MATERIALS AND METHODS

Plant materials and experimental design

Lettuce (*Lactuca sativa* L. cv. Vera) was planted in expanded polystyrene trays containing 128 compartments filled with vermiculite and irrigated with distilled water in the first five days. Seedlings were irrigated with the 0.5N Hoagland nutrient solution till 15 days. After that, seedlings with uniform seedling vigour were selected and transplanted to 3 L pots containing 0.5N Hoagland nutrient solution with different concentrations of Se.

The experimental design was a completely randomized factorial 7 x 2, in which the main factor was Se concentration in seven levels (0, 2, 4, 8, 16, 32 and 64 μmol L^{-1}) and the other factor was Se forms (sodium selenate – Na_2SeO_4, and sodium selenite – $Na_2SeO_35H_2O$, both from Sigma-Aldrich), with six replicates, totaling 84 plots. Each experimental unit was made up of one plant per pot.

Throughout the experimental period, the nutritive solution underwent constant aeration and pH was monitored daily at 6.0 ± 0.2 by addition of 0.1 mol L^{-1} NaOH or HCl. The solution was changed whenever the initial electrical conductivity dropped more than 30%. After 25 days of Se exposure, plants were harvested, and divided into shoots and roots.

During harvest, three replicates with five leaves each from the middle part of the plant were immediately wrapped in aluminum foil, submerged in liquid nitrogen and were stored in a freezer, at -80 °C, for enzyme assay. The

remaining plants (replicates) were dried in a forced-air drying oven at 65-70 °C until a constant mass of the root and shoot samples were achieved.

Antioxidant enzymes activity and lipid peroxidation measurement

In order to estimate the superoxide dismutase and catalase enzyme activities, frozen tissue was homogenized in a cooled 0.1 mol L^{-1} Tris–HCl buffer at pH 7.8 containing 1 mmol L^{-1} EDTA, 1 mmol L^{-1} dithiothreitol and 5 mL of 4% polyvinyl pyrrolidone per gram of fresh weight. The homogenate was filtered through a nylon mesh and centrifuged at 22000 x *g* for 30 min at 4 °C. The supernatant was used to measure enzyme activity.

Superoxide dismutase (EC 1.15.1.1) activity was assayed by monitoring photochemical inhibition of nitroblue tetrazolium (NBT) reduction (Beyer and Fridovich 1987). A 5 mL reaction mixture, containing 50 mmol L^{-1} Na_2CO_3 (pH 10.0), 13 mmol L^{-1} methionine, 0.025% (v/v) Triton X-100, 63 µmol L^{-1} NBT, 1.3 mmol L^{-1} riboflavin, and an appropriate quantity of enzyme extract was used. The reaction mixtures were illuminated for 15 min at photosynthetic photon flux density (PPFD) of 380 µmol m^{-2} s^{-1}. Non-illuminated mixtures were used to correct for background absorbance. One unit of SOD activity was defined as the amount of enzyme required to inhibit 50% of NBT reduction as monitored at 560 nm.

Catalase (EC 1.11.1.6) activity was tested by observing H_2O_2 consumption at 240 nm for 5 min (Rao et al. 1997). Reaction mixture (3 mL total volume) contained 25 mmol L^{-1} Tris-acetate buffer (pH 7.0), 0.8 mmol L^{-1} EDTA-Na, and 20 mmol L^{-1} H_2O_2, and the enzyme assay was carried out at 25 °C.

For the malondialdehyde (MDA) assay, 0.5 g of lettuce leaf was homogenized in 5 mL of 50 mmol L^{-1} buffer solution (containing 0.07%

$NaH_2PO_4.2H_2O$ and 1.6% $Na_2HPO_4.12H_2O$), ground with a cooled mortar and pestle, and centrifuged at 22000 x *g* for 30 min (4 °C). MDA concentration was calculated using the extinction coefficient of 155 mmol L^{-1} cm^{-1} (Fu and Huang 2001).

Selenium measurement

In this step, 500 mg of dried powder of plant materials was added with 10 mL of concentrated p.a. HNO_3 to Teflon PTFE flasks and digested at 0.76 MPa for 10 minutes in a microwave oven (CEM, model Mars 5). After cooling to room temperature, the extract was filtered (Whatman number 40 filter) and diluted by adding 5 mL of bi-distilled water.

Total Se in the extracts was determined in a PerkinElmer Analyst 800 atomic absorption spectrophotometer with electrothermal atomization by (pyrolytic) graphite furnace with transversal heating and automatic sampler. A hollow cathode Se bulb was employed as a radiation source, operating at 6.0 mA, with a 196.0 nm wavelength, and a 2.0 nm gap. The matrix modifier consisted of a solution containing 0.005 mg Pd + 0.003 mg $Mg(NO_3)_2$; argon (95% pure) was used as an inert gas in the graphite furnace. In these conditions, pre-treatment temperature was 1300 ºC and an atomization temperature was 1900 °C. Readings were carried out by peak absorption area, using a 5 s atomization time, taken out of the respective blanks of each sample digestion battery. Certified reference material (tomato leaf material, NIST 1573a) were included in each batch of samples for quality control, and the recovery percentages varied from 89.4 to 93.2%.

All data were subjected to a simple ANOVA at 95% confidence, using Sisvar 4.6 software (Build 6.1) and the graphs were done on Sigma Plot (version 11.0).

RESULTS AND DISCUSSION

Figure 1A shows that Se affected shoot biomass production, with an increase of 5.67 and 3.69% by selenate and selenite respectively, at low concentrations of up to 8 and 4 µmol L^{-1}. However, further increase in Se concentrations reduced shoot biomass production. Our results are in agreement with Fargašová (2003) who observed an inhibitory effect of Se on the mustard growth. In general, the biomass production was higher when selenate was supplied to the nutrient solution than selenite (Figure 1 A). Previous studies showed that selenate and selenite provide distinct responses in Se translocation (Zhang et al. 2003). Figure 1 B shows that Se translocation was higher when supplied as selenate. When lettuce received lower Se concentrations (2, 4, and 8 µmol L^{-1}), approximately 70% of the element applied as selenate and 50% as selenite were found in the shoots. When the plants were treated with higher concentration of Se, there was a small reduction in translocation in both Se forms. The difference between selenate and selenite is due to the high affinity between sulfate transporters and selenate, which facilitates its absorption and translocation (Zhang et al. 2003). As far as selenite absorption processes, little is known (Rosen and Liu 2009). There are indications that selenite transport by cellular simplasm actively takes place and that phosphate transporters act in the process, at least partially (Hopper and Parker 1999; Li et al. 2008). In addition, when selenite is absorbed by plants, it is rapidly converted to organic forms of Se in roots, which have low mobility in xylem (Li et al. 2008).

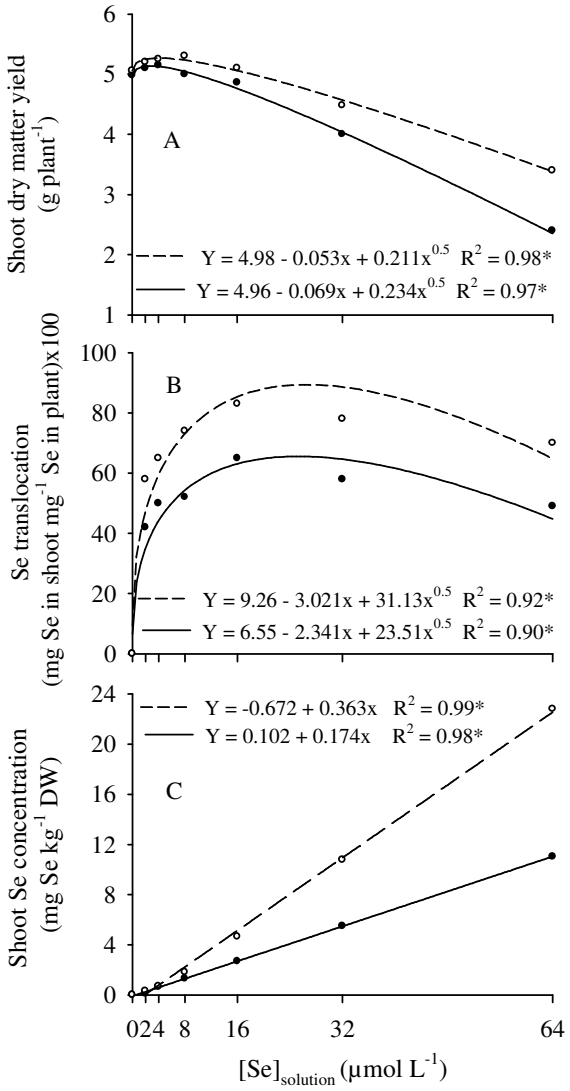

Figure 1. Shoot biomass (A), Se translocation (B) and shoot Se concentration (C) in lettuce plants treated with different concentrations and forms of Se. (● selenite ○ selenate) * $P < 0.05$.

Leaf Se concentration increased with an increase in Se concentration in the nutrient medium (Figure 1 C). These results corroborate those obtained in other works, which have reported that increasing doses of Se in medium culture can to cause a significant increase of Se content in agricultural crops (Ducsay et al. 2009; Broadley et al. 2010). Considering that Brazilian *per capita* consumption of fresh lettuce leaves is 33.3 g day^{-1} (Yuri et al. 2005), and that the recommended consumption of Se in adults is 50-70 µg day^{-1} (US Department of Agriculture 2001), the Se content obtained in this experiment with selenate without compromising production, met only ca. 5% of the recommended human Se intake (approximately 230 g FW $week^{-1}$ and considering that lettuce is composed of 96% water). Thus, the present study suggests a need for further research on biofortification of different crops with Se, in order to meet ideal Se consumption by Brazilians. These results shown in Fig 1 are conformity with the earlier result by Ríos et al. (2008).

Figure 2 shows changes in the activity of SOD, CAT and lipid peroxidation in lettuce leaves treated with an increasing concentrations Se, either as selenate or selenite. SOD activity was maximum at Se concentrations of 32.9 and 22.1 µmol L^{-1}, for selenate and selenite, respectively (Figure 2 A). That for CAT, the maximum activity was detected at concentrations of 12.3 and 20.8 µmol L^{-1} (Figure 2 B). During oxidative stress, excess reactive oxygen species (ROS) production causes membrane damage that eventually leads to cell death (Das et al. 1992, Montillet et al. 2005). In our experiment, increased lipid peroxidation at higher Se concentrations indicated the occurrence of oxidative stress (Figure 2 C). This might be one of thereasons for lowering lettuce production at higher Se concentrations (Figure 1A). To protect against ROS, plants have antioxidant enzymes such as SOD and CAT, as well as a wide array

of non-enzymatic antioxidants (Mittler 2002).

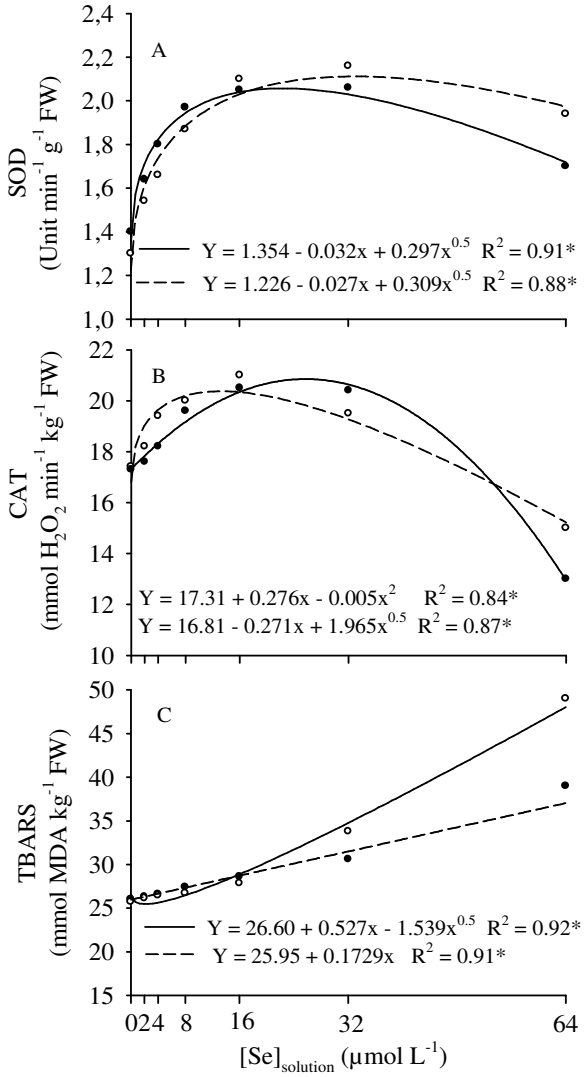

Figure 2. Activity of superoxide dismutase (SOD) (A), catalase (CAT) (B) and lipid peroxidation (TBARS) (C) in lettuce plants treated with different concentrations and forms of Se. (● selenite ○ selenate) * $P < 0.05$.

Se induced increase in SOD and CAT activity at low concentrations probably enhanced lettuce leaf production through the enhanced activity of antioxidants. Similar results were reported in ryegrass (Hartikainen et al. 2000).

Our results indicate for biofortification program with lettuce, application of Se as selenate at low concentrations would be more beneficial because it favors shoot biomass growth, Se translocation, and Se levels in the shoot biomass.

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ARTIGO 2

Selenium accumulation in lettuce germplasm

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Selenium accumulation in lettuce germplasm

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Abstract

Selenium (Se) is an essential micronutrient for animals and humans. Increasing Se content in food crops offers an effective approach to reduce the widespread selenium deficiency problem in many parts of the world. In this study, we evaluated thirty diverse accessions of lettuce (*Lactuca sativa* L.) for their

capacity to accumulate Se and their responses to different forms of Se in terms of plant growth, nutritional characteristics, and gene expression. Lettuce accessions responded differently to selenate and selenite treatment, and selenate is superior to selenite in inducing total Se accumulation. At least over 2-fold change in total Se levels between cultivars with high and low Se content was found. Synergistic relationship between Se and sulfur (S) accumulation was observed in nearly all accessions at the selenate dosage applied. The change of shoot biomass varied between lettuce accessions and the forms of Se used. The growth-stimulated effect by selenate and the growth-inhibited effect by selenite were found to be correlated with the alteration of antioxidant enzyme activities. The different ability of lettuce accessions to accumulate Se following selenate treatment appeared to be associated to an altered expression of genes involved in Se/S uptake and assimilation. Our results provide important information for the effects of different forms of Se on plant growth and metabolism. They will also be of help in selecting and developing better cultivars for Se biofortification in lettuce.

Keywords Lettuce; Selenium; Germplasm; Biofortification; Antioxidant enzyme activity; Gene regulation;

Abbreviations

Se, selenium; S, sulphur; Sultr, sulfate transporter; APS, adenosine 5′-phosphosulfate sulfurylase; APR, adenosine phosphosulfate reductase; SAT, serine acetyl transferase; APX, ascorbate peroxidase; CAT, catalase; GSH-Px, glutathione peroxidase; ROS, reactive oxygen species; SeMet, selenomethionine; qRT-PCR, quantitative reverse transcription-PCR; UPLC, Ultra Performance Liquid Chromatography

INTRODUCTION

Selenium (Se) is an essential micronutrient for humans and animals as it constitutes the key component of selenoproteins and selenoenzymes with important biological functions (Rayman 2002; Whanger 2004). The deficiency of Se in diets is considered to be the key factor in the development of serious illnesses in humans such as Keshan disease, a congestive cardiomyopathy (Yang et al. 1984). Moreover, studies indicate that Se deficiency weakens the immune system, increases viral infections, and promotes cancer (Beck et al. 2003; Diwadkar-Navsariwala et al. 2006). Because of large areas of soil that contains low levels of Se and consequently low levels of Se supply in food, Se deficiency is a widespread problem in many parts of the world. Combs (2001) reports that Se level in a population is highly correlated with Se content in agricultural crops. Thus, increasing Se content in food crops can have a positive impact on reducing Se deficiency in the world.

Selenium is obtained from dietary sources, including cereal grains and vegetables. Se biofortification in agricultural crops by means of Se fertilization or selection of crop genotypes with improved ability of accumulation via plant breeding provides a useful approach to increase the consumption of Se by animals and humans (Graham et al. 2007; White and Broadley 2009). The Finnish practice by adding sodium selenate to all multinutrient fertilizers offers one of the best examples of the agronomic approach (Hartikainen 2005). Through this program, the average Se intake in Finland increased from 25 μg day^{-1} to 110 μg day^{-1} following Se fertilization (Eurola et al. 1991). Although a number of recent works have demonstrated the effectiveness of Se fertilization in increasing Se content in a number of food crops (Chen et al. 2002; Pedrero et al. 2006; Broadley et al. 2010), only few studies have investigated the genetic

variation of crop species in accumulation of Se (Lyons et al. 2005; Zhao et al. 2009).

Given that Se is chemically similar to sulfur (S), plants and other organisms readily take up and metabolize Se via S uptake and assimilation pathway (Terry et al. 2000; Sors et al. 2005; Pilon-Smits and Quinn 2010). Se is acquired by plants from soils mainly as selenite and selenate. Plants uptake selenate through a process of active transport, which is mediated by sulfate transporters (Terry et al. 2000). The selectivity of these transporters for selenate and sulfate varies between plant species and with S nutritional status (White et al. 2004). Selenite is passively taken up into plants, and phosphate transporters are believed to partially act in the process (Hopper and Parker 1999; Li et al. 2008). The reduction of selenate and selenite to form selenoamino acids involves a number of key enzymes in S assimilation pathway, such as ATP sulfurylase (APS), APS reductase (APR), and serine acetyltransferase (SAT) in plants.

In contrast to the essentiality of Se in human and animal nutrition, Se is considered not essential for plants (Terry et al. 2000; Sors et al. 2005; Pilon-Smits and Quinn 2010). Nevertheless, several studies report the beneficial effect of Se at low concentrations in stimulating plant growth (Hartikainen et al. 2000; Rios et al. 2010). However, at high concentrations, Se inhibits plant growth. In general, selenate is less toxic to plants than selenite, but the effect of Se application to plants also depends on other factors, such as soil redox conditions, soil pH, and the presence of competing anions, e.g. sulfate and phosphate (Blaylock and James 1994; Dhillon and Dhillon 2003).

Lettuce is one of the most consumed leaf vegetables in many parts of the world. Lettuce provides a good dietary source for various phytochemicals,

such as phenolics and vitamin A, C, and E, as well as minerals. Beyond its basic nutrition, lettuce has been the target for Se biofortification in providing daily requirement of Se (Rios et al. 2008b; Ramos et al. 2010). Many different types of cultivated lettuce are known. They contain broad diversity in leaf color, shape, growth habit, and texture (Lebeda et al. 2007). The ability of different genotype of lettuce in accumulating Se, however, is unknown.

Exploiting genotypic variation is likely to be an effective method for Se biofortification in crops. The aim of this study was to evaluate the genotypic variation of lettuce germplasm in response to selenate and selenite treatment for Se biofortification in lettuce. Thirty heirloom and modern lettuce cultivars, plant introductions (PI), and landraces representing a broad range of market types were selected and used in the study. The effect of Se forms on plant growth, total Se and S levels, antioxidant enzyme activities, total amino acid content, and the expression of genes involved in Se/S uptake and assimilation were examined in attempting to provide the information for the selection of lettuce germplasm with substantial Se accumulation without negative effect on plant growth and nutritional characteristics.

MATERIALS AND METHODS

Lettuce germplasm

A population of thirty diverse accessions (twenty-nine named cultivars and PI251246) of lettuce (*Lactuca sativa* L.) were selected from lettuce genbank at the USDA, Salinas, California, and used in this study. The population includes the most widely grown iceberg and romaine-type lettuces, as well as those of green and red leaf, butterhead, and the lesser known types of Batavia, stem, Latin, lolla rossa, and oilseed lettuce (Table 1). Included are commercially

important cultivars, historically significant landmark cultivars found in the pedigrees of many modern cultivars, cultivars with unique morphology, and cultivars adapted to Northeast production conditions. Additional details on these accessions can be found at http://www.ars-grin.gov/, http://cuke.hort.ncsu.edu/cucurbit/wehner/vegcult/vgclintro.html, and http://compositdb.ucdavis.edu/database/lettcv2/display/. The phenotypes of 24-d-old young plants for these cultivars are shown in Fig. 1.

Plant growth and treatments

Seeds of each accession were sown in growth medium (Metro-Mix 360, Sun Gro Horticulture) and grown in a greenhouse with a 14 h light and 10 h dark photoperiod at 24 °C. The 20-day-old young seedlings were transplanted to 2.2 L pots containing Hoagland nutrient solution (Hoagland and Arnon 1950) with 40% ionic strength and grown hydroponically in the same greenhouse. Four days after transplantation, these plants were either continuously grown in the same nutrient solution or exposed to the nutrient solution containing 15 μM Na_2SeO_4 or 15 μM Na_2SeO_3 (Sigma-Aldrich). This concentration of Se was chosen as it showed to be the most suitable with minimal effects on plant growth in our preliminary studies with a few cultivars. The nutrient solution was changed twice each week. Throughout the experimental period, the nutrient solution underwent constant aeration, and pH was monitored and adjusted to 6.0 ± 0.2 by addition of 0.1 mol L^{-1} NaOH or HCl. After 2 weeks of Se treatment, a total of 360 plants (30 x 3 x 4; accessions x treatments x repeats) were harvested individually. The fresh weights of aerial part were weighed. The young leaf samples from each plant were immediately frozen in liquid nitrogen, ground to powder, and stored at -80 °C for enzyme assays, RNA extraction, and Ultra

Performance Liquid Chromatography (UPLC) analysis. For analysis of total Se and S, the leaf samples were dried at 65 °C in an oven for 6 days.

Table 1 Lettuce germplasm used in this study

ID	Line	Type	GRIN[1] accession number	Note
1	Salinas 88	Iceberg	W6 22150	Commercially important
2	Tania	Butterhead	PI596699	Commercially important
3	Vanguard	Iceberg	PI536812	Landmark cultivar
4	Empire	Iceberg	PI536778	Landmark cultivar
5	Grand Rapids	Green leaf	PI536792	Landmark cultivar
6	Cocarde	Red oak leaf	PI657644	novel type
7	La Brillante	Batavia		
8	Green Towers	Romaine	PI601336	Commercially important
9	Merlot	Dark red leaf		Used for baby leaf
10	Holborn Standard	Batavia	PI536856	Heirloom cultivar
11	Imperial 850	Iceberg / Batavia	PI536743	Landmark cultivar
12	Tall Guzmaine	Romaine		Landmark cultivar
13	Lolla Rossa	Lolla rossa	PI617943	Novel type
14	Oak Leaf	Green oak leaf	PI595576	Novel type
15	PI251246	Oil seed	PI251246	Egyptian land race
16	Balady Aswan	Stem	W6 29802	Egyptian land race
17	Valmaine	Romaine	PI543959	Landmark cultivar
18	Parade	Butterhead		
19	Salad Crisp	Iceberg	PI635076	Northeast adapted
20	Summertime	Iceberg	PI635072	Northeast adapted
21	Ithaca	Iceberg	PI536847	Northeast adapted
22	Blonde Lente	Blonde romaine	PI634670	
23	Ice Cube	Mini iceberg	W6 19082	
24	Ruebens Red	Red romaine		
25	Iceberg	Batavia	PI536860	Heirloom cultivar
26	Shining Star	Green leaf		Commercially important
27	Ruby	Red leaf	PI536761	Commercially important
28	Prizehead	Red leaf	PI536757	Landmark cultivar
29	Pavane	Latin		
30	Eruption	Red Latin		Used in baby leaf

[1]GRIN = Genetic Resources Information Network, http://www.ars-grin.gov/

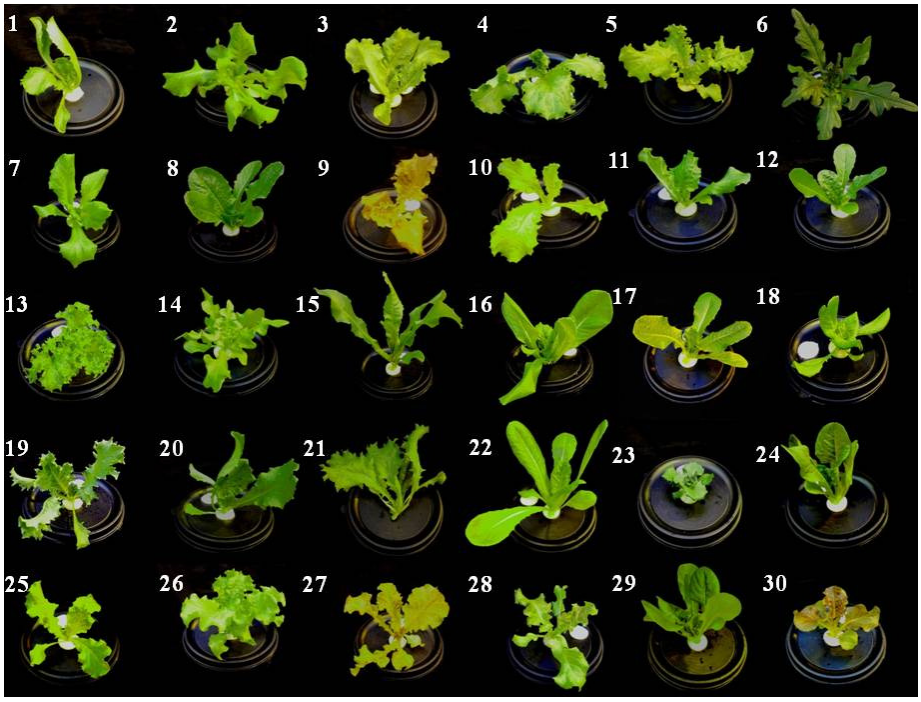

Fig. 1 Phenotypes of thirty lettuce accessions at 24 days of growth. The numbers correspond to the ID numbers in Table 1.

Analysis of total Se and S levels

Total Se and S contents in the samples were determined using an inductively coupled plasma (ICP) trace analyzer emission spectrometer (model ICAP 61E trace analyzer, Thermo Electron, San Jose, CA) essentially as described previously (Lyi et al. 2005). Dried tissues (approximately 200 mg) were weighed and acid digested in 2.0 mL HNO_3 with 2.0 mL $HClO_4$ at 120 °C for 1 h and then at 220 °C until $HClO_4$ fumes were observed. The instrument was calibrated with 7% $HClO_4$ as the low standard and 5.0 μg mL^{-1} of Se and

40.0 μg mL^{-1} of S in a multi-element standard as the high standard. The Se and S were determined using the 196.0-nm and 182.0-nm line, respectively.

Analysis of antioxidant enzyme activities

Antioxidant enzymes were extracted according to Grace and Logan (1996). Briefly, 200 mg leaves were ground with a pre-cooled mortar and pestle in 1.5 mL ice-cold extraction buffer containing 50 mM KH_2PO_4-KOH (pH 7.5), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.3% (w/v) Triton X-100, and 4% (w/v) insoluble polyvinylpolypyrrolidone (PVPP). The extract was kept on ice for 10 min and then centrifuged at 13,000 rpm for 10 min in a microcentrifuge at 4 °C. The supernatant was used immediately for measuring the following enzyme activities.

Ascorbate peroxidase (APX, EC 1.1.11.1) activity was measured by monitoring the decrease in absorbance at 290 nm (Nakano and Asada 1981). The assay mixture (1 mL) contained 50 mM Hepes-KOH (pH 7.6), 0.1 mM EDTA, 0.2 mM H_2O_2, 0.5 mM reduced ascorbate (AsA), and enzyme extract. The reaction was initiated by adding H_2O_2. The extinction coefficient of 2.8 mM^{-1} cm^{-1} was used for the calculation of enzyme activity.

Catalase (CAT, EC 1.11.1.6) activity was determined by monitoring the decrease of absorbance at 240 nm (Rao et al. 1997). The reaction mixture (1 mL) contained 100 mM potassium phosphate buffer (pH 7.0), 10 mL 10% (w/v) H_2O_2, and enzyme extract. The reaction was initiated by adding H_2O_2. The extinction coefficient of 39.4 mM^{-1} cm^{-1} was used for the calculation.

Glutathione peroxidase (GSH-Px, EC 1.11.1.9) activity was measured following the method of Flohe and Gunzler (1984) with slight modifications. The enzyme from 200 mg frozen leaf tissue was extracted in a 1.5 mL pre-chilled $KNaHPO_4$ buffer (pH 7.0). Following centrifugation at 12,000 rpm for 5

min, 0.2 mL of the supernatant was placed into a tube and mixed with 0.4 mL of 0.1 mM GSH (reduced glutathione, Sigma) and 0.2 mL of 67 mM KNaHPO4. The reaction mixture without enzyme extract was used as control. After pre-heating the mixtures in a water bath at 25 °C for 5 min, 0.2 mL H_2O_2 (1.3 mM) was added to initiate the reaction. The reaction was carried out for 10 min, terminated by adding 1 mL 1% trichloric acetic acid, and left on ice for 30 min. The mixture was then centrifuged for 5 min at 12,000 rpm, and 0.48 mL of the supernatant was added into a tube containing 2.2 mL of 0.32 M Na_2HPO_4 and 0.32 mL of 1.0 mM 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB). Following 5 min of incubation, the absorbance at wavelength 412 nm was measured. The enzyme activity was calculated as a decrease in GSH within the reaction time in comparison with that in the control.

Amino acid analysis by ultra performance liquid chromatography (UPLC)

The procedures for extraction and analysis of amino acids were preformed essentially as described previously (Lyi et al. 2005). The frozen samples (100 mg) were crush into powder at a speed of 4.0 m s^{-1} in a FastPrep FP120 instrument (Q-BIOgene) and extracted in 0.5 mL of 20 mM HCl containing 20 µL norleucine (250 µg mL^{-1}) as an internal standard. The extracts were centrifuged twice at 14,000 rpm for 10 min at 4°C, and the supernatants were transferred into new tubes. AccQ.Tag derivatives of extracted amino acids were generated using AccQ.Tag Ultra Derivatization Kit following the manufacturer's instruction (Waters). For derivatization, 70 µL of AccQ.Tag Ultra borate buffer was added to 10 µL of extract sample, followed by 20 µL of AccQ.Tag reagent solution. Derivatized amino acids were analyzed using an Acquity UPLC system equipped with a binary solvent delivery system and an auto sampler, and separated on an AccQ.Tag Ultra column (100 x 2.1 mm)

(Waters). Derivatized sample (5 μL) was eluted with a mixture of 10-fold diluted AccQ-Tag Eluent A (Waters) and 100% acetonitrile (eluent B) at a flow rate of 0.7 mL min^{-1} following a gradient of 99.9%:1% to 75%:25% eluent A:B. Derivatized amino acids were detected at 260 nm using a photo diode array detector. Amino acids in the samples were identified by co-elution with amino acid standard H (Pierce) and commercially available individual amino acids (Sigma). Concentration of amino acids in each sample was calculated based on peak areas and calibration curves generated with commercial standards.

RNA extraction, reverse transcription, and quantitative PCR

Total RNA from leaves of lettuce plants was extracted using Trizol reagent according to the manufacturer's instruction (Invitrogen, Carlsbad, CA). RNA (5 μg) was primed with oligo(dT) and reverse-transcribed into cDNA using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). The synthesized cDNA were diluted 10 times in water and their quality was checked based on amplification of lettuce *Actin* gene using primers listed in Table 2. Quantitative RT-PCR (qRT-PCR) was performed using the SYBR Green Universal Master Mix (PE Applied Biosystems) with gene-specific primers as listed in Table 2 in an Applied Biosystems 7900HT fast real-time PCR system. PCR program used was 50 °C for 10 min and 95 °C for 2 min followed by 40 cycles of denaturation for 15 s at 95 °C and annealing/extension at 60 °C for 1 min. The relative transcript levels were calculated as described previously (Lyi et al. 2007). The analysis of all gene expression was run in triplicate with two biological repeats.

Table 2 List of primers used in this study

Gene	Forward primer (5′-3′, top) reverse primer (5′-3′, bottom)	PCR size (bp)	Lettuce Unigene	Arabidopsis Gene	Expect Value[a]
Actin	TGGTAGGTATGGGCCAGAAA	180	TC17693	AT3G18780	8e-81
	GAACAGGGTGCTCTTCAGGA				
Sultr1;1	GACCTTGATGCAGCAATCCT	114	TC17575	AT4G08620	6e-85
	CGCAATGAGGAGACCAATTT				
Sultr2;1	CCATTGATCATGGGTGGAAT	199	TC27557	AT5G10180	3e-59
	GCAGACACCCAAAACAGCTT				
Sultr3;1	TTGCCCTTTCTGCCATTATC	126	TC22856	AT3G51895	6e-94
	TGCTAATGAAGGCAACACCA				
APS1	GATCCCTCCAGACCACAAGA	175	TC26942	AT3G22890	5e-149
	AATGGGTTCAGGGACCCTAC				
APS2	TCCACTTCCCTCTTGTTTCG	187	TC18561	AT1G19920	7e-75
	GCACCCACTCCAAATCAATC				
APR1	CGAAGATGGGCATCAAGAAT	158	TC24166	AT4G04610	6e-139
	CACTTGAACAACCGGAACCT				
APR2	GACCCGGTTTTTGAAGGAAT	142	TC26849	AT1G62180	8e-103
	TCGCACCCTATCGACACATA				
SAT1;1	AACCACTCGCCCTAGCCCTC	183	TC28318	AT5G56760	6e-91
	TAACTGCGGTCTCACCCACA				
SAT2;1	GGTGCAGGGAGTTGTGTTTT	126	TC17842	AT1G55920	6e-86
	TCAAACTTTGCTGGGGTTTC				
SAT3;1	TAACCCTAACTCGCCCACCT	115	TC17368	AT2G17640	6e-68
	AGCCTCACACTTGGCCTCTA				

[a] The amplifications of specific homologous genes in lettuce were verified by sequence PCR products and aligning the sequences with Arabidopsis genes

RESULTS

Lettuce accessions exhibit different growth responses to Se treatment

To examine the effect of Se on lettuce growth, 30 lettuce accessions were grown hydroponically in the Hoagland nutrient solution without or with 15 μM Na_2SeO_4 or Na_2SeO_3 for 2 weeks and their biomasses were measured. Depending on the form of Se application, different responses of the lettuce accessions in terms of shoot growth were observed and they can be divided into three groups ($P < 0.05$, Fig. 2). In the first group of seven accessions, plant growth was not affected by either selenate or selenite treatment at the dosage used. No significant difference in shoot fresh weight was observed between Se-treated and non-treated plants. In the second group of 18 accessions, shoot biomasses were similar between non-treated and selenate-treated plants, whereas plant growth was significantly inhibited when selenite was supplied. An average of 20% decrease in shoot biomass was noted, suggesting that these accessions were more sensitive to selenite. For the third group of five accessions, selenate treatment stimulated plant growth. An average of 13% increase in shoot biomass was observed in these accessions. Exposure of them to selenite caused no effect on plant growth. These results indicate that lettuce exhibits genetic variation for biomass production in response to different forms of Se treatment.

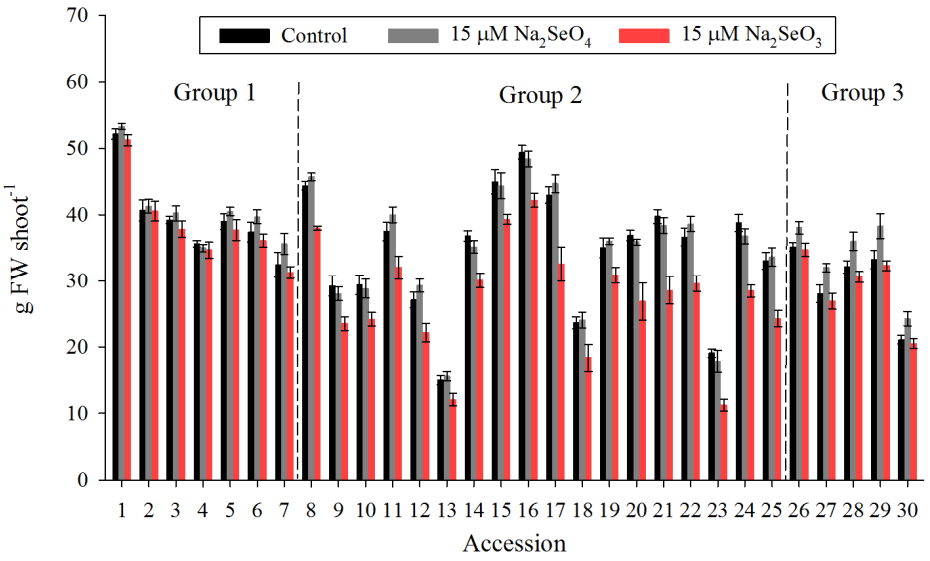

Fig. 2 Shoot biomass of 38-day-old lettuce accessions subjected to different forms of Se treatment for 2 weeks. The lettuce accessions were divided into three groups based on their different growth in response to selenate and selenite treatment. The accession numbers correspond to the ID numbers in Table 1. Error bars indicate standard error of the mean (SEM) (n = 4)

Lettuce accessions show different capacity in accumulating Se and S

To investigate the capacity of different lettuce accessions in accumulating Se and the effect of different forms of Se on Se and S accumulation, we examined the total Se and S levels in each accession following Na_2SeO_4 or Na_2SeO_3 application. Se was not detectable in Se non-treated lettuce. When plants were exposed to selenate and selenite, the lettuce accessions accumulated different levels of Se (Fig. 3a). The total Se levels showed over twofold difference between accessions containing high and low levels of Se. For all lettuce accessions, the total Se accumulation in leaves was higher when plants

were treated with selenate than with selenite. Although there was variation in the levels of Se accumulation treated with selenate and selenite among accessions, on an average, the selenate-treated plants contained 41% more Se than the selenite-treated plants.

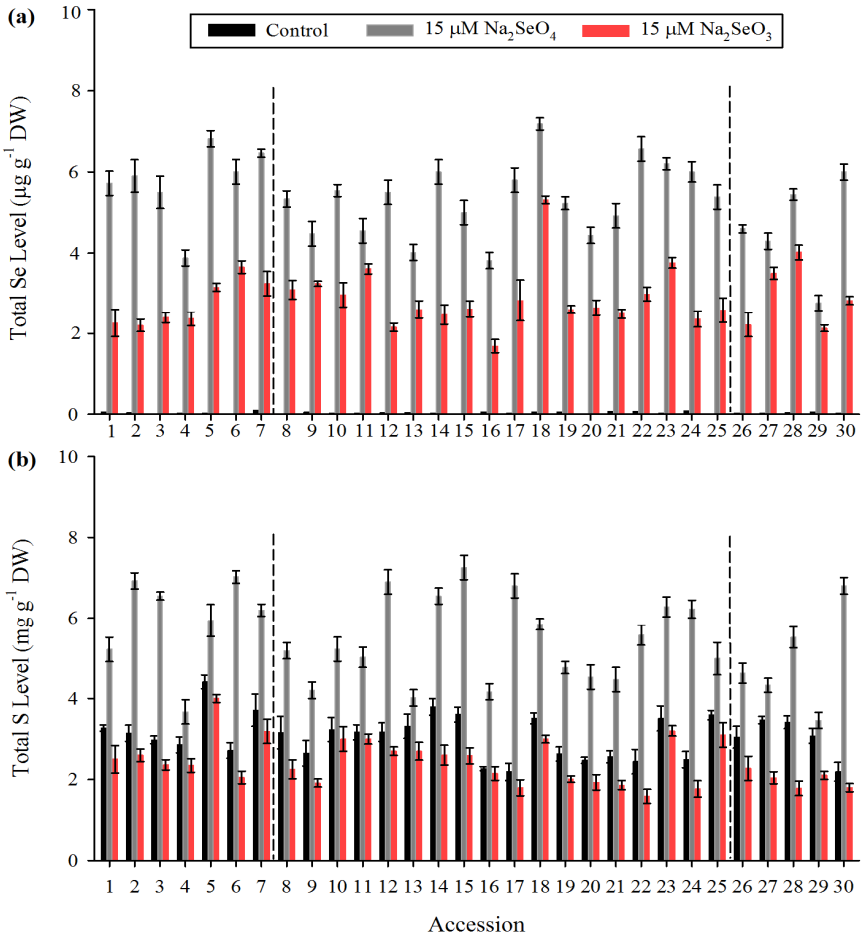

Fig. 3 Total Se (a) and S (b) level in lettuce accessions subjected to different forms of Se treatment. Error bars indicate standard error of the mean (SEM) (n = 4)

The different forms of Se also imposed different effect on S accumulation in the lettuce accessions. At the dosage of Se supplemented, selenate dramatically enhanced total S accumulation in nearly all accessions examined except for one accession (i.e. 29) (Fig. 3b). The extent in stimulating S accumulation varied among the lettuce accessions, and about half of them doubled S levels when exposed to selenate in comparison with controls. These results imply a synergism relationship between selenate and S at the dosage. Depending on the lettuce accessions, selenite had different effects on S accumulation (Fig. 3b). In some accessions, selenite exhibited no effect to total S accumulation, while in the others selenite treatment led to
decreased levels of total S accumulation. An average of 22% reduction in comparison with the controls was observed. The capacity of different lettuce accessions to accumulate Se and S appears not to be correlated with plant growth.

Effect of Se treatment on the activity of antioxidant enzymes

The effect of different forms of Se on the activities of several antioxidant enzymes, i.e. APX, CAT, and GSH-Px, in each lettuce accession was examined. In general, the activities of these enzymes varied depending on cultivars (Fig. 4). The biggest activity difference among the cultivars was observed with the CAT enzyme activity. When lettuce plants were exposed to Na_2SeO_4 or Na_2SeO_3, the activities of APX, CAT and GSH-Px showed no significant difference in response to selenate and selenite treatment in the first group of lettuce accessions (Fig. 4) like the case for plant biomass.

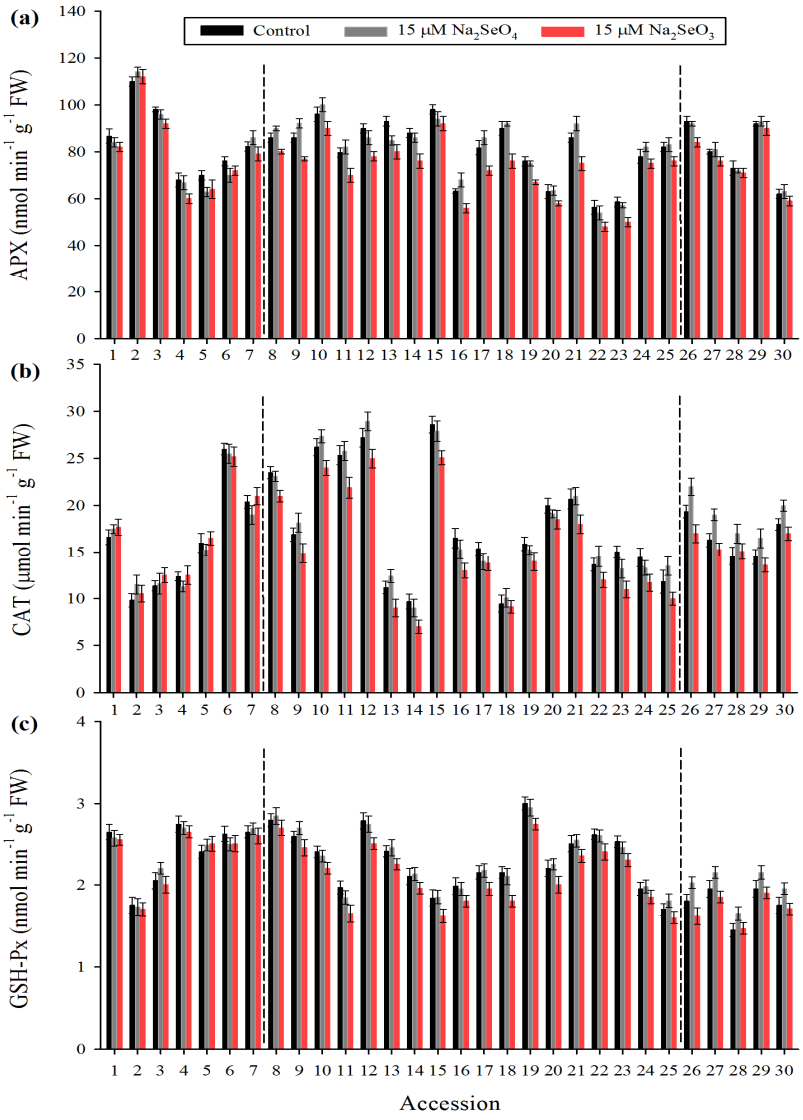

Fig. 4 Activity of ascorbate peroxidase (APX) (a), catalase (CAT) (b), and glutathione peroxidase (GSH-Px) (c) in lettuce accessions subjected to different forms of Se treatment. Error bars indicate standard error of the mean (SEM) (n = 4)

In agreement with reduced plant growth, a general decrease in the APX, CAT and GSH-Px activities was observed in the second group of accessions when selenite was added in the nutrient solution. In the case of APX, the decrease was statistically significant for nearly all accessions in this group. A significant increase in CAT and GSH-Px activities in response to selenate treatment was noted in the third group of accessions, while their activities remained similar to the controls when they were exposed to selenite, showing a similar pattern of response as plant growth. APX activity appeared not to be affected by both selenate and selenite treatment in this group. The higher CAT and GSH-Px activities correlated well with higher shoot biomass when lettuce accessions of this group were treated with selenate. These results indicate that alteration of antioxidant enzyme activities following Se treatment may exert direct effect on plant growth.

Se treatment does not dramatically affect amino acid accumulation

To examine the effect of Se treatment on nutritional quality, the free individual and total amino acid levels in these lettuce accessions were examined. Different accession accumulated different level of total amino acids (Fig. 5). In general, the most predominant amino acids in lettuce included glutamic acid, arginine, aspartic acid, serine, and threonine (Suppl. Fig. S1). Their contents varied greatly among accessions to a level of tenfold difference (Suppl. Fig. S1). The total amino acid contents appear not to be dramatically affected by either selenate or selenite treatment except a few accessions in the second group (Fig. 5). Although it is difficult to detect free cysteine due to its instability, the other S-amino acid methionine is readily detectable (Suppl. Fig. S1). When the plants were exposed to Se, the organic form of Se compound, selenomethionine (SeMet), was found (Suppl. Fig. S1). Moreover, SeMet

accumulation shared similar pattern as total Se levels when selenate was applied. SeMet has been reported as an effective chemopreventive agent (Combs and Gray 1998).

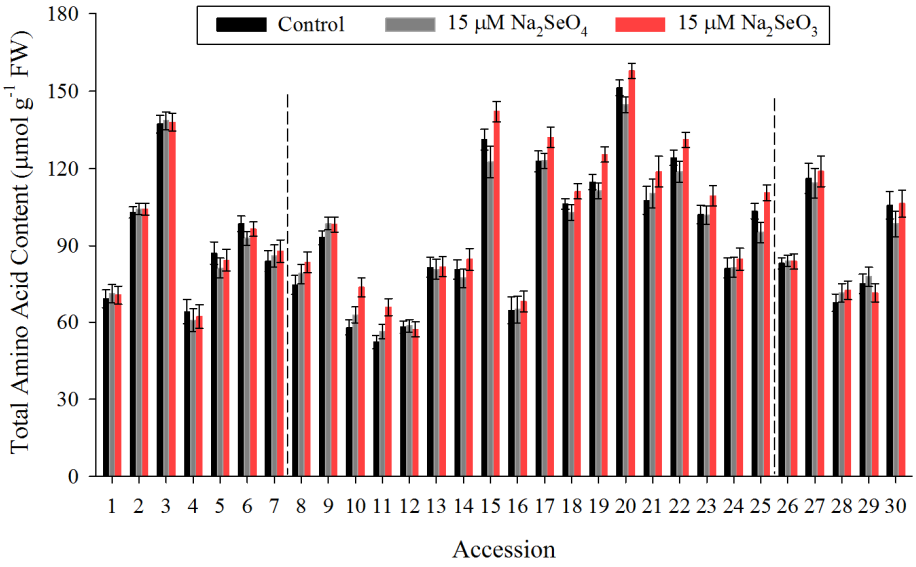

Fig. 5 Total free amino acid content in lettuce accessions subjected to different forms of Se treatment. Total levels of amino acids are the sum of histidine, serine, arginine, glycine, aspartic acid, glutamic acid, threonine, alanine, proline, tyrosine, valine, isoleucine, leucine, and phenylalanine. Error bars indicate standard error of the mean (SEM) (n = 3)

Expression of genes involved in S/Se transport and assimilation

Selenium is known to share the S transport and assimilation pathways. Thus, examination of expression of genes encoding the key steps in the pathways should help us in better understanding of Se metabolism in lettuce. To examine whether Se accumulation was correlated with the expression of genes

involved in S/Se transport and assimilation, we investigated the transcript levels of some crucial genes, i.e., *Sultr*, *APS*, *APR*, and *SAT* in six selected accessions which accumulated either high or low levels of Se following selenate treatment.

Sultr1;1, *Sultr2;1* and *Sultr3;1* are sulfate transporters with *Sultr1;1* representing high-affinity transporter in the plasma membrane, *Sultr2;1* being low affinity transporter; and *Sultr3;1* with unknown function (Takahashi 2010). As shown in Figure 6a-c, these sulfate transporters were found to express at high levels in plants with high Se content and at relatively low levels in plants with low Se content. When the lettuce plants were treated with 15 μM Na_2SeO_4, the transcript levels of *Sultr1;1* were significantly increased, whereas those of *Sultr2;1* and *Sultr3;1* remained at similar level as the non-treated plants. The results suggest an increase uptake by roots.

Following translocation into shoots, selenate is either stored in the vacuole of the cells or is further assimilated. ATP-sulfurylase (APS) is the first enzyme in the sulfate/selenate assimilation pathway and catalyzes the activation of selenate. *APS1* has been predicted to localize to plastids, whereas *APS2* may function in the cytosol (Rotte and Leustek 2000). The transcript levels of *APS* were similar between the accessions examined. However, when exposed to selenate treatment the transcript levels of *APS1* were significantly enhanced in accessions with high level of Se, but remained similar in those with low level of Se (Fig. 6d). No significant difference in the transcript levels of *APS2* were observed between accessions containing high or low levels of Se following Se treatment (Fig. 6e).

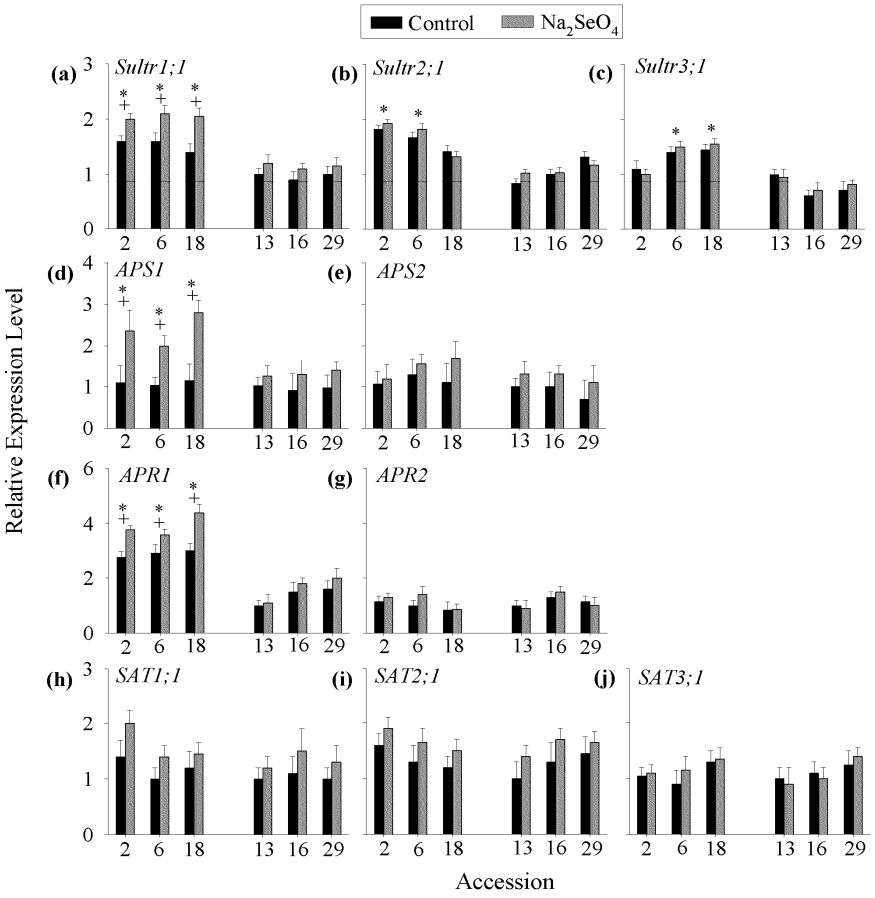

Fig. 6 Relative expression of genes involved in S transport and assimilation in lettuce accessions. Transcript levels of genes in leaves of lettuce cultivars with high and low Se content following 15 μM Na_2SeO_4 treatment were measured by qRT-PCR. The expression of cultivar 13 in nontreated samples was set to 1. Data are means of three technical trials with two biological repeats. Values marked by asterisks and plus indicate significantly different between plants with high and low Se levels and treated and non-treated samples, respectively ($P < 0.05$; $n = 3$)

In plant plastids, APS reductase (APR) catalyzes the reduction of activated selenate to produce sulfite. *APR1* was found to express highly in plants with high Se level and show relatively low expression in plants with low Se level (Fig. 6f). Se treatment significantly enhanced *APR1* expression only in those accessions with high Se levels. *APR2* transcript levels showed no significant difference between Se treated and non-treated samples in accessions with both high and low Se levels (Fig. 6g).

Serine acetyltransferase (*SAT*) catalyzes the formation of O-acetyl-L-serine (*OAS*) from serine and acetyl-CoA, which represents a key metabolic regulatory step in selenoamino acid assimilation (Saito 2004). Examination of the transcript levels of *SAT1;1*, *SAT2;1* and *SAT3;1* in these accessions showed no significant difference of expression between Se treated and non-treated plants (Fig. 6h-j).

DISCUSSION

Lettuce is a widely consumed vegetable in the world, and encompasses broad genetic and morphology diversity. The capacity of lettuce to accumulate Se in the edible part is important in determining the effectiveness of a biofortification program in the crop. Se content varied greatly between the accessions evaluated. We found that there was at least over two fold change in total Se levels between those accessions with low and high levels of Se accumulation. A dozen of accessions showed higher capacity in accumulating more Se than the others.

Selenium accumulation in lettuce was affected by the chemical species of Se. Selenate application was more effective than selenite in inducing Se accumulation in edible part of lettuce plants for all accessions examined. The

result is consistent with other studies of different crops in showing that selenate is superior to selenite in inducing Se accumulation in shoots of plants (Hopper and Parker 1999; Chen et al. 2002; Zhang et al. 2003; Cartes et al. 2005; Sharma et al. 2010). Rios et al. (2008b) and Ramos et al. (2010) also showed that for the same concentration of Se applied, selenate promotes higher accumulation of Se in lettuce plants. The different accessions, however, responded differently to the same concentration of selenate and selenite treatment, resulting in different levels and ratio of Se accumulation. While many plants accumulate mainly selenate from selenate supplement and predominantly organic Se from selenite treatment (Whanger 2002), the speciation of Se in lettuce accessions remains to be determined.

Since Se is an analog of S, antagonistic relationship between selenate and sulfate are generally reported in plants when high dosages of selenate or sulfate are used (Zayed et al. 1998; White et al. 2004; Lyi et al. 2005). The results from the present study showed that selenate in nutrient solution increased total S level in nearly all accessions tested in spite with different extent. These results reflect a synergistic instead of antagonistic relationship between the two elements at the concentration of 15 μM selenate. Our data agree with studies showing that selenate application at low levels could promote S accumulation in plants (White et al. 2004; Lyons et al. 2005; Lefsrud et al. 2006). On the other hand, selenite exhibited no effect on or decreased total S levels in the lettuce accessions we tested. Rios et al. (2008a) reported that the application of selenite even at high dosages does not alter total S level in lettuce shoot. The decreased S levels in some lettuce accessions imply diverse genotypic variation of lettuce in response to selenite.

Selenium application is known to have both positive and negative effects on plant growth and development depending on the forms and dosages used. Our results with respect to shoot biomass reveal significant differences based on the Se forms and accessions grown. In general, the results indicate that selenate is less toxic than selenite to plant growth as shown in other studies (Hopper and Parker 1999; Chen et al. 2002; Cartes et al. 2005; Pedrero et al. 2006). A previous study in lettuce reports that the levels of Se accumulation increase with increased amount of Se application and lettuce can tolerance up to 40 μM selenate and 5 μM selenite without negative effects on plant growth and metabolism (Rios et al. 2008b). In agreement with this study, we found that at the Se dosage used the shoot biomasses of the majority accessions showed no visible detrimental change when exposed to selenate. In contrast, the growth of many accessions was reduced following treatment with selenite. The different effect of selenate and selenite on plant growth could be caused by distinct mechanisms of metabolism of different Se forms (Sors et al. 2005). Selenate is translocated to the shoots mainly in the inorganic form, whereas selenite is rapidly converted into selenoamino acids in roots (de Souza et al. 1998; Zayed et al. 1998) and translocated to the shoots in organic forms that are readily incorporated into proteins in replacement of S to cause toxicity to the plant (Hopper and Parker 1999). The group of accessions whose growth was stimulated by selenate exhibited no inhibition of growth when exposed to selenite, indicating a general higher tolerance to Se. While selenite at concentration of over 10 μM is noted to inhibit lettuce growth (Rios et al. 2008b), it is clear from our results that some lettuce accessions can tolerance high selenite levels.

Selenium treatment has been shown to affect antioxidant enzyme activities, which in turn influences plant growth (Hartikainen et al. 2000; Djanaguiraman et al. 2005; Rios et al. 2008b). Our experiments showed that the growth stimulated effect of selenate in the third group of lettuce accessions was correlated with enhanced activities of CAT and GSH-Px, while the growth-inhibited effect of selenite in the second group of accessions was associated with the general reduction of enzymatic activities of APX, CAT and GSH-Px. The data are consistent with other studies showing that a decline in shoot biomass is associated with a decrease in these enzyme activities and the growth-stimulated effect of Se is related to its antioxidant function (Xue et al. 2001; Rios et al. 2009).

Amino acids have many functions in metabolism and therefore are important nutrients. Although the lettuce accessions exhibited a large variation in total free amino acid content, growing lettuce accessions in nutrient solution containing 15 μM selenate or selenite did not significantly modify the total amino acid levels in nearly all cultivars as in other studies (Frias et al. 2010) except a few showing increased total amino acid contents when treated with selenite. In these few cultivars, the increased amino acid accumulation was associated with significantly inhibited growth. A similar result was reported in a previous study (Rios et al. 2008a). The growth inhibition effect by selenite is suggested to be due to its promotion of the most selenocysteine formed to be used for the formation of non-functional proteins, which gives greater phytotoxicity and therefore lower plant growth (Rios et al. 2008a).

The genotypic variation of lettuce cultivars led to Se accumulation at different levels when exposed to Se. The different ability to accumulate Se in response to selenate treatment was found to be associated with altered

expression of *Sultr1;1*, *APS1* and *APR1*. The higher expression of these genes in the high Se cultivars implies a greater uptake for both selenate and sulfate in roots (Takahashi 2010; Shinmachi et al. 2010) and an increased assimilation in plastids (Rotte and Leustek 2000; Sors et al. 2005).

In summary, by examination of diverse lettuce accessions, we found genotypic variation of lettuce germplasm upon selenate and selenite treatment in terms of Se accumulation as well as plant growth, total antioxidant enzyme activities, and amino acid contents. Our research will be helpful in selecting and developing better cultivars with relatively higher ability to accumulate Se without negative effects on plant growth and other nutritional characteristics. In agreement with a previous report (Rios et al. 2008b), selenate would be the most suitable for lettuce biofortification. Lettuce cultivars in groups 1 and 3 should have higher capacity in tolerance to Se and increased antioxidant levels.

Acknowledgement

We thank Dr. Xiangjun Zhou for help with gene expression analysis, Mr. Laurence Heller for help with amino acid analysis. S.J.R thanks Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for granting the doctorate scholarships (regular and sandwich program).

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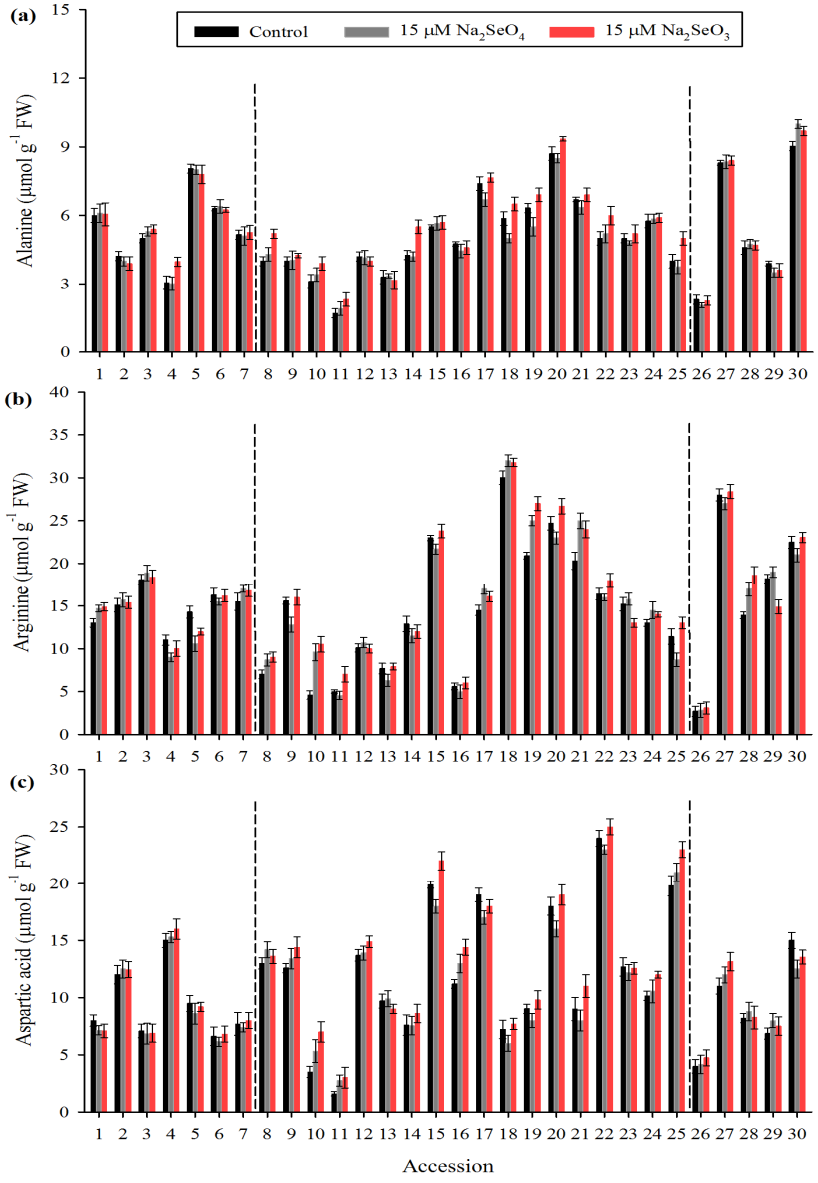
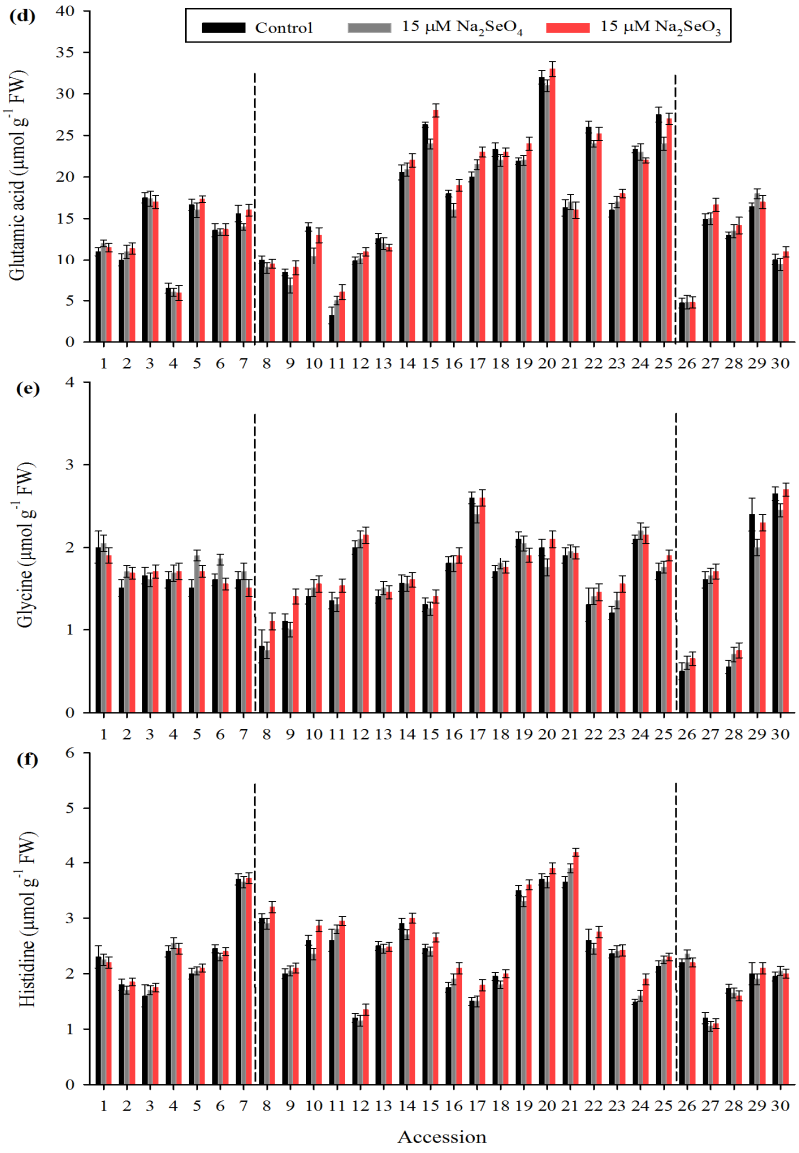
Supplementary Figure S1

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15 µM Na_2SeO_3
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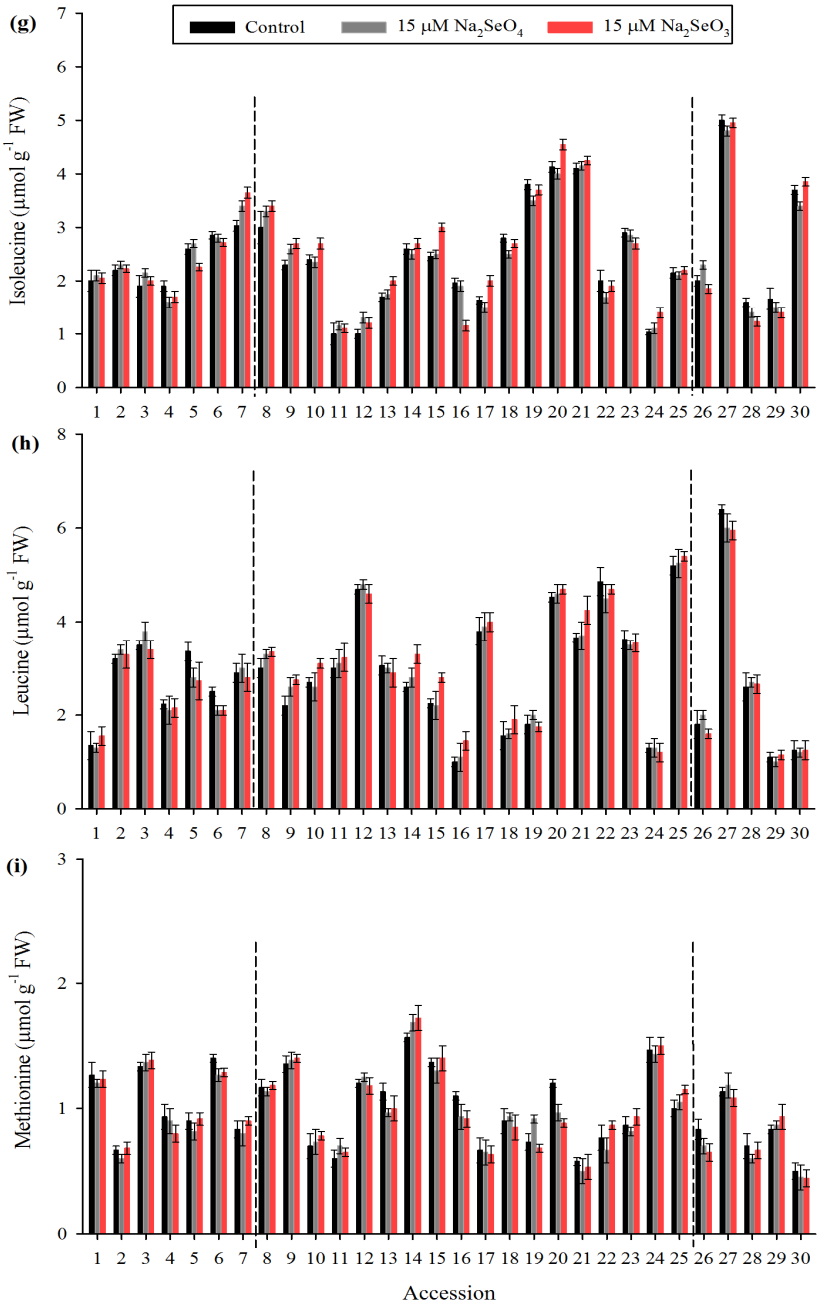
(e)
Glycine (µmol g^{-1} FW)
(f)
Histidine (µmol g^{-1} FW)
Accession


(g)
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15 µM Na_2SeO_3
Isoleucine (µmol g^{-1} FW)
(h)
Leucine (µmol g^{-1} FW)
(i)
Methionine (µmol g^{-1} FW)
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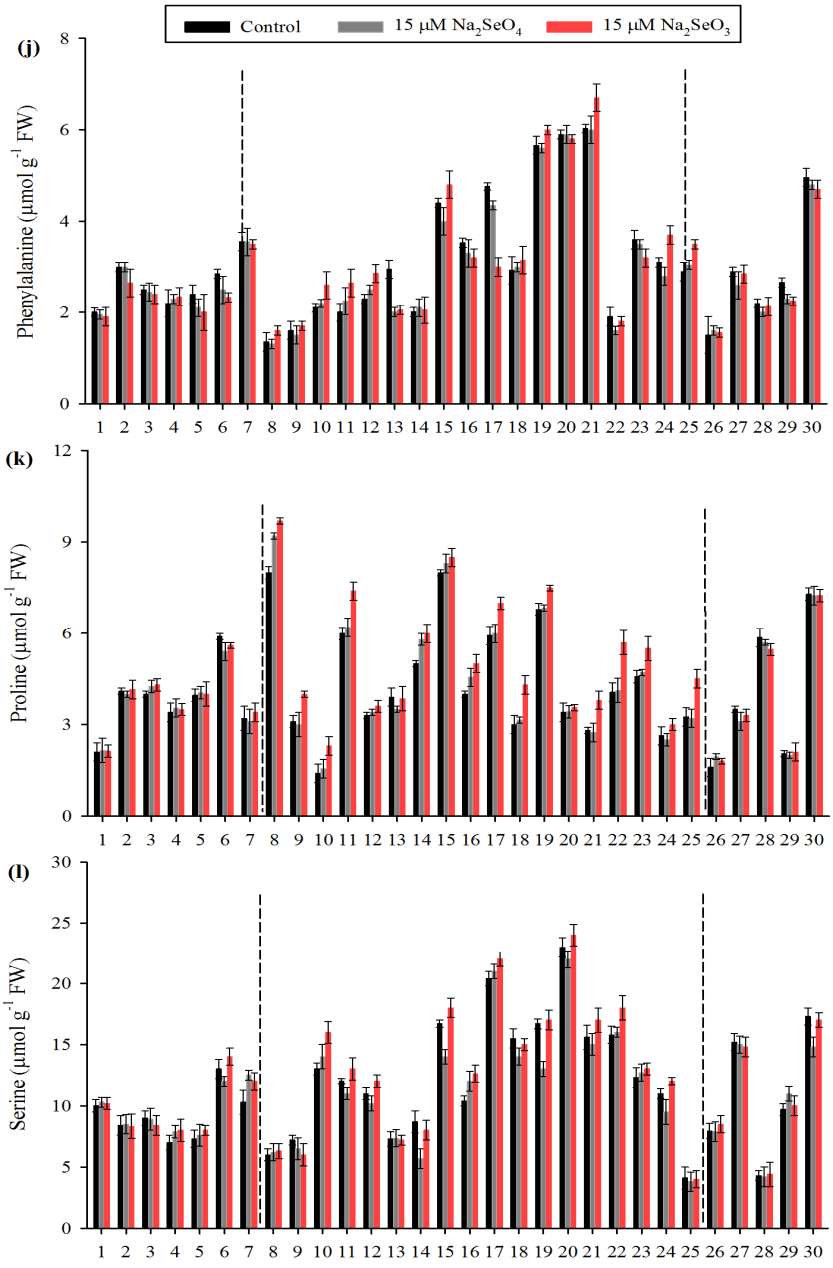
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(j)
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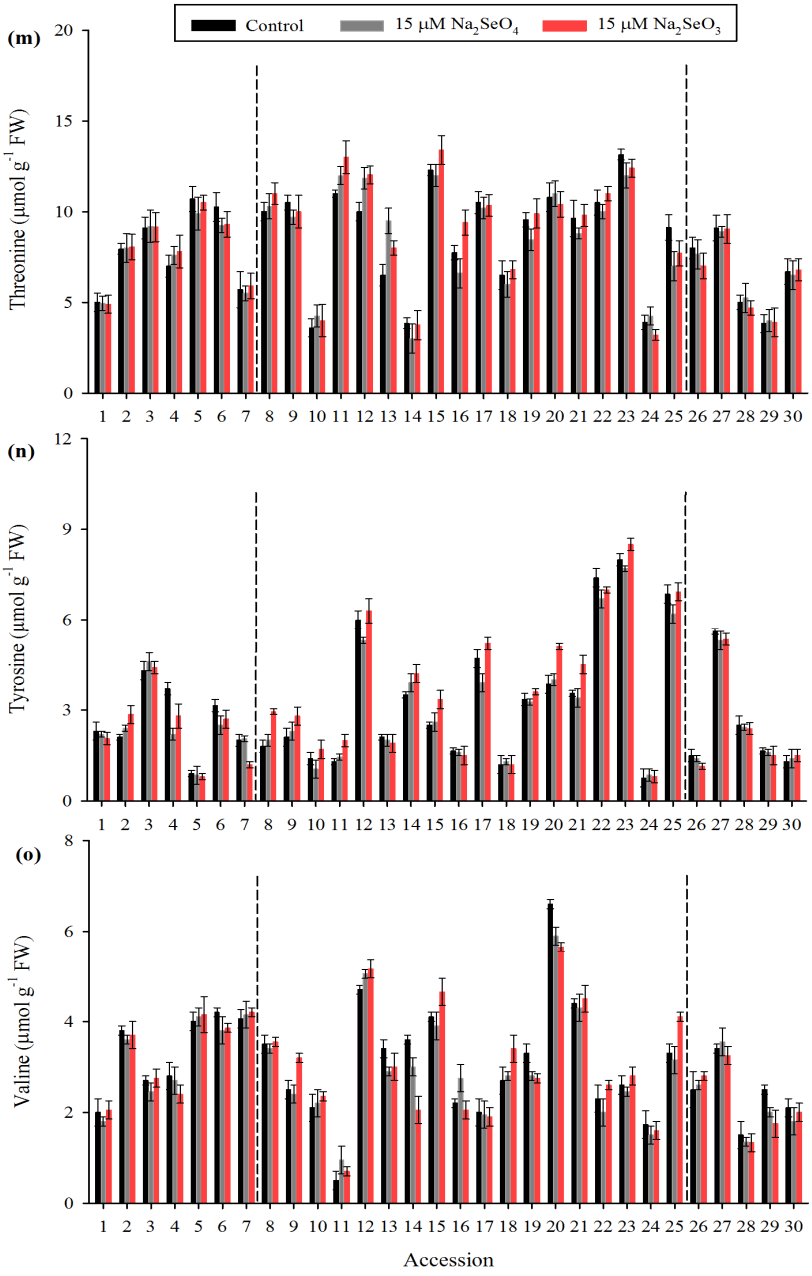
(k)
Proline (µmol g^{-1} FW)
(l)
Serine (µmol g^{-1} FW)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15 µM Na2SeO3
(m)
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(n)
Tyrosine (µmol g-1 FW)
(o)
Valine (µmol g-1 FW)
Accession

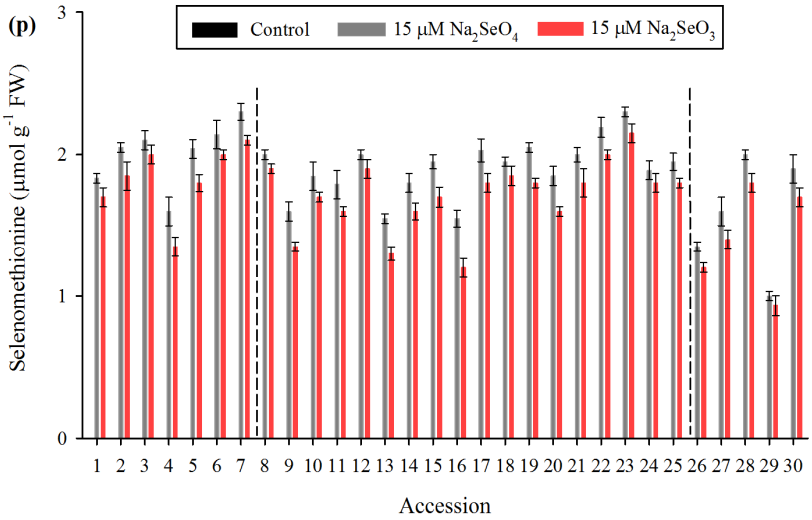

Figure S1. Contents of alanine (a), arginine (b), aspartic acid (c), glutamic acid (d), glycine (e), histidine (f), isoleucine (g), leucine (h), methionine (i), phenylalanine (j), proline (k), serine (l), threonine (m), tyrosine (n) valine (o) and selenomethionine (p) in lettuce cultivars subjected to different forms of Se treatment. Error bars indicate standard error of the mean (SEM) (n = 3)

ARTIGO 3

Evaluation of genotypic variation of broccoli (*Brassica oleracea* var. *italic*) in response to selenium treatment

(Artigo apresentado na íntegra, conforme publicado conforme as normas do *Journal of Agriculture and Food Chemistry*, mar., 2011, DOI: jf-2010-04731f. Disponível em: http://pubs.acs.org/doi/abs/10.1021/jf104731f)

Evaluation of Genotypic Variation of Broccoli (*Brassica oleracea* var. *italic*) in Response to Selenium Treatment

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Abstract:

Broccoli (*Brassica oleracea* var. *italic*) fortified with selenium (Se) has been promoted as a functional food. Here we evaluated 38 broccoli accessions for their capacity to accumulate Se and for their responses to selenate treatment in terms of nutritional qualities and sulfur gene expresion. We found that total Se

content varied with over 2-fold difference among the leaf tissues of broccoli accessions when the plants were treated with 20 μM Na_2SeO_4. Approximately half of total Se accumulated in leaves was *Se*-methylselenocysteine and selenomethione. Transcriptional regulation of adenosine 5′-phosphosulfate sulfurylase and selenocysteine *Se*-methyltransferase gene expression might contribute to the different levels of Se accumulation in broccoli. Total glucosinolate contents were not affected by the concentration of selenate application for the majority of broccoli accessions. Essential micronutrients (*i.e.* Fe, Zn, Cu, and Mn) remained unchanged among half of the germplasm. Moreover, total antioxidant capacity was greatly stimulated by selenate in over half of the accessions. The diverse genotypic variation in Se, glucosinolate, and antioxidant contents among accessions provides the opportunity to breed broccoli cultivars that simultaneously accumulate Se and other health benefit compounds.

Keywords: Selenium; glucosinolate; broccoli; *Brassica oleracea* var. *italic*; germplasm; antioxidant; gene expression

INTRODUCTION

Selenium (Se) is an essential micronutrient for animals and humans, and has been implicated to have important health benefits. In addition to its roles in improving immune function, in reducing viral infection, and in slowing down the aging process (*1*), Se at supranutritional levels has been shown as a cancer preventative agent in reducing the incidence of prostate, colon, and lung cancer (*2*). Although

the SELECT trial (the Selenium and Vitamin E Cancer Preventive Trial) using selenomethionine (SeMet) shows that this particular form of Se does not prevent prostate cancer in relatively healthy men (*3*), other studies have demonstrated that some methylated selenoamino acids can offer chemoprotectative effects against cancer with low body accumulation to avoid Se toxicity (*4-6*). Because of large areas of soil in many parts of the world that contains low levels of Se and consequently low levels of Se supply in food, Se deficiency is a widespread problem. Thus, increasing Se intake through foods, especially cultivated Se-accumulating crops, such as Brassica vegetables, is the most effective and safe way in providing functional forms of Se and in reducing Se deficiency in the world (*4, 7*).

Glucosinolates (GLS) are sulfur (S)-containing secondary metabolites that are present primarily in the Brassicaceae family (*8*). A substantial amount of evidence has linked ingestion of a GLS rich diet with reduced risk of cancer (*9*). The cancer preventive effect of GLS is due to the breakdown products of GLS, which act as inducers of phase II detoxification enzymes in protecting against cancer (*9, 10*).

Broccoli (*Brassica oleracea* L. var. *italica*) contains multiple nutrients including vitamins and minerals, as well as many health beneficial secondary metabolites and antioxidants (*11*). The consumption of broccoli has been steadily increasing for the last decade, due in part to its health promoting properties (*12*). Broccoli can accumulate high levels of functional forms of Se (*13*), and is also a rich dietary source of GLS as

well as their bioactive degradation metabolites (*11*). Thus, broccoli has been promoted to serve as a functional food against cancer (*4, 14*). Given that Se is an analog of S and shares S uptake and assimilation pathways (*15, 16*), antagonistic relationship between Se and S metabolisms are generally reported when plants are exposed to high levels of selenate or sulfate (*13, 17*). Thus, it is not surprising to find that high levels of Se fertilization results in low levels of GLS accumulation (*18, 19*). However, a number of studies have shown a synergistic instead of antagonistic relationship between Se and S metabolism under certain conditions in plants (*20, 21*). Indeed, Hsu et al. (*22*) has recently shown that it is possible to produce Se fortified broccoli that simultaneously accumulates with high levels of Se and GLS.

Only few studies have investigated the genetic variation of crop species in accumulation of Se (*20, 23*). A large variation in GLS content in Brassica vegetables has been shown (*24, 25*) and genetic variation was reported to be the most important factor in determining GLS content (*14*). However, genotypic variation of broccoli accessions in accumulating Se and its effect on GLS and other nutrient contents have not been studied. Thus, the aim of this study was to evaluate the genotypic variation of broccoli germplasm in response to selenate treatment. The effects of selenate treatment on total Se accumulation and GLS synthesis, as well as on plant growth, total S level, Se containing amino acid contents, macro- and micronutrient concentrations, antioxidant levels, and the expression of genes involved in Se/S uptake and assimilation were examined.

Exploiting genotypic variation is likely to be effective in providing information for breeding varieties that simultaneously accumulate high levels of Se and GLS, and contribute nutritional improvements in broccoli.

MATERIALS AND METHODS

Plant Materials and Experimental Design. Thirty eight available accessions of broccoli were obtained from the Plant Genetic Resources Unit at Geneva, NY. Seeds of each accession (**Table 1**) were planted and grown in a greenhouse as described previously (*20*). Uniform 3-week-old young seedlings were selected and transplanted into 2.2 L pots containing Hoagland nutrient solution with 40% ionic strength (*26*). The seedlings from each accession were divided into two treatment groups and each with four replicates. One week after transplantation, one group of these plants were exposed to 20 μM Na_2SeO_4 (Sigma-Aldrich), and the other as control sets that received no Se treatment. The nutrient solutions were changed twice each week. After 2 weeks of Se exposure, a total of 304 plants (38 x 2 x 4; accessions x treatments x repeats) plants were harvested individually and the fresh weights of aerial part were weighed. The young leaf sample from each plant was either dried for elemental analysis or immediately frozen in liquid nitrogen and stored at -80°C for RNA extraction and metabolite analyses. As the broccoli accessions develop florets at different time, we chose to do the comparative analyses on leaf samples that had the same developmental stage.

Table 1. Broccoli accessions used in this study

ID	Name	GRIN[1] accession number
1	Green Sprouting Early CT Strain	G 21111
2	Purple Sprouting Late	G 28836
3	Purple Sprouting Late Improved	G 28837
4	White Sprouting Improved	G 28840
5	Cavolo Ramoso Calabrese Precoce	G 28848
6	Broccoli Neri	G 28852
7	Broccolo Natale Pied Grande Liscio	G 28853
8	Cavolo Broccolo Di Sarno	G 28855
9	Cavolo Broccolo Marzullo	G 28873
10	Cavolo Broccolo Natalino	G 28880
11	Broc 3	G 30009
12	-	G 30014
13	Packman F1	G 30778
14	Cavolo Broccolo Precoce	G 30928
15	Atlantic	G 30929
16	Cavolo Broc Verde Calabrese Precoce	G 30933
17	Cavolo Broccolo Di Sarno	G 30934
18	Purple Sprouting Early	G 30937
19	Persius F1	G 32206
20	Wintergarden F1	G 32209
21	Purple Sprouting Xmas	G 28832
22	Purple Sprouting Early	G 28833
23	Late Purple Sprouting	G 28835
24	White Sprouting Early	G 28838
25	Cavolo Broccolo Natalino Di Sarno	G 28854
26	Cavolo Broccolo Bronzino Di Albenga	G 28859
27	Cavolo Broccolo Verde Calbrese	G 28865
28	Cavolo Broccolo Frevarota	G 28872
29	Cavolo Cavolina Rizza	G 28878
30	Pinnacle F1	G 30414
31	Premium Crop F1	G 30415
32	Zeus F1	G 30416
33	Xmas Purple Sprouting	G 30775
34	Broccoli Grande Precoce	G 30931
35	Cavolo Broccolo Ramoso Calabrese	G 30932
36	Big Sur F1	G 32208
37	De Cicco	G 32213
38	Romano	PI 231210

[1]GRIN = Genetic Resources Information Network (http://www.ars-grin.gov/npgs/searchgrin.html)

To explore whether the data obtained from leaf samples would be applicable to florets, some accessions were allowed to grow until florets and floret samples were analyzed for the correlation of accumulation of metabolites between leaves and florets of broccoli.

Elemental Analysis by an Inductively Coupled Plasma (ICP) Trace Analyzer. Dried tissues (approximately 200 mg) were weighed and acid-digested in 2.0 mL HNO_3 with 2.0 mL $HClO_4$ at 120 °C for 1 h in capped tubers and then at 220°C until $HClO_4$ fumes were observed. Total Se and other element contents were determined by ICP trace analyzer emission spectrometer (model ICAP 61E trace analyzer, Thermo Electron, San Jose, CA) as described previously (*13*).

Quantification of Se-methylselenocysteine (SeMSCys) and SeMet. Extraction, AccQTag derivatives, and analysis of SeMSCys and SeMet were performed according to the method described previously (*20*) with slight modifications. Leaf tissue materials (100 mg) were extracted overnight at 4°C in 50 mM HCl (10:1, v/w) and centrifuged at 12,000*g* to remove cell debris. AccQTag derivatized SeMSCys and SeMet were separated on an AccQ.Tag Ultra column (100 x 2.1 mm) using an Acquity UPLC system (Waters). SeMSCys and SeMet levels were calculated based on peak areas and calibration curves generated from commercial standards (Sigma-Aldrich).

Analysis of Total GLS Level. Extraction and analysis of GLS were performed as described (*27*) with some modifications. The frozen samples

(200 mg) were crushed into powder at a speed of 4.0 m s^{-1} in a FastPrep FP120 instrument (Q-BIOgene) and extracted in 1.5 mL of 80% MeOH preheated to 75-85°C. After incubation at 80°C for 15 min, the extracts were centrifuged at 12,000*g* for 3 min and the supernatants were added to the DEAE Sephadex A-25 columns. Sulfatase (15 U, Sigma) was added to each column and left at room temperature in the dark overnight. Desulphoglucosinolates were eluted with 80% MeOH and water, Speedvac-dried, and resuspended in 300 µL water. The sample (5 µL) was analyzed using an Acquity UPLC system on a HSS T3 column (1.8 µm, 100 x 2.1 mm) (Waters) and eluted by a mobile phase consisting of solvent A (water) and solvent B (100% acetonitrile) with a linear gradient from 0% to 90% of solvent B at a flow rate of 0.65 mL min^{-1} for a total 4 min. Quantification of the total glucosinolate contents in samples was achieved by comparison of the total peak areas with a calibration curve constructed from commercial sinigrin standard (Sigma-Aldrich).

Ferric Reducing Antioxidant Power (FRAP) Assay. Extraction and analysis of total antioxidant activity were performed as described (*28*) with some modifications. The frozen samples (100 mg) were crushed and extracted in 1.0 mL of Milli Q water, followed by centrifugation at 12,000*g* for 10 min. An aliquot of 5 µL supernatant was added to 2 mL of FRAP reagent and incubated at 37˚C for 30 min. The absorbance of the reaction mixture at 593 nm was measured before and after 30 min of incubation. Quantification of the total antioxidant activity was expressed

as mmol Fe^{+2} g^{-1} of fresh weight. All the samples were measured in triplicate.

RNA Extraction, Reverse Transcription, and Quantitative PCR Analysis. Total RNA from leaves of broccoli plants was extracted and reverse-transcribed into cDNA according to the procedure described previously (*20*). Quantitative RT-PCR (qRT-PCR) was performed using the SYBR Green Universal Master Mix (PE Applied Biosystems) with gene-specific primers as listed in **Table 2**. PCR program used was 50°C for 10 min and 95°C for 2 min, followed by 40 cycles of denaturation for 15 s at 95°C and annealing/extension at 60°C for 1 min. Analysis of all gene expression was run in triplicate with two biological repeats.

Statistical Analysis. All results were analyzed using analysis of variance (ANOVA) and significantly different means between treatments were separated with the Student's t-test at the 0.05 significance level of probability. All results were expressed as means with corresponding standard errors.

Table 2 List of primers used in this study

Genes	Forward primer (5′-3′, top) reverse primer (5′-3′, bottom)	PCR Size (bp)	GenBank Accession
BoActin	CTGTGACAATGGTACCGGAATG ACAGCCCTGGGAGCATCA	62	AF044573
BoSultr1;1	AAGCAGTTCATGCTCGGTCT AGCGAGCTTAGCGTATCCAA	150	AJ311388
BoSultr1;2	GATTCTGCTGCAAGTGACGA ACGCGAATGATCAAGATTCC	126	AJ416460
BoSultr2;1	GTTTCGCTTCTGCTTTCGTC AGCCATGAACCCAACAAGAG	186	DQ091257
BoAPS1	AGACGACGAGCAAAAGGCTA GGTTGTACCCCATGTTCTGG	145	FN641890
BoAPS2	CGTTGACACTCCCATCACTG TTGATCGGAGGAAGAGGATG	199	FN641891
BoAPS3	TGAAACAGCACGAGAAGGTG ACGTTTCTCCACAGGGTGAC	197	FJ626851
BoAPR1	TGAGGAGCTAGCGAAGAAGC CGTCTTCGGCTCCACTAAAG	110	FN641892
BoAPR2	TCTTTGGTTACCCGTGCTTC GGAGAAGCCTCTTCCAGCTT	107	TC111450
BoAPR3	TTCCCTTCCTCAGAGCTCAA TCCTTTGCAACTGACTGCAC	149	TC135211
BoSAT1;1	ATATCCATCCAGCAGCGAAG CTGTCTCCGCAAGCTTTACC	148	TC130359
BoSAT2;1	AAGAGACCCAGCTTGCGTTA GCAAAGCGAGGATCTTTCTG	122	TC117378
BoSAT3;1	TCATGGAACTGGAGTGGTCA CTTCGCCTATTTTGGGATGA	126	TC155320
BoSMT	AGATTCTGAAGAAGCGGCCTA CCACCCACTCCTTCCGTTCAG	178	AY817737
BoHMT1	TTCAGGAATGCCTTGAAACC TTAGCTTTTCCGTCCCACAC	169	DQ679980

RESULTS AND DISCUSSION

Broccoli Growth. In our previous study, we have shown that broccoli plants accumulate approximately 10-fold higher levels of total and

organic forms of Se in shoot when treated with selenate than selenite (*13*). At the concentration of 20 μM Na_2SeO_4, broccoli accumulates substantial levels of Se without negative effects on plant growth (*13*). Thus, this concentration of selenate was chosen to evaluate genotypic variation of broccoli accessions in response to selenate treatment. As shown in **Figure 1,** broccoli accessions exhibited different shoot fresh weights. No significant difference in growth was observed between selenate-treated and non-treated plants for all accessions (**Figure 1**), indicating a capacity of complete tolerance at the Se dosage used.

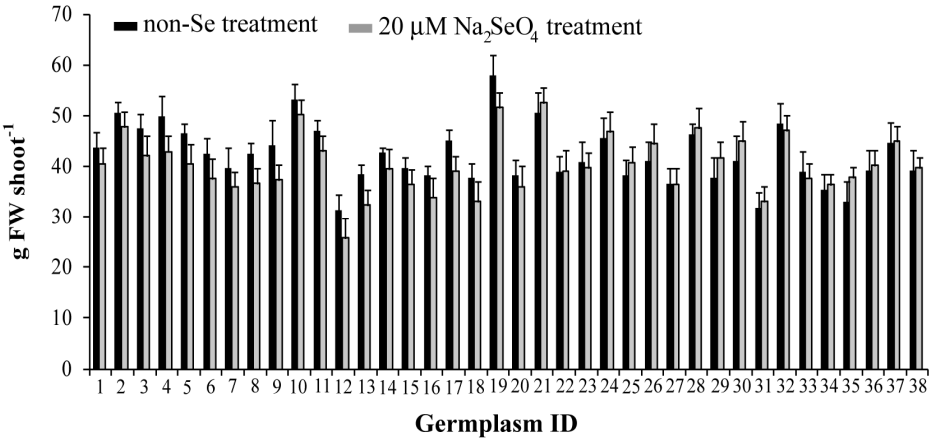

Figure 1. Shoot biomass of 6-week-old broccoli accessions without and with 20 μM Na_2SeO_4 treatment for 2 weeks. The germplasm numbers correspond to the ID numbers in Table 1. Error bars indicate standard error of the mean (n=4).

Total Se and S Contents. Broccoli as well as some other Brassica vegetables belongs to Se secondary accumulator, which can accumulate substantial levels of Se when grown on media containing low to moderate levels of Se. When broccoli accessions grew in control nutrient solution without selenate supplement, Se was undetectable. When plants were treated with 20 μM Na_2SeO_4, different levels of Se were accumulated in the broccoli germplasm (**Figure 2A**). The range of Se accumulation varied from 801.2 to 1798.4 μg g^{-1} dry weight, showing an over 2-fold difference in total Se levels between broccoli accessions. While wheat (*Triticum* spp.) germplasm exhibits no significant difference in accumulating Se (*23, 29*), lettuce (*Lactuca sativa*) accessions show over 2-fold difference (*20*). In comparison with those Se non-accumulating crops, broccoli accumulated hundreds fold more Se, which makes it an excellent supplemental food source in areas of low Se intake. In our previous study, we examined Se accumulation in leaves and florets following different concentration of Se treatment. We have shown that the levels of total Se accumulation in leaves correlate well with those in florets of broccoli (*13*). Thus, variation of Se levels in leaves should be a good indication of levels in florets of broccoli accessions, and a 2-fold change of Se levels in broccoli should exert different impact in providing dietary Se.

Since Se and S share the same uptake and assimilation pathways in plants (*16*), the S levels were also examined in these broccoli accessions following selenate treatment. The total S content varied among

accessions, which appears to have no correlation with plant growth. In all accessions, S levels were not decreased when plants were exposed to 20 μM Na_2SeO_4, indicating no antagonistic relationship between Se and S accumulation at the dosage used. Among the accessions, 7 lines (i.e. 2, 6, 11, 20, 28, 30, and 35) instead showed enhanced S levels with an average of 19.6% increase when Se was applied in nutrient solution (**Figure 2B**). This result implies a synergism relationship between Se and S metabolism at the dosage in these accessions, suggesting a different capacity of them in uptaking and metabolizing S in the presence of Se. The data also agree with other studies showing that selenate application at suitable levels could promote S accumulation in some plants (*17, 20, 22*).

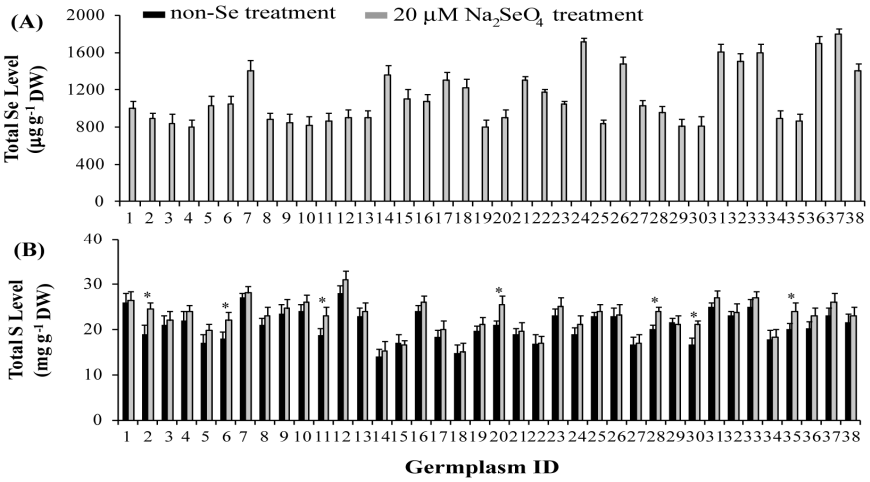

Figure 2. Effect of selenate treatment on total Se (A) and S (B) accumulation in broccoli accessions. Values marked by asterisks indicate significant difference between Se nontreated and treated samples ($p<0.05$) ($n=4$).

Accumulation of Organic Forms of Se. Se-methylselenocysteine has been reported to be an effective form of Se in serving as chemopreventive agent (*5, 30, 31*). Among various crop species, broccoli accumulates high level of Se with SeMSCys as well as SeMet that account for the majority of organic forms of Se (*13, 31*). To examine the accumulation of organic Se species in these broccoli accessions, we extracted and analyzed SeMSCys and SeMet contents. While SeMSCys and SeMet were absent in plants without Se treatment, they are readily detectable when grown in Se-containing nutrient solution. The levels of SeMSCys and SeMet varied among accessions (**Figure 3**). A general correlation of SeMSCys with total Se accumulation was observed. Analysis of the amounts of organic Se species accumulated in these broccoli accessions revealed that approximately 43% of the total Se accumulation was SeMSCys and SeMet, which represented an average of 26% and 17%, respectively, of the total Se levels following 20 μM Na_2SeO_4 supplement for 2 weeks. Our previous study has shown that the SeMSCys level is highly correlated between leaves and florets in broccoli and its accumulation increases with increased levels of selenate supply (*13*). We also observed that SeMSCys content increased with increasing length of selenate exposure (unpublished data). Thus, high percentage of conversion into organic Se species may be obtained by extending the period of Se treatment. Higher percentages of SeMSCys and SeMet accumulation have been also reported in a few S-accumulating crop species, such as radish (*Raphanus*

sativus), garlic (*Allium sativum)*, onions (*Allium cepa)*, and leeks (*Allium ampeloprasum* var. *porrum)* (*31, 32*).

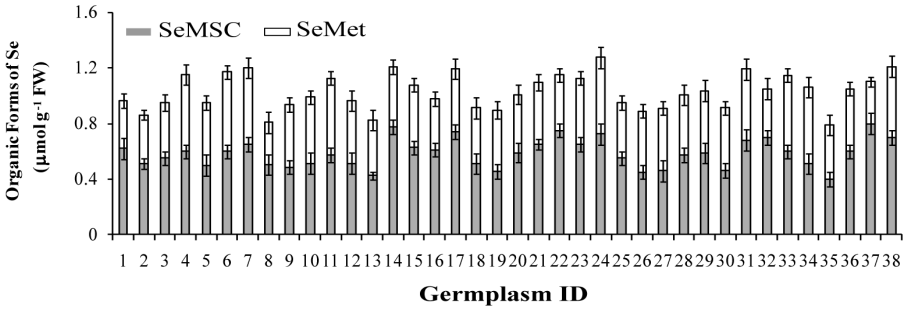

Figure 3. SeMSCys and SeMet accumulation in broccoli accessions treated with 20 µM Na_2SeO_4 for 2 weeks. Error bars indicate standard error of the mean(n=4).

Other Essential Nutrient Accumulation. It is well established that Se affects S uptake and assimilation. To investigate whether exposure of broccoli accessions to selenate influenced nutrient balance and whether there was genotypic variation in their responses, we evaluated the levels of macronutrients (Ca, Mg and P) and micronutrients (Fe, Zn, Cu, and Mn) in plants without and with selenate treatment. No significant difference in these macronutrient contents were observed between Se-treated and non-treated plants at the dosage applied (data not shown). In contrast, micronutrient accumulation in leaf samples of broccoli accessions responded differently to selenate treatment. Approximately half of accessions remained unchanged and the other half showed decreased levels of micronutrients when exposed to 20 µM Na_2SeO_4 ($P < 0.05$; **Figure 4**).

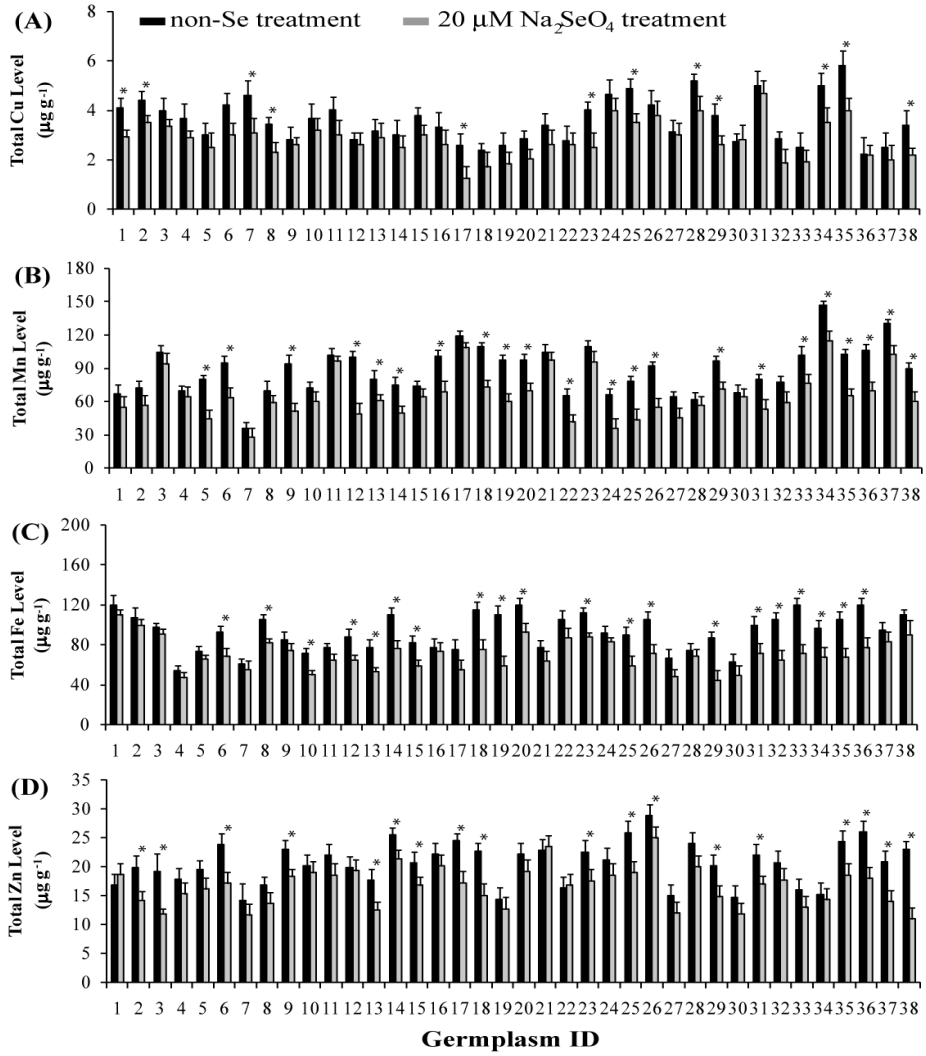

Figure 4. Contents of micronutrient Fe, Zn, Cu, and Mn in broccoli accessions treated with and without 20 μM Na_2SeO_4. Values marked by asterisks indicate significant difference between Se nontreated and treated samples ($p<0.05$) (n=4).

The decreased levels of micronutrient accumulation upon Se treatment have been reported for Mn, Fe and Zn in kale (*Brassica oleracea* Acephala Group) (*33*) and for Fe and Mn in leaf tissue of rapid-cycling *Brassica oleracea* (*34*). The uptake of Mn, Cu, Fe and Zn is reported to be inhibited by increasing level of Se treatment in *Sinapis alba* seedlings (*35*). It is clear that by examining genotypic variation of broccoli accessions, different capacity in uptake and accumulation of these essential micronutrients was found among germplasm when treated with selenate.

Glucosinolate Levels. Broccoli along with other Brassica vegetables contains specific phytochemicals of GLS, which have been proposed to confer protection against certain degenerative diseases such as cancer (*9, 36*). Glucosinolates are S-containing metabolites, thus, their levels of accumulation in plants are influenced by Se status. High concentration of Se can suppress GLS accumulation in plants (*18, 19*). For simultaneous enrichment of GLS and Se level, we evaluated GLS levels in broccoli accessions and assessed genotypic variation of GLS levels in response to selenate treatment. As shown in **Figure 5,** an approximately 3-fold variation in GLS levels was observed between leaf samples of broccoli accessions. Similarly, much wide variation in GLS content in broccoli florets has been shown (*25*). While 13 accessions had reduced levels of total GLS content when plants were exposed to 20 μM Na_2SeO_4, the majority of broccoli accessions contained similar levels of total GLS

following Se treatment. A recent study shows that selenate application even at 40 μM does not affect total GLS level in a broccoli variety (*22*). We examined total GLS in florets of 8 accessions that had similar stage of floret development. As the case for Se accumulation, a high degree of correlation (Pearson R=0.88, $p<0.05$) in total GLS contents between leaves and florets was observed (**Supporting Information Figure S1**). Clearly, genotypic variation in GLS contents among broccoli accessions in response to Se treatments was present. Thus, it is possible to select and breed cultivars with high levels of Se accumulation without negative effect on GLS contents.

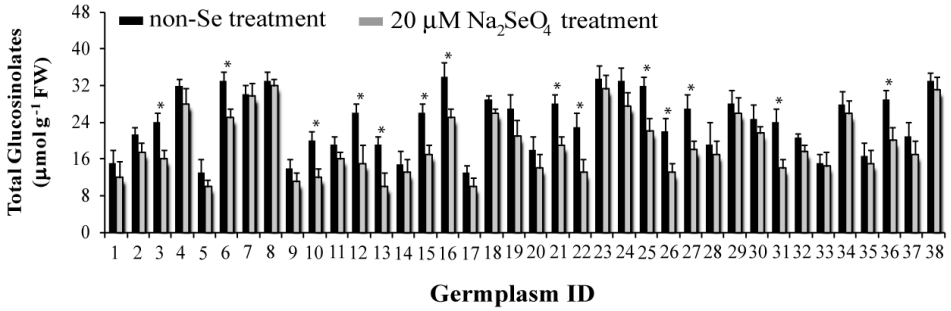

Figure 5. Total glucosinolate contents in broccoli accessions without and with selenate treatment. Values marked by asterisks indicate significant difference between Se nontreated and treated samples ($p<0.05$) (n=4).

Total Antioxidant Activity. Increasing antioxidant levels in food crops can reduce the risk of a number of chronic disease (*37*). Broccoli is a rich source of antioxidants comprising of ascorbic acid, tocopherol, phenolics,

and carotenoids. Se often acts as an antioxidant in plants (*38*). To examine the response of broccoli germplasm to selenate treatment in inducing antioxidant production, the total antioxidant activity was measured. Various levels of antioxidants were observed in broccoli accessions as shown in other studies (*39*). Selenate treatment increased total antioxidant capacity in over half of the germplasm (**Figure 6**). Some of the accessions, *i.e.* 8, 11, 20, 23, 25, 27, 32, and 34 exhibited an approximately 2-fold enhancement. A high degree of correlation (Pearson R=0.95, $p<0.05$) in total antioxidant capacity and its response to Se stimulation was also obtained between leaves and florets of the selected accessions tested (**Supporting Information Figure S2**).

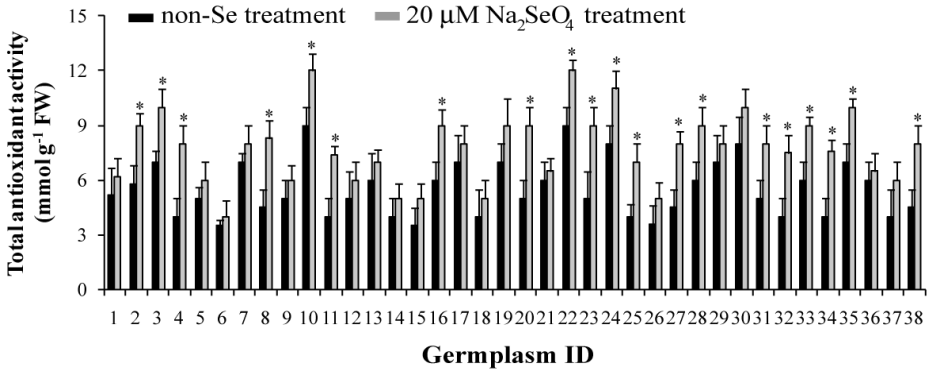

Figure 6. Total antioxidant levels in broccoli accessions without and with selenate treatment. Values marked by asterisks indicate significant difference between Se nontreated and treated samples ($p<0.05$) (n=3).

Increased total antioxidant activity in response to Se treatment has been reported in a number of Se-enriched plants, such as in garden cress

(*Lepidium sativum*) (*40*) and lettuce (*41*). The diverse response of broccoli germplasm in promoting antioxidant production along with varied Se levels offers the opportunity for enhancing their health-promoting properties.

Expression of Genes Involved in Se Metabolism. Plants take up selenate, the major soluble form of Se in soil, via sulfate transporters and metabolize it through the S assimilation pathway (*15, 16*). To gain a better understanding of Se assimilation in broccoli, we investigated the transcript levels of a number of key genes in S/Se uptake and assimilation pathways. As shown in **Figure 7**, no significant difference in expression was observed for all genes examined between the eight selected accessions that accumulated high and low levels of Se. While Se treatment did not dramatically alter the transcript levels for most of the genes examined, significantly high expression of *ATP sulfurylase 1 (APS1),* a plastidic isoform of *APS,* and *selenocysteine Se-methyltransferase (SMT*) were observed in those accessions that accumulated high levels of Se.

ATP sulfurylase is the first and rate-limiting enzyme in the S assimilation pathway (*42*). Selenate application significantly increased the expression of *APS1* in those accessions which accumulated more Se (**Figure 7D**), indicating the important role of APS1 in controlling Se accumulation in Broccoli. Indeed, previous reports show that alteration of *APS* expression dramatically enhances Se metabolism in plants (*43*).

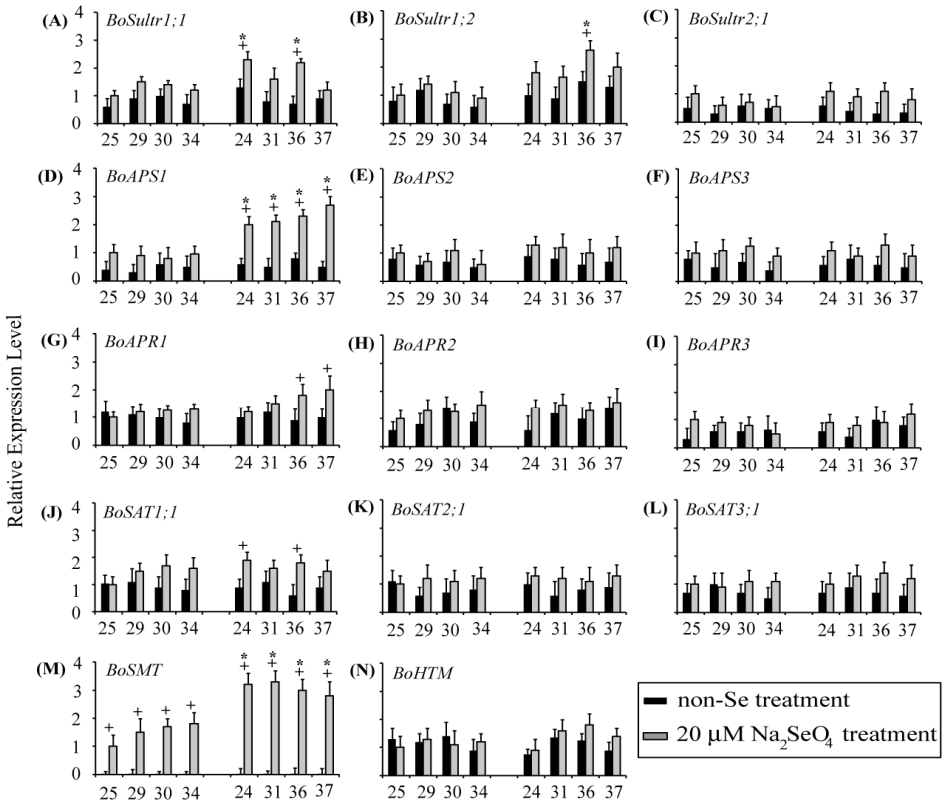

Figure 7. Relative expression of genes involved in Se/S transport and assimilation in broccoli accessions. Transcript levels of genes in broccoli accessions with low (25, 29, 30, 34) and high (24, 31, 36, 37) Se contents upon 20 μM Na_2SeO_4 treatment were measured by qRT-PCR. The expression of accession # 25 in Se treated sample was set to 1. Data are means of 3 technical trials with 2 biological repeats. Values marked by asterisks and plus indicate significantly different between plants with high and low Se levels and between treated and non-treated samples, respectively ($P < 0.05$; $n = 3$).

Selenocysteine Se-methyltransferase represents a key enzyme responsible for SeMSCys production. The expression of *SMT* in broccoli can be dramatically induced upon selenate treatment (*13*). In comparison with non Se-treated plants, high levels of expression of *SMT* were observed when exposed to selenate (**Figure 7M**).

Significantly high expression was seen in accessions accumulating more Se, implying a greater potential for SeMSCys synthesis. Overexpression of *SMT* can increase organic Se species accumulation in both Arabidopsis and Indian mustard (*44, 45*). The results suggest that *APS1* and *SMT* expression may be important for high levels of Se accumulation in leaves of broccoli.

As Se secondary accumulators, all broccoli accessions accumulate high levels of Se but genotypic variation among broccoli germplasm was observed. Se application at proper concentration did not exert negative effect on plant growth and total S level. Broccoli germplasm exhibited various responses in organic Se species production, micronutrient accumulation, GLS synthesis, and total antioxidant contents following Se treatment. The diversity in broccoli germplasm offers the opportunity to develop varieties with high levels of Se and GLS production, as well as other nutritional qualities. A general correlation between total Se levels and organic forms of Se was observed in broccoli accessions. Some accessions accumulate high levels of both Se and GLS (e.g, accession ID 7, 16, 18, 21, 23, 24, 36, and 38). A majority of broccoli accessions have the capacity to simultaneously accumulate Se and GLS without

antagonistic effect (e.g, accession ID 4, 7, 8, 18, 19, 23, 24, 29, 30, 34, and 38). Among them, many also produced enhanced levels of total antioxidants in response to Se treatment. Thus, this study provides important information for breeding of varieties with enhanced health benefits and as a Se supplemental food source in areas of low Se intake.

ABBREVIATIONS USED

Se, selenium; S, sulfur; Ca, calcium; Mg, magnesium; P, phosphorus; Cu, copper; Fe, iron; Mn, manganese; Zn, zinc; GSL, glucosinolate; Sultr, sulfate transporter; APS, adenosine 5′-phosphosulfate sulfurylase; APR, adenosine phosphosulfate reductase; SAT, serine acetyl transferase; SMT, selenocysteine Se-methyltransferase; HTM, homocysteine S-methyltransferases; SeMSCys, Se-methylselenocysteine; SeMet, selenomethionine; qRT-PCR, quantitative reverse transcription-PCR; ICP, inductively coupled plasma; UPLC, ultra performance liquid chromatography

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Supporting Information

Figure S1

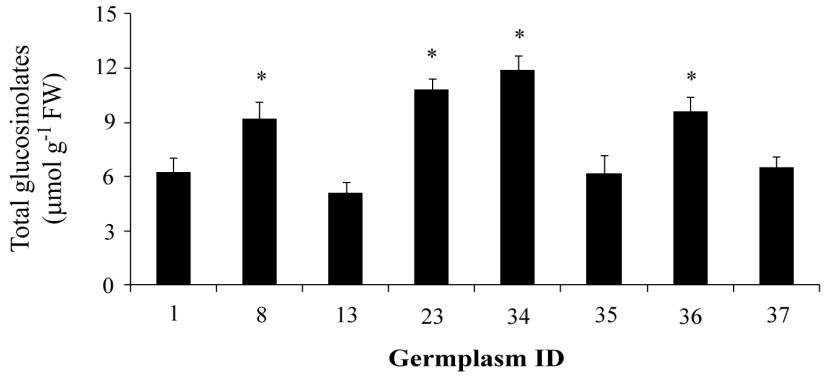

Figure S1. Total glucosinolate contents in florets of selected broccoli accessions without selenate treatment. Error bars indicate standard error of the mean (n=4).

Figure S2

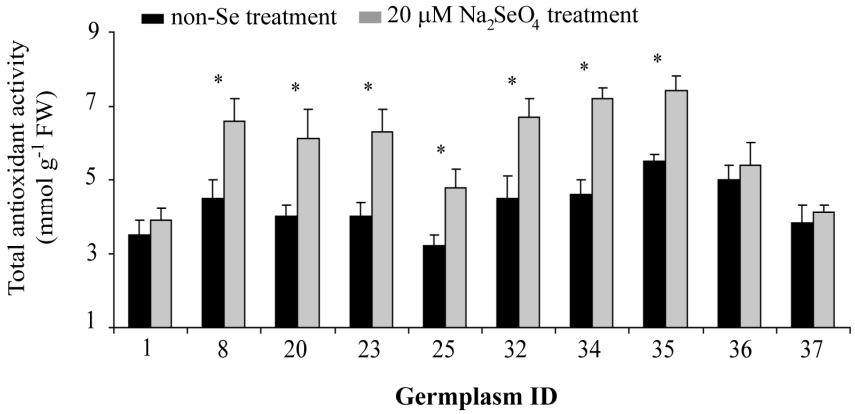

Figure S2. Total antioxidant levels in florets of selected broccoli accessions without and with selenate treatment. Values marked by asterisks indicate significant difference between Se nontreated and treated samples ($p<0.05$). Error bars indicate standard error of the mean (n=3).