SELENIUM SPECIATION IN ARABIDOPSIS THALIANA

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Master Thesis

Selenium Speciation in Arabidopsis Thaliana

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Preface

This thesis was performed as a master thesis as a part of the MSc in Radioecology at the Department of Plant and Environmental Science (IPM) at the Norwegian University of Life Science (UMB). This thesis presents a study of selenium speciation in Arabidopsis thaliana and the protective role in plants.

I appreciated all the supports and guidance from my supervisors. I thank Professor Brit Salbu, for her very helpful advices and sharing the time and knowledge on my master project. I thank Professor John Einset, for his the great help on the experiment and the interesting discussions. I thank researcher Espen Govasmark for his patience and detailed guidance in the experiments and the composing of thesis. I couldn't make it without them and sincere thanks them for the constant helps and encouragements.

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At last, I send my thanks to all my friends who give me a colorful student life. It's a great time for me to study in UMB, and all these memories will remain in my heart.

Abstract

Selenium has been proved as an essential micronutrient and is beneficial to animals and humans. It is a structural component of the important antioxidant enzyme, glutathione peroxidase, which catalyzes reactions to detoxify reactive oxygen species. However, the essentiality of Se in plants remains controversial and the protective role of Se in plants has rarely been investigated. In this study, Arabidopsis thaliana was grown in controlled environments having selenate or selenite enriched medium, and the effects of Se on plant growth, Se distribution and Se species were investigated. The ^{60}Co gamma facility was used as the gamma radiation source to facilitate oxidative stress in plant. A sequential extraction procedure including an enzymatic digestion step was developed for the fractionation of different Se species in Arabidopsis. High performance liquid chromatography interfaced with inductively coupled plasma mass spectrometry (HPLC-ICP/MS) was selected for the separation and speciation of Se using anion exchange and reversed phase.

From the observed results, gamma irradiation and higher medium Se concentrations were toxic to the plants, but irradiated plants treated with 25µM selenate were shown to be protected. Selenate was transported and distributed mainly in plant leaf while selenite was located in plant roots in higher amounts. The anion exchange chromatogram showed that selenomethionine (SeMet) was the dominant organic Se species in both selenate and selenite treated plants. Selenate was the second most abundant Se form in selenate treated plants; a certain amount of selenite was detected in selenite treated plants but it was converted to SeMet readily. A small amount of Se-methylselenocysteine was found in all the Se enriched plants. Due to instrumental problems, only few Se species were identified using reversed phase.

Content

Abbrevations

Se	Selenium
SeMet	Selenomethionine
SeCys	Selenocysteine
SeMeCys	Selenium-methylselenocysteine
SeOMeCys	Oxidized Selenium-methilselenocysteine
SeOMet	Oxidized Selenomethionine
GSH	Glutathione
GPx	Glutatione peroxidase
NADPH	Nicotinamide Adenosine Dinucleotide Phosphate
ROS	reactive oxygen species
ICP/MS	Inductive Coupled Plasma Mass Spectrometry
HPLC	High Performance Liquid Chromatography
SOD	Superoxide dismutase
GSSG	Glutathione disulfide
MDA	Monodehydroascorbate
DHA	Dehydroascorbate
DDT	Dithiothreitol
IAM	Iodoacetamide
DW	Dry weight
SEC	Size Exclusion Chromatogram

Introduction

Selenium

Selenium (Se) is a metalloid element with the atomic number 34 and atomic mass of 78.96, discovered as a byproduct of sulfuric acid production by *Jöns Jakob Berzelius* (Sweden) in 1817. Selenium is in the group 16 in the periodic table and its chemical properties are similar to sulfur and tellurium. The oxidation states of Se exist as +6, +4, 0 and -2. Selenium has six stable isotopes (^{74}Se, ^{76}Se, ^{77}Se, ^{78}Se, ^{80}Se and ^{82}Se) and three radioactive isotopes (^{72}Se, ^{75}Se and ^{79}Se).

Selenium occurs rarely in the elemental form in the nature. The natural occurrence of Se is in numbers of inorganic forms, such as selenides (Se^{-2}), selenite (Se^{4+}), and selenate (Se^{6+}). Selenite (Se^{+4}) and selenate (Se^{+6}) are the main inorganic Se species present in soil and are easily leached or transferred by particulate runoff. Volatile Se species, such as dimethylselenide (DMSe), dimethyldiselenide (DMDSe), are examples of two organic Se form(Zayed, Lytle et al. 1998).

For living organisms, two organic Se species – selenomethionine (SeMet) and selenocysteine (SeCys), are very important. SeMet can be generated from inorganic Se species by microorganisms and plants. SeCys is produced in animal bodies under nucleic control (UGA codon encodes the SeCys) and also thought to be synthesized in plants by a pathway similar to bacteria (Terry, Zayed et al. 2000). Both SeMet and SeCys are incorporated into proteins in animal bodies and some species of plants(Suzuki 2005).

Selenium metabolic pathway

The metabolic pathway for Se is presented in Figure.1. Inorganic Se species, selenite and selenate, can be reduced to organic forms. In the reduction from selenate to selenite, selenate is coupled to ATP by ATP sulfurylase to form the adenosine phosphoelenate (APSe), and APS reductase reduces it to selenite. However, the conversion from selenate to selenite appears to be a rate-limiting step in the Se assimilation pathway (Pilon-Smits and Quinn 2010)

as most plants supplied with selenate accumulate predominantly selenate, while plants supplied with selenite accumulate organic Se. Selenite is readily reduced to selenodiglutathione (Gs-Se-Gs) by glutathione (GSH). Then, by the reduction with NADPH, Gs-Se-Gs is reduced to selenol (Gs-SeH), and subsequently to GSH-conjugated selenide by GSH reductase (Terry, Zayed et al. 2000).

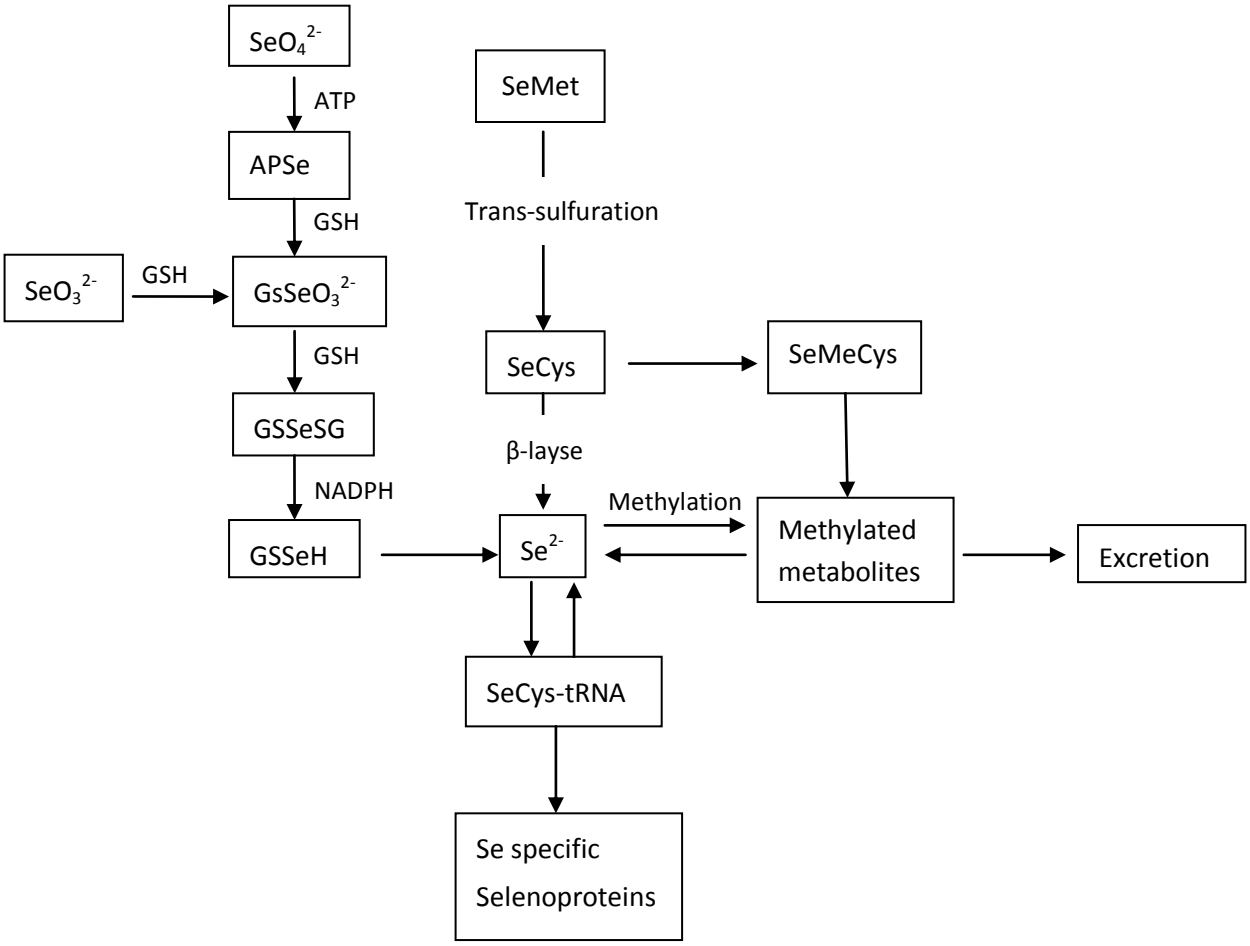


Figure. 1 Brief description of the Se metabolic pathways *(Terry, Zayed et al. 2000; Suzuki 2005)*

Organic Se compounds in nutritional sources are mainly Se containing proteins include SeMet, SeCys or methylated Se-species. SeMet is considered to be converted to SeCys through the trans-selenation pathway; subsequently, the β-layse converts SeCys into selenide which is thought to be the primary route of the conversion from selenoamino acid to selenide (Suzuki 2005). After the reduction of Se compounds to selenide, it may be methylated to methylselenol, or converted into selenophosphate to synthesize the SeCys-tRNA and finally synthesized selenoproteins (Suzuki 2005; Pilon-Smits and Quinn 2010). On the other hand, SeCys can also

be methylated to selenium-methylselenocysteine (SeMeCys) by SeCys methylatransferase, and then maybe converted into methylselenol by the β-layse.

Deficiency and toxicity of selenium

Selenium is confirmed as an essential micronutrient and a benefit for animal and human health. Deficiency of Se in human body may result in the oxidative damage and some diseases. Keshan disease, one of the best known diseases caused by the Se deficiency in human body, was found in the Se deficient area in China which is a wide belt extending from northeast to southwest China. The deficiency is because of low-Se soils and thus low Se concentrations in food and the occurrence of Keshan disease was reduced by Se-supplementation. Keshan disease is charactwerized as an abnormality of heart muscle. Additional symptoms include coughing, breathing difficulty, vomiting, anorexia, swollen body and emphysema.

At higher dosages, Se may be toxic for all organisms. The range between trace nutritional requirement and toxicity is very narrow. For animals, the toxicity level is 2 to 5 mg Se kg^{-1} dry weight (DW) forage feed which is 20000 times higher than the minimal nutritional recommended concentration of 0.05 to 0.10 mg Se kg^{-1} DW forage (Wilber 1980; Wu, VanMantgem et al. 1996). For human health, the toxicity of Se has been confirmed to cause hypochromic anemia and leucopenia; other syndromes, such as nail damage and hair, were also found from the long-term workers employed in the manufacture of Se rectifiers(Rosenfeld and Beath 1964). Selenium contaminated water at the natural wildlife refuge in California was considered as the agent responsible for the mortality, growth defects and reproductive failure of fish (Saiki and Lowe 1987).

In 1973, a main physiological function of Se was found for Se as an essential structural component of the important antioxidant enzyme, glutathione peroxidase (GPx) (Rotruck, Pope et al. 1973), which catalyzes the reduction of reactive oxygen species (ROS) by reduced glutathione (GSH) in order to protect cells against oxidative damage.

Protective role of selenium

The generation and scavenging of ROS in organisms

Reactive oxygen species are composed of free radicals, oxygen ions, and peroxides, both organic and inorganic. Reactive oxygen species molecules are e.g. hydrogen peroxide (H_2O_2), radicals like hydroxyl radical (·OH), ions like hydroxyl ion (OH^-), and both ion and radical like the superoxide anion ($\cdot O^{2-}$).

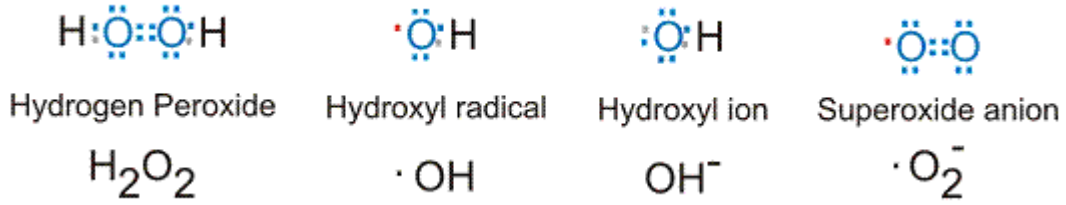


Figure.2 Examples of ROS. *(Held 2010)*

In organisms, ROS are inevitable byproducts of the normal metabolism of oxygen localized in different cellular compartments (Foyer, Descourvieres et al. 1994). Mitochondria are considered to be a major site of ROS production, according to an endogenous and continuous physiological process under aerobic conditions (Fleury, Mignotte et al. 2002). Reactive oxygen species can also be converted from ground state oxygen by the reaction of energy transfer or electron transfer. Energy transfer leads to the singlet oxygen, whereas electron transfer may change the valence of atoms, which results in sequential reduction to superoxide, hydrogen peroxide, and hydroxyl radical (Klotz 2002) as presented in Figure 3.

Some ROS species are quite unstable, such as superoxide and hydroxyl radicals (.OH). Hydroxyl radical is considered to be the most chemically active which can react with almost all the biomolecules in organisms (proteins, DNA, lipid, organic acid etc.), and also contributes to the transformation of non-free radical reagents to free radical reagents. Whereas, some ROS such as hydrogen peroxide are freely diffusible and comparatively long-lived (Finkel and Holbrook 2000).

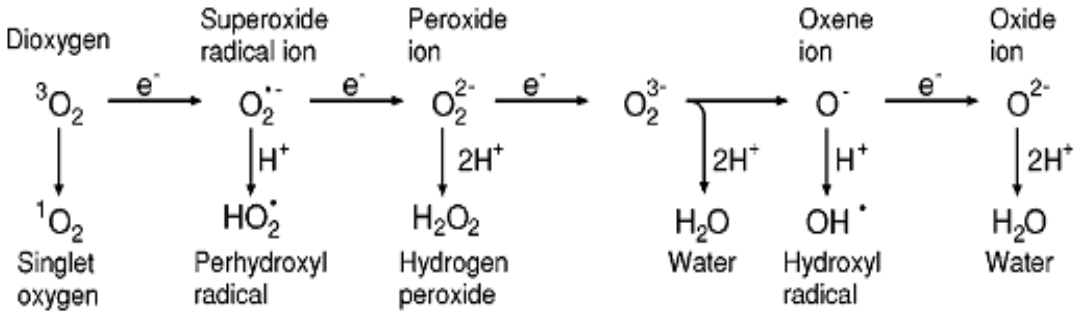


Figure.3 Generation of different ROS by energy transfer or sequential univalent reduction of ground state triplet oxygen *In* (Apel and Hirt 2004)

The scavenging of ROS and the protective role of Se

In physiological steady state condition, ROS can be scavenged by relevant protective mechanisms (the antioxidant defense system) in cells to maintain a non-toxic concentration of ROS. The antioxidant defense system is mostly composed of two components – the antioxidants (vitamins C, vitamin E, glutathione and thioredoxin etc.) and the antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidise e.g.). When ROS production exceeds the body's natural antioxidant defense level, the extra ROS induces oxidative stress which results in damage to biomolecules such as lipids, proteins and DNA (Apel and Hirt 2004).

Nonenzymatic antioxidants include the major cellular redox buffers ascorbate and GSH, and also tocopherol (vitamin E), flavonoids, alkaloids, and carotenoids (Apel and Hirt 2004). Glutathione is oxidized by ROS, forming oxidized glutathione (GSSG). For example, the sequential reaction of GSH reduced hydroxyl radical to superoxide anion and formed a GSSG (Arteel and Sies 2001), and then superoxide anion is catalyzed by superoxide dismutase (SOD) to produce H_2O_2 , which could be readily decomposed by catalase. Ascorbate is oxidized to monodehydroascorbate (MDA) and dehydroascorbate (DHA), and then, GSSG, MDA and DHA can be reduced to regenerate GSH and ascorbate through the ascorbate –glutathione cycle (Apel and Hirt 2004).

One of the important ROS scavenging mechanisms is the catalysis reaction of the antioxidant enzyme, GPx, which contains a selenocysteine residue at the active site (Arteel and Sies 2001).

This reaction was thought to go through 3 steps (equation 1, i-iii) which include the decomposition of organic hydroperoxide into selenic acid and corresponding alcohol (equation 1, i), the sequential reduction by GSH (equation 1, ii) and (equation 1, iii), which reduce the selenic acid to produce the glutathione disulfide (GSSG) and regenerate the selenol. The summarized reaction (equation 1, iv) is the catalysis by GPx, and then, glutathione reductase reduces the GSSG to GSH by the expense NADPH (equation 1, v) to complete the GSH cycle (Arteel and Sies 2001).

Equation 1 ROS scavenging mechanism catalyzed by GPx *(Arteel and Sies 2001)*

(i) $R\text{-}SeH + ROOH \longrightarrow R\text{-}SeOH + ROH$

(ii) $R\text{-}SeOH + GSH \longrightarrow R\text{-}Se\text{-}SG + H_2O$

(iii) $R\text{-}Se\text{-}SG + GSH \longrightarrow R\text{-}SeH + GSSG$

(iv) $ROOH + 2GSH \xrightarrow{GPx} GSSG + H_2O + ROH$

(v) $GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+$

The antioxidant property was also shown by other selenoproteins. Selenomethionine has a GPx-like role in the catalytic reaction which catalyses by selenomethionyl residue in proteins (Assmann, Briviba et al. 1998). Another important selenoprotein, thioredoxin reductase, reduces lipid hydroperoxides by the participation of NADPH. The thioredoxin system was also thought to effectively reduce more antioxidants. Other selenoproteins, such as selenoprotein P and ebselen (a mimic of glutathione peroxidase and can scavenge H_2O_2), was also reviewed to play protective role in the defense of oxidative stress(Arteel and Sies 2001).

Selenium and human health

The Norwegian daily Se intake recommendation for adults is 40 to 50 µg day^{-1} (Helsedirektoratet 2005) whereas The Food and Nutrition Board of the National Academy's Institute of Medicine recommended daily intake of Se at 55µg per day. Appropriate doses of supplemental Se to humans can enhance cellular defense against reactive damage and prevent

certain types of cancers. A randomized trial in China reported that Se supplementation can decrease the overall cancer mortality (Blot, Li et al. 1993). In the study of 1312 participants with a prior history of skin cancer(Clark, Combs et al. 1996), Se intake of 200μg day^{-1} in the form of selenized yeast reduced cancer mortality and lowered the incidence of some types of secondary cancer (e.g. prostate, lung ,colon); however, no differences in skin cancer rates were found. Additionally, the Selenium and Vitamin E Cancer Prevention Trial (SELECT), based on the presumed antioxidant and anticancer properties of these agents that inhibit specific cellular processes in the development of cancers, suggested that both Se and vitamin E can potentially prevent prostate cancer (Klein, Lippman et al. 2003).

The source Se for human is mainly from the daily dietary. Compared to inorganic Se, organic Se is believed to be safer and more bioavailable as a dietary supplement than inorganic Se (Abdulah, Miyazaki et al. 2005). Nowadays, Se sources in the human diet are nuts, cereals, meat, eggs and dairy products. Another attractive Se source is the selenized yeast due to its low cost and high content of SeMet (Rampler 2009).

Different Se species have different contributions for the prevention of cancers. In a feeding study with rats (Ip, Birringer et al. 2000), supplementation of selenized garlic (73% gamma-glutamyl-Se-methylselenocysteine) to the diet at different levels consistently caused a lower total tissue Se accumulation compared to selenized yeast (85% SeMet). However, selenized garlic was much more effective in suppressing the development of premalignant lesions and the formation of adenocarcinomas in the mammary gland of carcinogen-treated rats. Another study reported that only 1-3 mg kg^{-1} of Se-methyselenocysteine could be a better chemopreventive agent than either SeMet or selenite(Ip, Birringer et al. 2000). Methylselenic acid, a new Se species of interest with regard to chemopreventive activity, was reported to significantly suppress the development of human premalignant breast cancer in a time-dependent manner with a much lower level (5-10μmol L^{-1}) than SeMeCys (200μmol L^{-1}) (Dong, Ganther et al. 2002).

Selenium in plants

Because of the benefit from Se to animal and human health, Se concentrations in daily diets have gained more attention recently. Nevertheless, Se has not been proven to be essential for higher plants (Shrift 1969; Terry, Zayed et al. 2000), though there is evidence that Se is required for the optimal growth of algae (Price, Thompson et al. 1987; Fu, Wang et al. 2002; Novoselov, Rao et al. 2002).

Selenium availability in plants

Plants vary in the abilities to accumulate Se in their tissues. Some plants are able to accumulate large amounts of Se when they grow on seleniferous soils. These species are Se accumulators and include a number of species of *Astragalus, Stanleya, Morinda, Neptunia, Oonopsis,* and *Xylorhiz*a (Terry, Zayed et al. 2000). Selenium accumulators can accumulate high levels of Se ranging from hundreds to several thousand milligrams Se kg^{-1} DW in their tissues. For the nonaccumulators (Brown and Shrift 1982), such as most forages and crop plants, as well as grasses, they do not accumulate Se much above a ceiling of 100 mg Se kg^{-1} DW even when grown on seleniferous soils(Agalou, Roussis et al. 2005). Marschner et al (1995) showed that crop plants grown on nonseleniferous soils typically have Se concentrations ranging from 0.01 to 1.0 mg kg^{-1} DW. Burau (Burau, McDonald et al. 1988) found that tissue Se levels in 17 different crops exhibited rarely exceeded 1 mg Se kg^{-1} DW.

Not all plants species on seleniferous soils are Se accumulators. Astragalus contains both Se-accumulating species and nonaccumulating species and these can grow next to each other on the same soil (Ernst 1982) and several species plants growing on seleniferous soil had markedly different tissue Se concentrations (Trelease and Beath 1949). There is another category of plants that called secondary Se accumulators (Brown and Shrift 1982). They grow on soils of low-to-medium Se content and accumulate up to 1000 mg Se kg^{-1} DW (Terry, Zayed et al. 2000).

Uptake and transport in plants

The translocation of Se from root to shoot is dependent on the form of Se supplied. Selenate is transported much more easily than selenite, and thus plant roots accumulate more Se when applied selenite than selenate (Li, McGrath et al. 2008) . The mechanism of selenate uptake by plants has been well reported. Selenate is taken up via the high affinity sulfate transporter in the root plasma membrane and competes with the uptake of sulfate (Terry, Zayed et al. 2000). Sulfur starved wheat plants showed about 10 fold increase in selenate uptake and up-regulated expression of sulfate transporter gene leading to increasing uptake of selenate. However, the mechanism of selenite uptake is less well known. In the study of rice *(Oryza sativa)* (Zhang, Shi et al. 2006), little effect of selenite uptake was found when plants were treated with sulphate. In the study of Li et al. (2008), sulfur starvation also showed no effect on selenite uptake by wheat plants, while the P starvation resulted in a 60% increase in selenite uptake by wheat; and the presence of phosphate inhibited selenite influx into root which decreased the affinity of selenite significantly. Although no evidence showed the selenite uptake is mediated by the membrane transporter (Terry, Zayed et al. 2000), selenite uptake is probably via the phosphate pathway.

The shoot Se/root Se ratio is much higher when supplied with selenate than supplied with with SeMet and selenite (Zayed, Lytle et al. 1998). A study of time-dependent kinetics of Se uptake by Indian mustard showed that only 10% of the selenite taken up by plant was transported from root to shoot, whereas, selenate was rapidly transported into shoots (De-Souza, Pilon-Smith et al. 1998). Selenite uptake by root can be assimilated to organic form, and transported as seleno-amino acid.

The distribution of Se in plants varies according to different species, development phase, and physiological condition (Trelease and Beath 1949). For Se accumulators, Se is concentrated in young leaves during the early stage of growth; during the reproductive stage, high levels of Se are found in seeds while the Se content in leaves is rapidly reduced (Ernst 1982). For non-accumulators (such as cereal crop plants), when mature they often show about the same Se content in grain and in root, with smaller amounts in the stem and leaves(Beath 1937)

Selenium essentiality and detoxification in plant

The essentiality of Se as a micronutrient for higher plants is not clear now. In order to investigate the essentiality of Se in higher plants, several studies have been done to investigate if there are essential selenoprotein in plants. The detection of selenoproteins in pants was conducted by testing for the presence of a family of selenoproteins, such as GPx (Eshdat, Holland et al. 1997). From another investigation (Sabeh, Wright et al. 1993), a 16-kd tetrameric protein, which is concluded as a GPx selenoprotein similar to that found in mammals, was found in *Aloe vera*, but the molecular evidence suggests that, the GPx-like enzymes appeared in higher plants may not be selenoproteins (Terry, Zayed et al. 2000). Analysis of genes isolated from plants with significant sequence homology to animal GPx genes(Criqui, Jamet et al. 1992; Holland, Benhayyim et al. 1993) indicates that the plant genes exhibit only one subgroup of animal GPx (the phospholipid hydroxyperoxide GSHperoxideases) (Eshdat, Holland et al. 1997). On the other hand, the investigations on gene decoding (change the genetics code to synthesis proteins) suggests that part of machinery for synthesizing selenoproteins may be present (Hatfield, Choi et al. 1992).

Although evidence for the essentiality of Se in plants is lacking, several results have shown that the addition of Se contributes to the detoxification of oxidative stress. An investigation on the impact of Se in lettuce found that the antioxidative effect on plants was increased when applied at 0.1mg Se kg^{-1} (Xue, Hartikainen et al. 2001). Guo et al. (1998) indicated that Se increases the concentration of GSH and enhances the activity of GPx in plant seeds and seedlings, such as wheat, rice and cucumber. By investigating of cole and soybean, Xue et al. (1993) suggested that the addition of Se may enhance the GPx system in plants.

Analytical method for selenium speciation

The inductively coupled plasma – mass spectrometry (ICP-MS) was developed decades ago and has been commercially available for over 20 years. ICP-MS is a widely used technique for general speciation analysis, as well as Se, due to its several advantages (lower detective limits, multi-isotope detection etc.).

In ICP-MS, the inductively coupled plasma gives a high efficiency of atomization and ion formation and the mass spectrometry offers specific and sensitive detection capability. The plasma is generated by the sparked argon stream in a quartz torch with certain radio frequency electromagnetic field. Under the induced magnetic field, the plasma is accelerated and struck with the gas atoms to make a further ionization so that the plasma is maintained. For aqueous samples, the liquid is first nebulized into an aerosol, and then, carried by argon gas to go through the central tube in the quartz torch. The ions generated from the plasma are sampled by the mass spectrometry to complete the determination (B'Hymer and Caruso 2006).

Because of the mass dependent measurements of MS, the determination of Se is always interfered by numbers of argon containing polyatomic species. For the most abundant Se isotope, ^{80}Se (49.8% abundance), the argon dimmers could be the major interference due to the same atomic mass which overlaps the mass signal. Other interferences can derive from the plasma entrained atmospheric gas (O_2, N_2 etc.), solution (water, organic), and matrix (acid, buffers, salts etc) (B'Hymer and Caruso 2006). For example, the polyatomic ion of chloride (CCl_2^+), possibly from the HPLC mobile phase, may interfere with the determination of ^{82}Se. The most frequently encountered interferences for different Se isotopes are listed in Table 1.

Table 1 Spectral interference in Se determination by ICP-MS

Se isotope	Interference
74	$^{38}Ar^{36}Ar^+$, $^{37}Cl_2^+$, $^{40}Ar^{34}S^+$
76	$^{40}A^{r36}Ar^+$, $^{40}Ar^{36}S^+$, $^{31}P_2{}^{14}N^+$
77	$^{40}Ar^{36}ArArH^+$, $^{38}Ar_2H^+$, $^{40}Ar^{37}Cl^+$
78	$^{40}Ar^{38}Ar^+$, $^{31}P_2{}^{16}O^+$
80	$^{40}Ar_2^+$, $^{79}BrH^+$
82	$^{40}Ar_2H_2^+$, $^{12}C^{35}Cl_2^+$, $^{34}S^{16}O_3^+$, $^{81}Br^+$

To eliminate interferences, collision/reaction cell technology is commonly applied. This cell consists of a multipole located before the analyzer quadrupole. The cells are pressurized with a gas or a mixture of gases which takes part in the collision/reaction with the focused ions. In the collision cell, the interference ions collide with an inert gas (Helium, argon etc.) for dissociating it (unlikely) or reducing its energy, then the lower energy interference is screened from the quadrupole mass analyzer. In the reaction cell, the interference or the analyte ions react with a

gas that help to prevent interfering ions from spectrally overlapping with the target isotopes (B'Hymer and Caruso 2006).

For the separation of Se compounds in Se speciation analysis, high performance liquid chromatography (HPLC) is an often used technique. Considering that many Se compounds of interest are non-volatile, the derivatization step is required prior to the gas chromatographic analysis. However, HPLC requires no derivatization to create volatility but only need the sample to be soluble. The coupling of HPLC to ICP-MS is a successful technique for Se speciation which achieves the both the low detection limits and high performance separation of Se species (B'Hymer and Caruso 2006).

Depending on the physical-chemical properties of the compounds of interest, different separation mechanisms can be considered. For example, the size exclusion chromatography (SEC) separates Se compounds depending on the particle size, eluting the large particles faster and retaining the smaller ones (no chemical interaction in SEC). In this study, we applied anion exchange chromatography and reversed phase chromatography for Se speciation.

Anion exchange chromatography

Anion exchange chromatography separates ions and polar molecules based on the charge properties of the molecules. The stationary phase surface has ionic functional groups that interact with the analyte ions of opposite charge. In the anion exchange column, the stationary phase retains anions using positively charged groups. Contrarily, the stationary phase in cation exchange column is negatively charged to retain cations. The mixture of ions is separated depending on the strengths of the interactions between the ions in the mobile phase and the functional groups on the stationary phase.

Reversed phase chromatography

Reversed phase chromatography is probably the most extensively used method for Se speciation. Due to the fact that the stationary phase is less polar than the mobile phase, the polar compounds are eluted first and the non-polar species are retained. Mixtures of water or

aqueous buffers and organic modifiers are used for the elution and separation of analytes. The most commonly used organic solvents are acetonitrile, methanol, or tetrahydrofuran. Methanol is usually used as the organic modifier to maintain the plasma stability for ICP-MS detector. In this study, we added a small percentage of heptafluorobutyric acid (HFBA) in water or methanol as the mobile phase which has been reported that help improve the resolution of many organic Se species (Kotrebai, Tyson et al. 2000).

Objective

Selenium has been proved to play a protective role in many organisms, but the protective function in plants has not been investigated. The objectives of this study are:

(1) Investigate the protective role of Se for the ROS scavenging in plant. (Results were not presented because the ROS measurement is inconsistent and thus not successful. It was caused by an unknown problem.)

(2) Develop a method for Se speciation analysis in Arabidopsis and identify the Se species in Arabidopsis thaliana to provide the data for the further studies of the protective function of Se in plants.

Materials and method

Growing conditions

Wild type Arabidopsis thaliana was used in this study. Treatment to the samples is described in Table 2. Arabidopsis seed was germinated in 8.5 cm diameter plastic petri dishes with growing culture containing Murashige and Skoog medium, 30 g L^{-1} sucrose and 8g L^{-1} agar with different Se supplements. Considering that the distribution is various for different Se species, two kinds of selenic salt -sodium selenite (Se^{+4}) and sodium selenate (Se^{+6}) were used, respectively. The culture conditions were set into control group, 1 µM and 25 µM Se (selenite or selenate) supplement groups, and three replicates for each group. Plants were incubated in a controlled environment at 24°C, 16 hours photoperiod and an 80 µM m^{-2} s^{-1} light intensity. The irradiation was applied through the whole growing period with 60 mGy h^{-1} gamma radiation. Growing period was 3 weeks (from seeding to harvest).

Table 2 Description of sample treatments.

[a] Se Species / [b] Se Concentration	Se +4		Se +6	
Control	[c] γ	-	γ	-
1 µM	γ	-	γ	-
25 µM	γ	-	γ	-

a. Se species indicate the growing medium was supplemented with selenite or selenate.
b. Se Concentration indicated the Se concentration supplemented in the growing medium.
c. "γ" or "–"indicate exposing or not exposing to gamma radiation.

Radioactive source

The ^{60}cobalt (^{60}Co) facility at UMB was used as the gamma radiation source to facilitate the oxidative stress to the plants. The facility was set in 2003 with ^{60}Co pellets enclosed in a lead

chamber. The activity of the source was 82.4 GBq during the experiment. The dose rate was adjusted by changing the distance between samples and the ^{60}Co source (Fig. 4).

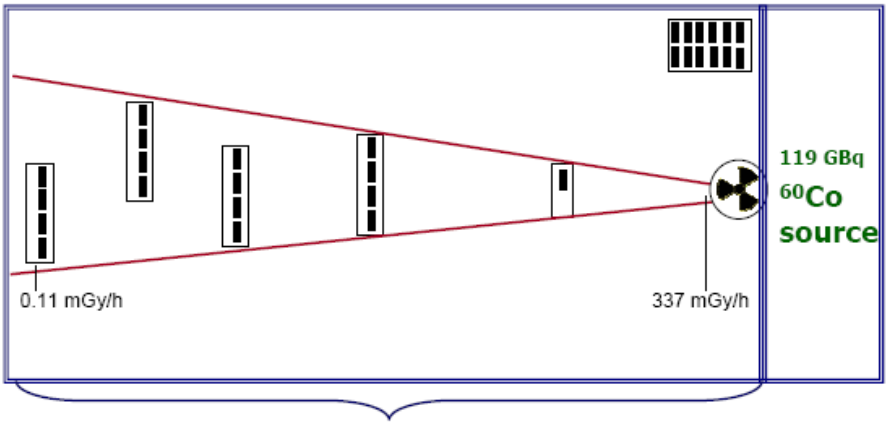


Figure 4 Dose rate was adjusted by the distance between samples and source

Standards and reagents

Selenium standards of Se^{+4} (Na_2SeO_3), Se^{+6} (Na_2SeO_4), selenomethionine (SeMet) and selenomethyl-selenocysteine (SeMeCys) were purchased from Sigma Aldrich (Oslo, Norway). Oxidised SeMet (SeOMet) and the oxidized SeMeCys (SeOMeCys) were prepared by oxidizing SeMet and SeMeCys with 3% (v/v) hydrogen peroxide (H_2O_2) and evaporate at 60℃. Other chemicals and enzymes (protease and lipase) were purchased from Sigma-Aldrich and Fluka. The water used in this experiment is obtained from the Milli-Q (MQ) system. In the enzymatic digestion, dithiothreitol (DTT), iodoacetamide (IAM) and the enzymes were prepared with 0.1M Tris-HCl (pH 7.5) solvent. The pH of anion exchange HPLC mobile phase was adjusted to pH 5.1 (HNO_3).

Sample preparation

Harvest

Plant was separated into root and leaf parts at harvest. To clean the growth medium agar which remained on the plants, 20mM Ethylenediaminetetraacetic acid (EDTA) solution was applied to wash the plants for 30 minutes, and the plants were freeze dried.

Total Se concentration determination

A certain amount of each sample was taken and the DW was determined. The samples were placed in a Teflon tube, and 40 µl Tellurium as internal standard, 2ml distilled ultrapure nitric acid (HNO_3) and 1.5 ml MQ water were added to each tube. The samples were digested using an ultraclave (UltraCLAVE 3, Milestone) for 40 min at 240°C, diluted to 6 ml and measured by ICP-MS.

Sequential Extraction

Sonication and ball mill grinding are considered give a higher extraction yield than other methods of tissue disintegration. During sonication, however, the formation of aerosols may cause loss of material and this technology also produces the potential risk of contamination. For the above reasons, ball mill grind was selected for homogenization of the plants. In the ball mill grinding process, dried plant tissues are extracted with 2ml 0.1 M ammonium acetate (NH_4AC) in a ball mill with Zirconium balls for 1 min at 30 Hz. The sealed capsules effectively lower the risk of contamination.

Enzymatic digestion

DTT (Dithiothreitol) is a low molecular weight redox reagent. It is frequently used for reduction of disulfide bonds in proteins. In this experiment, generally, it was used to prevent *intramolecular* and *intermolecular* S-S, Se-Se or Se-S bonds from forming between cysteine residues of proteins.

Under the condition of proteolytic digestion, selenocysteine undergoes a risk of oxidation. A derivatization reaction with IAM (iodoacetamide) can prevent this process by replacing the hydrogen from –SeH and alkylate the cysteine residues.

In our experiment, 2ml 0.1M Tris-HCl (without urea), 40 µl 0.2M DTT (in 0.1M Tris-HCL pH7.5) and 55µl 0.5Mol IAM were added to each homogenized sample, and incubated at 25°C in dark for 2 hours. To eliminate the excess IAM, then, 500ul 0.2M DTT was added to each sample and

shook the samples using the roller bed for 1 hour at room temperature. 30 µg protease XIV and 20µg lipase dissolved in 1ml 0.1M Tris-HCl (pH 7.5) was added to each sample. Then, the samples were incubated in a shaking water bath over night (12-17 hours) at 37°C.

Filtration

After the enzymatic digestion, all the samples were filtered by using syringe membrane filter at 0.45µm. The filtrates were centrifuged at 10,000 x g for 10 minutes. The supernatants were separated and repeated the centrifugation once more. Then, the supernatants were stored at -20°C until analysis by HPLC-ICP/MS.

The coupling of HPLC and ICP MS

Selenium speciation was conducted with the HPLC coupled ICP/MS. In this study, fractionation was carried on by a Model 1100 HPLC with the anion-exchange column (Hamilton PRP X-100) fitted with a guard column or reverse -phase column (C8 Altima). The ICP-MS (Elan 6000 - Perkin Elmer) is a type of ICP/MS which do not have the advantage of collision or reaction cell as decribed earlier. The sample was introduced by a peristaltic pump connected a micromist nebulizer (glass expansion, romainmoitier, Switzerland) with the spray chamber. The signal of m/z 82 was collected. The outlet from HPLC was connected directly with the peristaltic pump by a narrow tube and the interest fractions were delivered into the nebulizer.

Chromatographic conditions

The optimized chromatographic HPLC conditions for anion exchange and reversed phase are showed in Table 3. ICP-MS detection of Se was carried out using ^{82}Se isotope.

Table 3 chromatographic conditions

Separation mechanism	Anion exchange	Reversed phase
Column	Hamilon PRP X-100	C8 Altima/Alltech
Mobile phase	A: 25mM ammonium acetate, pH 5.1(HNO3) B: 250mM ammonium acetate pH 5.1(HNO3)	A: 0.1% HFBA in water B: 0.1% HFBA in methanol
Elution program	0-5min: 100% A 5-10min: 0-100% B 10-25min: 100% B 25-35min: 0-100% A	0-5min: 5% B 5-30min: 5-40% B 30-35min: 40% B 35-37min:40-5% B 37-40min: 5% B
Flow rate	1.5 ml/min	0.9 ml/min
Injection volume	200ul	200ul

Results

Observational growth different of Arabidopsis

There were significant differences in growth and development of Arabidopsis between Se and gamma radiation treatments as presented in figure 5. The control group (Fig.5 a) developed normally, looking healthy and green with fully developed leaves. The control group exposed to gamma radiation (Fig.5 b) developed abnormally with small and more yellowish leaves. The Arabidopsis plants grown in 1 µM Se (Fig.5 c,e) enriched medium were smaller and more yellowish than the controls, and when these plants were exposed to gamma radiation (Fig.5 d,f), they grew abnormally with yellowish leaves. However, one replicate of gamma irradiated plants with 1µM selenite treatment (Fig.5 f) was developed as the plants grew in growth chamber (Fig.5 e). Therefore, it could not accurately indicate the visual difference between these two groups. No visual differences of growth between the 1 µM Se selenite and selenate treated plants. Increasing the medium selenate (Fig.5 g) concentration to 25 µM, a great inhibition of the growth was presented as they did not develop after germination. Arabidopsis plants grown in the 25 µM selenite medium (Fig.5 i) developed similar to the plants in the 1µM selenite medium. Exposing the 25 µM selenate plants to gamma radiation (Fig.5 h) increased the growth performance of approximately half of the plants, whereas the 25 µM selenite (Fig.5 j) treated plants either did not germinate or grew poorly.

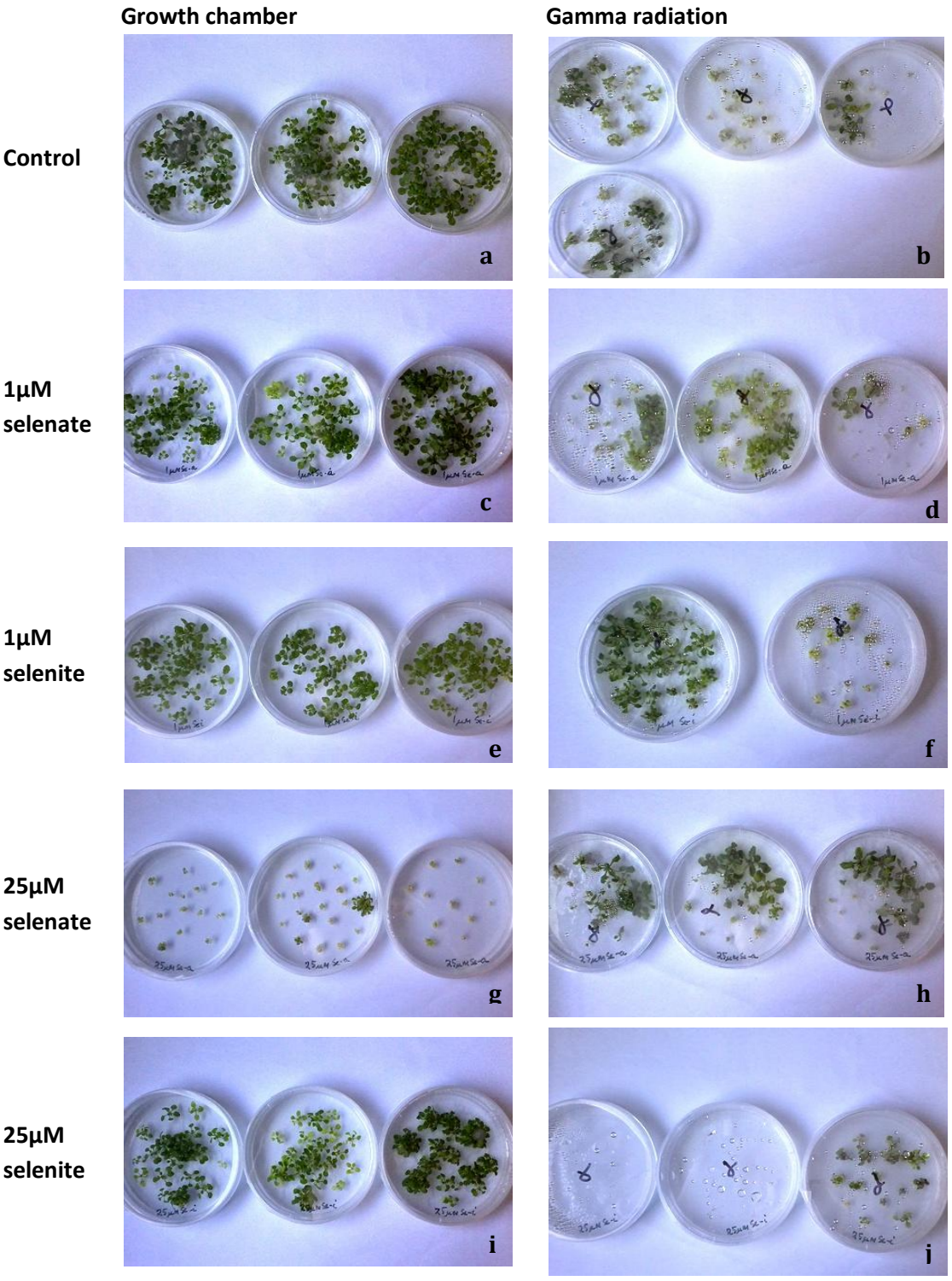


Figure 5 Arabidopsis growth difference when harvest

Selenium translocation in Arabidopsis

The Se concentrations of control leaf and roots were 0.31±0.08 µg g^{-1} and 0.39±0.07µg g^{-1}, respectively (Table 4). Both leaf and root Se concentrations increased with increasing Se

concentration of the growth medium, except with 25 µM selenate treated roots, which is lower than 1 µM selenate treated root. All selenite treated plants had higher Se concentrations in the root than leaf whereas selenate treated plants had higher Se concentrations in leaves (Table 4). Root Se concentrations were higher in selenite treated plants than in selenate treated plants, whereas it was higher in leaves of the selenate treated plants.

Table 4 Total Se concentration in root and leaf *(µg Se g^{-1} DW)*

Treatments	Growth Chamber (µg Se g^{-1})		Gamma Irradiation (µg Se g^{-1})	
	Leaf	**Root**	**Leaf**	**Root**
Control	0.31±0.08	0.39±0.07	0.19±0.04	0.39±0.15
1 µM Se Ⅵ	17±8.7	6.2±2.0	9.1±4.1	9.9±5.1
1 µM Se Ⅳ	4.8±0.83	17±2.5	9.0±0.35	25±3.7
25µM Se Ⅵ	316	0.65	286±65	122±167
25µM Se Ⅳ	112±16	532±255	76	210

Within the selenite treatments, plants exposed to gamma radiation had a lower Se concentration in the leaves but a higher Se concentration in roots. Within the selenate treatments, the gamma irradiated plants grown in 1 µM Se medium had the higher Se concentration in root than leaf, while the irradiated plants grown in 25 µM selenate medium had the higher Se concentration in leaves.

Total Se concentrations in the whole plants are presented in Table 5. The total Se concentration in the control group was 0.34µg g^{-1} and Arabidopsis plants developed normally (Fig.5 a). Plants exposed to gamma radiation had total Se concentration of 0.25 µg g^{-1} and the plants developed abnormally with yellowish leaves (Fig.5 b). When plants were treated with selenate or selenite enriched medium, the total Se concentration increased with the increasing medium-Se concentration. However, the plants growth performance in growth chamber decreased with increasing medium-Se concentration (Fig.5 c,e,g,h). Exposing to gamma radiation, the total Se concentration of 1 µM selenate treated plants is 9.10µg g^{-1}, and the plants developed abnormally with yellowish leaves (Fig.5 d). Contrarily, the 25 µM selenate treated plants had

much higher total Se concentration (202 μg g^{-1}) and developed better with green leaves (Fig.5 h). For gamma irradiated 1 μM selenite treated plants, the total Se concentration is 14 μg g^{-1} and the plants developed abnormally with yellowish leaves (Fig.5 f). Increasing the medium selenite concentration to 25 μM, the total Se concentration of those germinated plants was 108 μg g^{-1} while the other plants did not germinate (Fig.5 j).

Table 5 Total Se concentration *(μg Se g-1 DW)*

Total Se (ug g^{-1})	**GC**	**Gamma**
Control	0.34	0.25
1uM Se6	12	9.10
1uM Se4	9.6	14
25uM Se6	198	202
25uM Se4	210	108

Leaf/root Se ratios of the plants in growth chamber was about 2.71 for 1 μM selenate treatment and 0.27 for the 1 μM selenite treatments (Table 6). When the medium Se concentration increased to 25 μM, the leaf/root Se ratios of selenate treatments was about hundredfold higher than 1 μM treatments while it was about 0.21 for selenite treatment.

Table 6 The leaf/root Se concentration ratio in the Arabidopsis plants

Se leaf/Se root	**GC**	**Gamma**
1 μM Se^{+6}	2.7	0.92
1 μM Se^{+4}	0.27	0.36
2 5 μM Se^{+6}	486	2.3
2 5 μM Se^{+4}	0.21	0.36

Speciation of Se by anion exchange and reverse phase

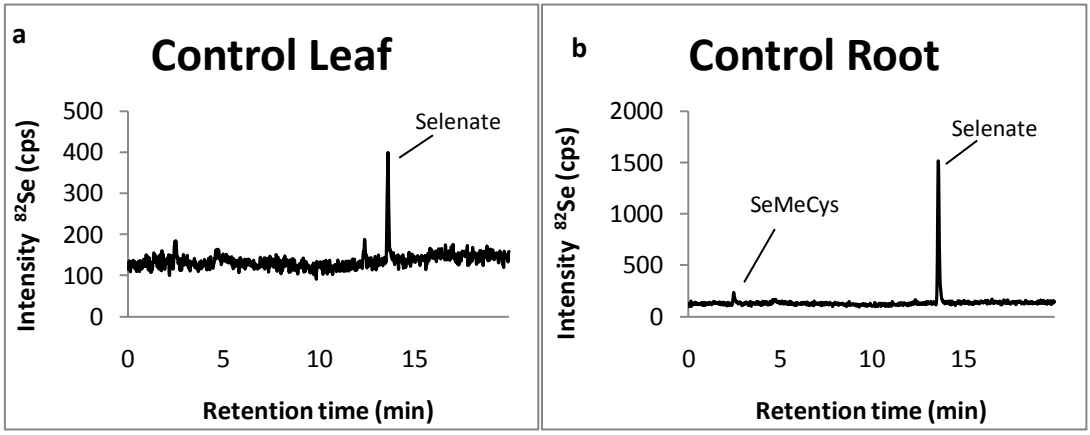
The identification of Se species in this investigation was performed by HPLC-ICP MS and based on the matching of retention time of Se standards: selenate, selenite, SeMet, SeMeCys, oxidized SeMet and oxidized SeMeCys. Identification of Se species was performed by anion exchange and reversed phase HPLC-ICP/MS. The retention time of six standards are presented in Table 7. All Se standards eluted within 15 minutes in both anion exchange and reversed phase. The 25μM selenate and 25μM selenite treatments extracts were selected for Se speciation because of the high Se concentration.

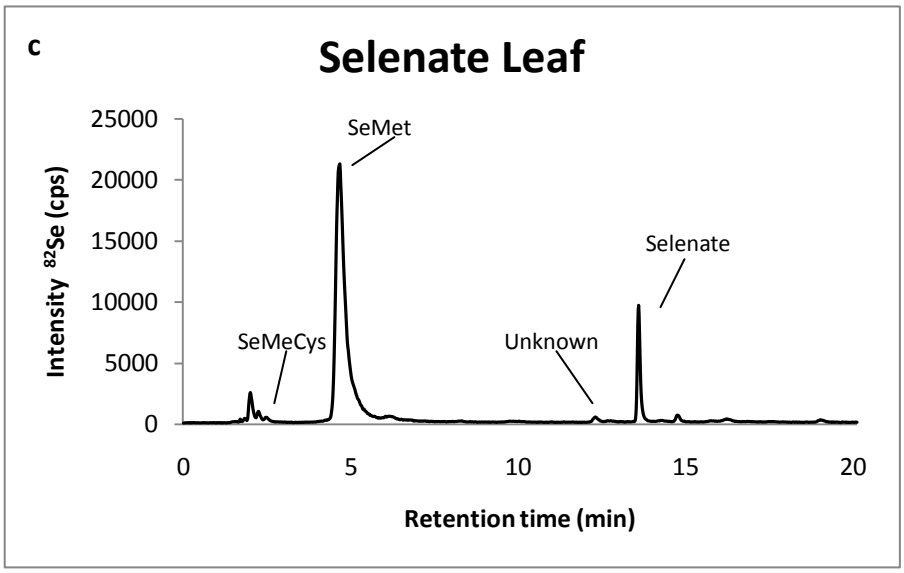
Table 7 Selenium standards retention time (min)

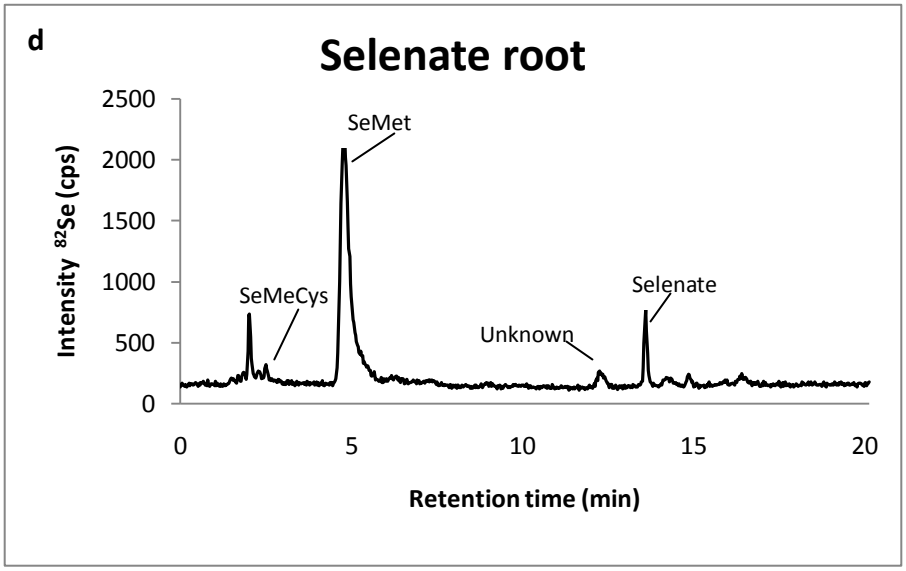
	SeIV	SeVI	SeMet	SeMeCys	SeOMet	SeOMeCys
Anion exchange	5.26	13.63	4.78	2.53	1.88	1.80
Reversed phase	4.76	2.83	-	11.9	6.56	3.18

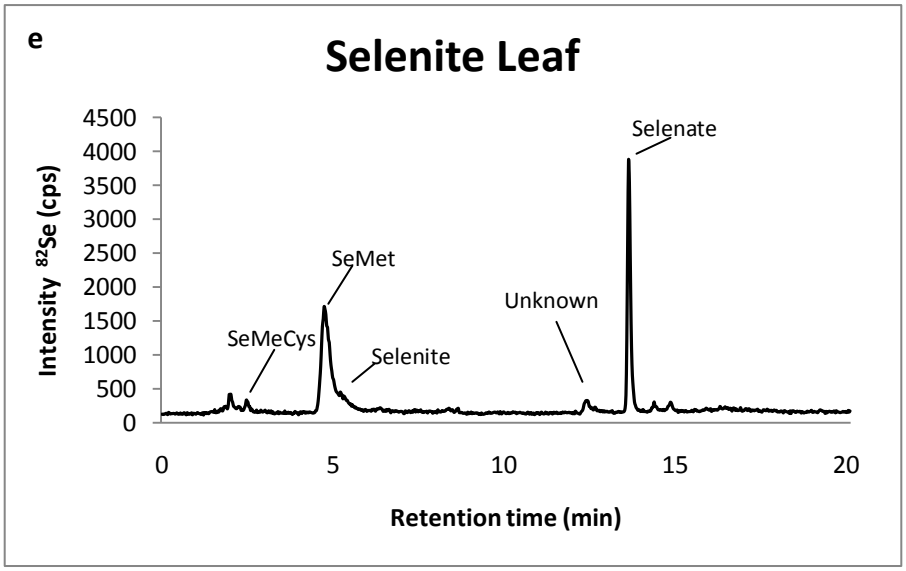
Anion exchange analysis

The anion exchange Se chromatograms are presented in Figure. 6. In control groups (Fig.6 a,b), a big peak eluted at the same retention time as for selenate in leaf and root. In the control root, another small peak eluted as SeMeCys. In 25µM selenate groups (Fig.6 c,d), eight peaks in leaf and nine peaks in root were found in the chromatograms. According to the standard chromatograms of six selenium species, three peaks eluted at the similar time as for SeMeCys, SeMet and selenate were identified, respectively, in both leaf and root. The first two peaks in the chromatograms were in the range of oxidized selenium compounds (etc. oxidized SeMet and oxidized SeMeCys). Due to the weak ionic property of these Se species, the peaks in the anion exchange chromatograms were closed to the column void and the low resolution also resulted in the difficulty to spate these peaks. In 25µM selenite groups (Fig.6 e,f), SeMeCys, SeMet and selenate peaks were also identified. A small Se peak eluted in the tail of SeMet peak was at the same retention time as for selenite (5.26 min). However, the oxidized selenium compounds at the beginning of the chromatograms could not be distinguished as the reason mentioned above. Furthermore, a high intensity peak at the retention time of 12.37 min was not identified.




c
Selenate Leaf
Intensity ^{82}Se (cps)
25000
20000
15000
10000
5000
0
SeMet
Selenate
SeMeCys
Unknown
0
5
10
15
20
Retention time (min)


d
Selenate root
Intensity ^{82}Se (cps)
2500
2000
1500
1000
500
0
SeMet
SeMeCys
Selenate
Unknown
0
5
10
15
20
Retention time (min)


e
Selenite Leaf
Intensity ^{82}Se (cps)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Selenate
SeMet
Unknown
SeMeCys
Selenite
0
5
10
15
20
Retention time (min)

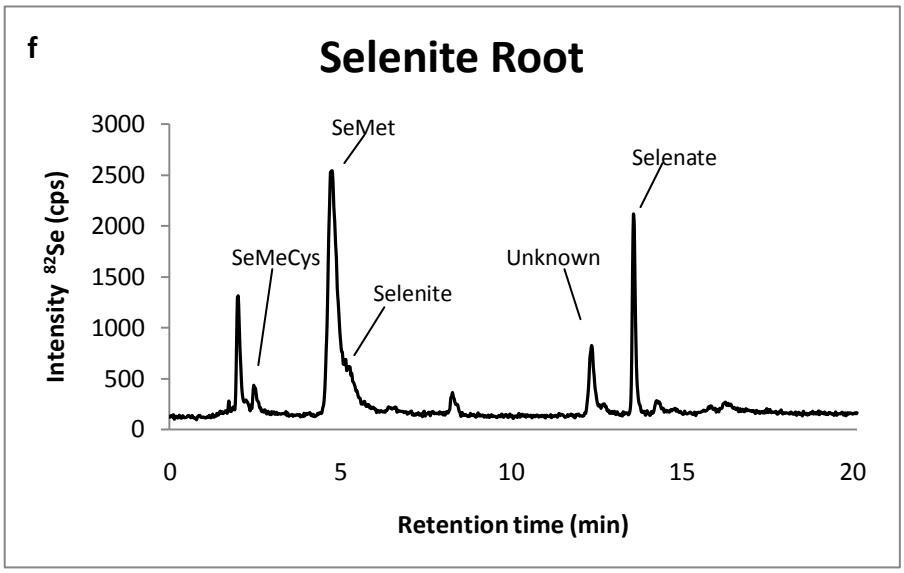
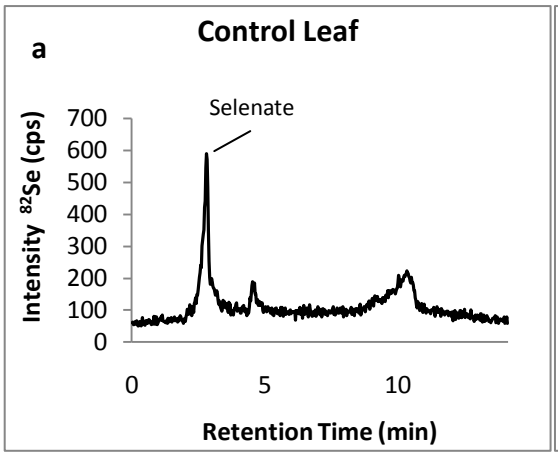


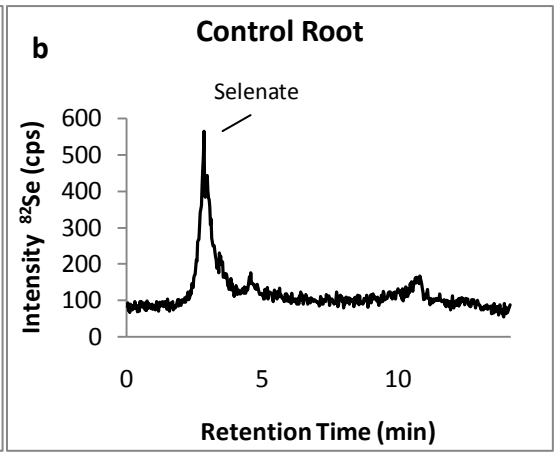
Figure. 6 Anion exchange chromatogram of Se speciation in Arabidopsis leaf and root extracts. (Note the scale differences in y-axis)

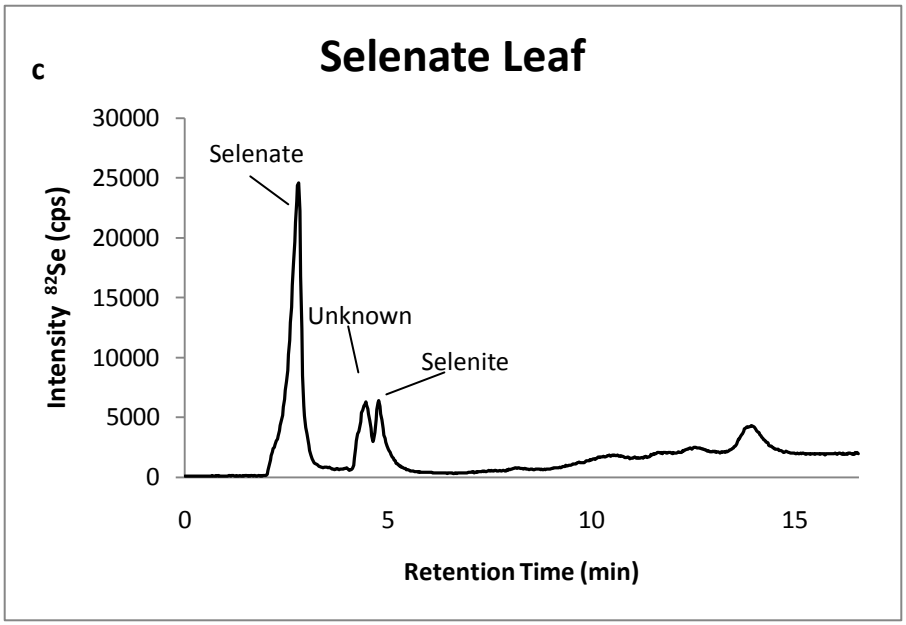
A similar result was also obtained from a previous experiment for Se speciation in selenate treated Arabidopsis separating by anion exchange HPLC (not showed). In this experiment, the samples were extracted using ammonium acetate and the Se species was analyzed according to the retention time of a 6 Se species containing standard chromatograms. Selenate was detected both in root and leaf samples. Due to the close retention time, Selenocysteine and SeOMet peaks were difficult to distinguish. SeMeCys was detected in the root sample, though it disappeared in the leaf sample.

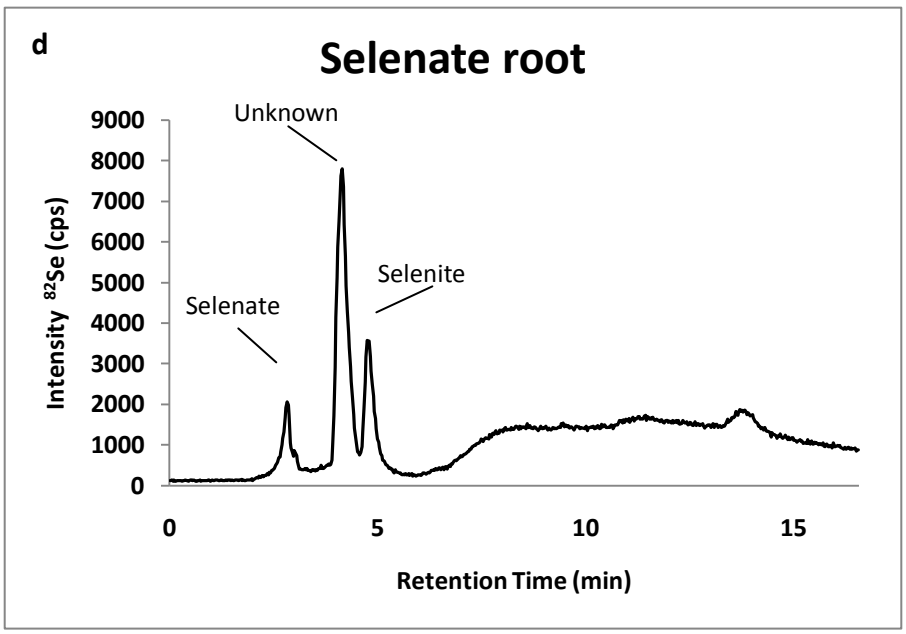
Reversed phase analysis

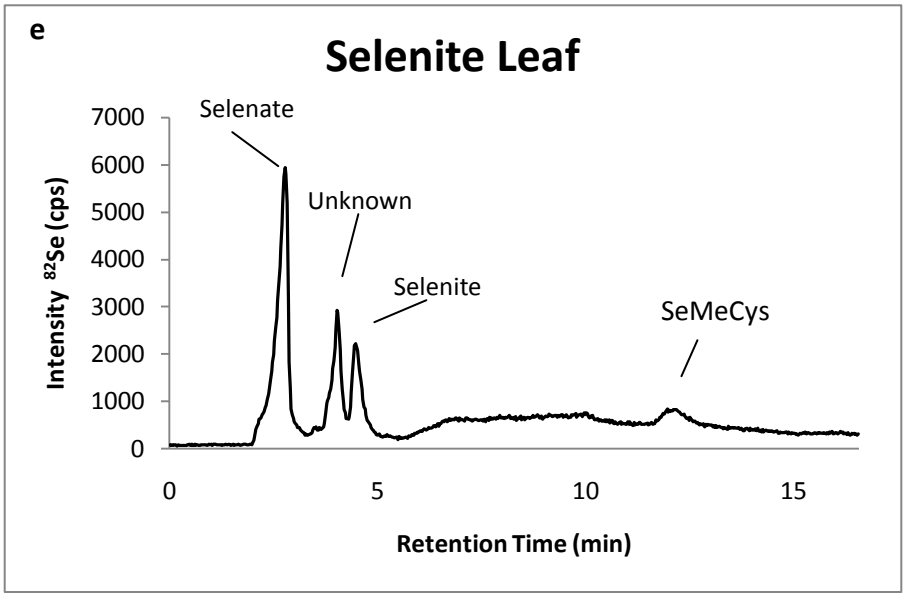
The results of reversed phase chromatograms are presented in Figure. 7. In control group, three peaks appeared in leaf and four peaks appeared in root at very low concentrations. In 25µM selenate and 25µM selenite groups, four peaks were obtained in both leaf and root. The peak eluted at the similar retention time as for selenate (2.83 min) in all groups. Selenite peak (around 4.76 min) was identified in all the Se treated plants. The unknown peak eluted before selenite presented in each samples except the control groups. In selenite treated groups, SeMeCys peaks eluted both in leaf and root.


a
Control Leaf
Selenate
Intensity ^{82}Se (cps)
700
600
500
400
300
200
100
0
0
5
10
Retention Time (min)


b
Control Root
Selenate
Intensity ^{82}Se (cps)
600
500
400
300
200
100
0
0
5
10
Retention Time (min)


c
Selenate Leaf
Selenate
Unknown
Selenite
Intensity ^{82}Se (cps)
30000
25000
20000
15000
10000
5000
0
0
5
10
15
Retention Time (min)


d
Selenate root
Unknown
Selenite
Selenate
Intensity ^{82}Se (cps)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0
5
10
15
Retention Time (min)



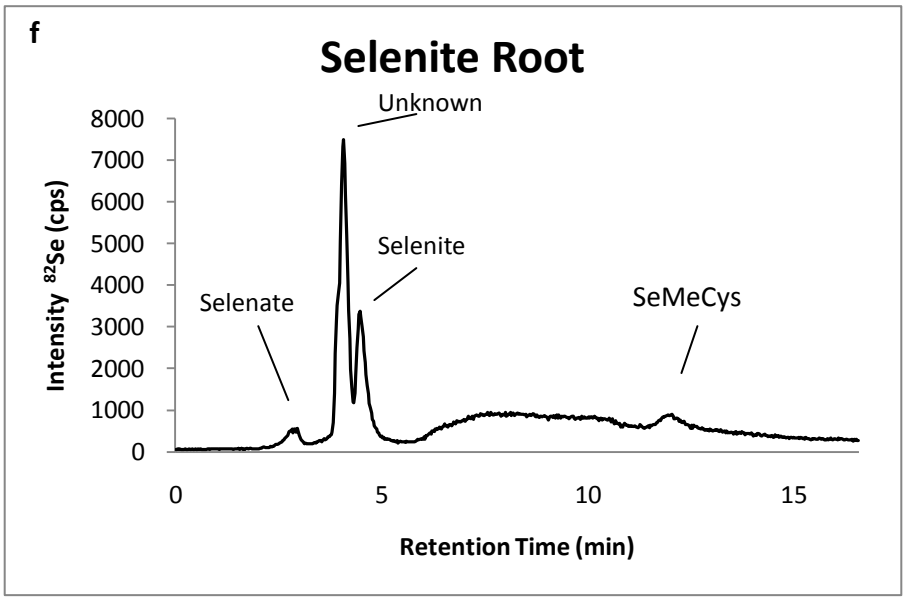


Figure. 7 Reversed phase chromatogram of Se speciation in Arabidopsis leaf and root extracts. (Note the scale differences in y-axis)

Discussion

Observational growing difference

Compared to Arabidopsis plants in growth chamber, plant growth in all groups, except the irradiated plus 25 µM selenate group, were negatively affected by gamma radiation (Fig. 5). Similar results have been found by Wi et al. (2007) who reported that Arabidopsis seedling growth was significantly decreased by high dose gamma exposure of 50 Gy while the seedling growth was slightly enhanced by low dose (1 to 2 Gy) gamma exposure. Vandenhove et al. (2010) estimated that a 10% reduction of Arabidopsis growth could be caused by the dose rate of 60 and 80 mGy h^{-1}. In their studies, gene expression analysis showed a significant transient and dose dependent change in ROS-producing NADPH oxidase (RBOHC) expression which indicated the ROS production induced by gamma irradiation. In the present investigation, however, the ROS measurement (result not showed) found no effect of ROS on the gamma irradiated plants, while the Se applied at the toxic level induced ROS.

On the other hand, ROS concentration has also been tested for plants exposed to low dose (< 1Gy) gamma irradiation (results were not showed) and treated with 0, 0.1 µM and 1 µM selenate. When gamma irradiation dose increased from 0 to 1Gy, the leaf ROS concentration showed a slight decrease in each group. Therefore, low dose gamma irradiation might be thought to protect plants from oxidative stress. For the interesting result of 25 µM selenate treated plants, more work is required to investigate the mechanisms of gamma radiation enhance Arabidopsis growth when high concentrations of selenate are supplied.

Arabidopsis exposed to higher Se concentrations in growth medium were negatively affected as their growth performance decreased (Fig.5). These plants exhibited more yellowish, much withered and smaller size compare than the control groups. These phenomena are the same as the symptoms of Se toxicity in plants (Terry, Zayed et al. 2000). The shoot Se concentration was reported to reduce 10% of yield ranged from 2mg kg^{-1} in rice and 330mg kg^{-1} in clover in nonaccumulators by Mikkelson et al. (1989). In the present study, the leaf Se concentration

(Table 4) was shown to be much higher than in the study of Mikkelson and the growth of Se treated plants was also inhibited more than 10% compare to the controls.

The effect from selenate and selenite supplement to plant growth also varies, which is in accordance with others (Terry, Zayed et al. 2000). In our study, the visual plant growth was reduced more when supplied with selenate than selenite. Similar results were also reported on rapeseed plants by Sucheta et al. (2010) who found the selenate treated rapeseed plants growth were inhibited significantly compared to controls and selenite treatment where the dry matter content of leaves was lower than in selenate treated plants.

Se distribution in Arabidopsis

The plant Se translocations varied with different Se forms. Selenate was more transportable than selenite, which is in accordance with others (Terry, Zayed et al. 2000).

The Se leaf/Se root ratio in our study (Table 6) is consistent with the Zay (Zayed, Lytle et al. 1998) who reported that the Se shoot/Se root ratio ranged from 1.4 to 17.2 when selenate was supplied and less than 0.5 for selenite supplement. According to Zay's study, selenate treated plants also showed the highest accumulation of Se in shoots, followed by SeMet, then selenite; and roots accumulated the highest Se amount when supplied with SeMet, followed by selenite, then selenate. Such distribution of Se is due to that selenate is easily transported from root to leaf, whereas selenite is readily transformed into organic Se when it was taken up by plant root (Ramos, Faquin et al. 2010). For the irradiated groups, leaf Se/root Se ratio was 0.92 for 1 µM selenate treatment and 0.36 for 1 µM selenite treatment. Compared to the non-irradiated 1 µM selenate treatment, the lower ratio of the irradiated plants might be caused by gamma irradiation which may have affected plant growth. Plants exposed to 25 µM selenate or selenite had leaf Se/root Se ratio of 2.34 and 0.36, respectively. Although the 25 µM selenate treated plants in growth chamber have the highest leaf Se/root Se ratio, their leaves developed much smaller and abnormally compared to irradiated plants (Fig 5 g, h). Therefore, gamma radiation probably reduces the selenate toxicity in a certain extent in Arabidopsis, but it may also be because of the medium Se differences and /or small differences in growth conditions that are

expressed because of high to toxic Se concentrations in the plants. To support the above results and discussion, more work is required to investigate on the Se effect on gamma irradiated plants.

Se speciation

Anion exchange

Numbers of Se-containing compounds were detected in plant using HPLC-ICP/MS, though some of the eluted peaks remain unidentified which is in accordance with others (Gergely, Kubachka et al. 2006; Li, McGrath et al. 2008; Mounicou, Dernovics et al. 2009). In the present study, a certain amount of selenate was detected in selenate treated plants, as expected. The most abundant organic Se species found in both leaf and root was SeMet and its amount was approximately two fold of selenate. This is in agreement with Lee et al. (2000) who found SeMet as the dominant Se form in both root and shoot of selenate enriched pickleweed plants while selenate was the second most abundant Se species. In a study of wheat (Li, McGrath et al. 2008), it was reported that only selenate was detected in the root, shoot and xylem water extracts in wheat plants treated with selenate. After transportation in the xylem, the Se is incorporated into proteins as SeMet. Thus, the concentration of SeMet in the plant seems to be dependent of the time after plant selenate uptake.

In the chromatograms of selenite treated plants, several peaks were presented. The dominant peak in the Se-chromatogram was at the same time as selenate, which probably resulted by the contamination in the anion exchange column or an unknown interference (e.g. $^{40}Ar_2H_2^+$, $^{12}C^{35}Cl_2^+$, $^{34}S^{16}O_3^+$, $^{81}Br^+$). This unknown peak did also occur in the controls. When the same samples of control group were measured one more time (another day), this selenate peak were at the back ground level (result not showed), and the intensity of selenate became ca. 4 times lower than SeMet in other selenate treated replicates. Due to the problem of the instrument, however, the measurement could not be continued and the result presented here were with the unknown peak.

Similar as Kahakachchi and Hua-Fen Li's (Kahakachchi, Boakye et al. 2004; Li, McGrath et al. 2008) research, the extracts of selenite treated plant contained more Se species, such as the organic species (e.g. SeMet, SeMeCys), oxidized Se compounds and the inorganic species (e.g. selenite). Compare to the minor species of selenite, organic Se (SeMet) remain the major species and showed a much higher intensity in root samples. This is probably due to the conversion from selenite to organic Se forms (most SeMet) in plant roots. The identification of a small amount of SeMeCys in root and leaf samples is similar to the research of Li et al. (2008) who found SeMeCys as minor organic Se species in the selenite treated plants. However, SeMeCys was not detected in the Indian mustard plants by (Kahakachchi, Boakye et al. 2004) even they used the protease to hydrolyze the samples. The oxidized organic Se peaks eluted at the beginning of the chromatograms were difficult to distinguish due to the same retention times. Additionally, some unidentified Se species were also detected. The unidentified Se peak which eluted before selenate (12.37 min) is probably an anion Se species, which showed a high intensity in the selenite treated root extracts but a much lower intensity in the other samples.

Reversed phase analysis

The reversed phase chromatograms in our study gave a poor result for the identification of SeMet in the standard chromatogram (not showed), and some unexpected peaks were eluted in the control groups.

The selenate peak in the control groups and other samples, as discussed in anion exchange chromatography, could be caused by the impurity in the instrument. On the other hand, as Mounicou (2009) mentioned, selenate was eluted close to the column void which might contain some unresolved chromatographic signals of other ionic species.

In SeMet standard chromatogram, the SeMet could not be significantly found as other standard, and even, the dominant peak was selenate which might be caused by the high concentration of the selenate standard just measured before SeMet. In all the Se treated samples, however, a high intensity unknown peak was presented at 4.16 min. According to the result from anion

exchange, large amount of SeMet was found in all the measured samples. Hence, the unknown peak was supposed to be SeMet and the standard chromatogram of SeMet might get mistakes.

In anion exchange chromatograms, small amounts of SeMeCys were detected in all the Se treated samples. However, the small peak of SeMeCys was only found in selenite treated plants while no SeMeCys peak was found in selenate treatments. This could be because the intensity in the background was so high that the SeMeCys peak disappeared in the background.

The high intensity of selenite in the selenate treated samples was confusing. In anion exchange chromatograms, no selenite peak was found in the selenate treatments. Thus, the selenite could not be in the samples. In control groups, there was a peak, much smaller than the selenate peak, which eluted at the similar time as selenite (4.58 min) which might be due to the impurity in the instrument or unknown interferences. Accordingly, the selenite peak in selenate treatments might be caused by the impurity. However, if the large amount of selenite was the result of an impurity, this still needs to be confirmed.

Considering the long period (about 4 months) for the sample storage and the repeated measuring of the same samples (defreeze and refreeze frequently), some changes (impurity or other reaction in the samples e.g.) could have resulted. Hence, those changes in the sample probably caused the poor results of the speciation. Although the reversed phase HPLC was considered to have a high resolving power for selenoamino acids, in the present study, the peaks in the chromatogram were not well distinguished (broad peak area). This might be due to the lower amount of certain Se species in the samples.

Conclusion

This study showed that Arabidopsis thaliatha could accumulate a certain amount of Se when treated with selenate or selenite enriched growth medium. The distribution of Se in plant was in agreement with previous studies - selenate was transported and distributed in plant leaf while selenite was located in plant root. Arabidopsis plants treated with higher Se concentrations were shown to be negatively affected in plant growth and development. However, the 25 μM selenate treatment might protect plants from radioactive stress. Selenium speciation analysis by anion exchange HPLC-ICP-MS could identify four Se species (selenate, selenite, SeMet, SeMeCys), and the identification of other peaks needs more Se standards. Reversed phase HPLC gave a poor result in this study, which probably was because of long storage period or the repeated measuring of samples.

For the further study of the protective role of Se in plants, more work is required and the analytical methods need to be improved. For Se speciation, in this case, a collision or reaction cell is needed to be able to analyze the samples at lower Se concentration with a higher resolution. Using a Size Exclusion Chromatogram (SEC) prior to the sample analyses is a preferred procedure to fractionate the samples, and the optimization of the chromatographic elution strategy could be optimized; however, the low amount of the samples did not allowed us to do these procedures which require more sample materials. Thus, the experiment should be started with some plants, which is expected to have similar matrix as Arabidopsis and give more biomass and high Se concentration, to gain more experiences for the future analysis of Arabidopsis.

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