

Strontium toxicity in *Arabidopsis thaliana* disrupts calcium homeostasis and induces oxidative stress

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Research Article

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Abstract

Strontium (Sr), a nonessential element for plants, shares key chemical similarities with calcium (Ca) and is becoming increasingly prevalent in the environment. However, the phytotoxic effects of Sr remain insufficiently characterized. In this study, we examined the effects of Sr on *Arabidopsis thaliana* grown under both Ca-sufficient and Ca-deficient conditions. At high concentrations (10 mM), Sr markedly inhibited both root and shoot growth, disrupted Ca homeostasis, and promoted the accumulation of reactive oxygen species. Exposure to Sr also impaired cuticular layer formation in leaves and reduced anthocyanin content, indicating compromised stress responses. Circular dichroism spectroscopy revealed that Sr alters the secondary structure of peptide hormones, suggesting that it interferes with signaling pathways. Genetic screening of ethyl methanesulfonate-mutated *Arabidopsis* lines revealed mutations in OPT6 and CNGC9, implicating these transporters in Sr uptake and toxicity. Collectively, our findings demonstrate that Sr cannot substitute for Ca in physiological functions and exerts toxicity through oxidative stress, membrane damage, and disruption of peptide signaling.

1 Introduction

Strontium (Sr) is present in the Earth's crust at ~ 370 ppm (Turekian and Wedepohl 1961). In European topsoil, the median Sr concentration is 89 mg/kg, with reported values ranging from 8–3120 mg/kg (Salminen 2005). In plants, Sr is a nonessential element, and high toxicity has not been reported. The absorption rate of Sr by plants varies among species and depends on soil properties (Uchida 2007a, b). Sr and calcium (Ca) are alkaline earth metals, and Sr shares similar chemical properties with Ca. Ca is involved in salt and insect stress responses and has been reported to be transported over long distances within minutes (Choi 2014). Plants with high Ca accumulation also exhibit high Sr accumulation (Tsukada 2005, Uchida 2007b). We previously reported that tomato plants accumulate both Ca and Sr (Nagata 2019). Several plant species, including *Arabidopsis* (Kanter et al. 2010), soybean (Sowa 2014, Wojciak-Kosior 2016), and *Amaranthus* (Chu 2015), have been reported to accumulate higher levels of Sr in shoots than in roots.

Sr has the potential to disturb the absorption and distribution of Ca. Ca increases mechanical strength by cross-linking pectin during the formation and maintenance of cell walls (Ezaki et al. 2005). In the cell wall, Ca interacts with the carboxyl groups of pectin as Ca^{2+} to form intermolecular cross-links and gel pectin (Jarvis, 1984). The cuticular layer is a waxy tissue that forms on the surface of the leaf cell wall and consists mainly of lipids, specifically cutin and wax (Kolattukudy, 1980). Sr may influence multiple morphological processes; however, its effects on leaf morphogenesis have not been examined in detail. Toluidine blue stains leaves lacking an intact cuticular layer (Tanaka et al. 2004). Therefore, we examined whether Sr affects cuticular layer formation in leaves.

Reactive oxygen species (ROS) homeostasis plays a central role in plant growth and development. At low concentrations, ROS function as internal signaling molecules (Mittler, 2017). However, at high concentrations, ROS cause oxidative damage to proteins, lipids, and other macromolecules (Apel and

Hirt, 2004, Jin et al. 2021). NADPH oxidase, also referred to as respiratory burst oxidase homolog (RBOH), has been identified as an enzyme responsible for generating superoxide anion radicals in *Arabidopsis thaliana* (Kaya et al. 2019). Superoxide anion radicals are induced by cadmium (Cd) stress (Abozeid et al. 2017). On the basis of these observations, we hypothesized that Sr also induces superoxide anion radical production.

Casparian strips are known to regulate mineral absorption, including Ca uptake (Li et al. 2017). These structures are formed by the deposition of hydrophobic lignin within the cell wall. Li et al. reported that suberin accumulation is affected by Ca availability and visualized suberin deposition in roots via the use of Fluorol Yellow 088 (Li et al. 2017). It is therefore possible that Sr affects Ca uptake in roots through changes in lignin and suberin deposition. In addition, Ca ions play an important role in root tip growth, suggesting that Sr interferes with amyloplast formation during columellar cell maturation. We therefore examined the effects of Sr on the development of these tissues.

In this study, we hypothesized that Sr does not function as a Ca substitute, induces ROS generation, and involves specific transporters in its uptake. Accordingly, we pursued three objectives: to elucidate the effects of Sr under Ca-deficient conditions, to assess whether ROS production contributes to Sr toxicity, and to sequence the genomes of selected mutant strains. We therefore focused on Ca-associated toxicity and sought to identify genes related to Sr toxicity via the use of randomly mutagenized lines generated by ethyl methanesulfonate (EMS). Finally, AlphaFold2 and PyMOL were used to predict the three-dimensional structures of proteins encoded by mutated genes identified in the variant strains.

2 Materials and methods

2.1 Plant material and growth conditions

Seeds of the *Arabidopsis thaliana* (L.) Heynh., Col-0 line were obtained from Inplanta Innovations, Inc. (Kanagawa, Japan) and surface sterilized in 70% (v/v) ethanol for 2 min, followed by treatment with 10% (v/v) commercial bleach containing detergent (Kitchen Haite, Kao, Tokyo, Japan), which includes sodium hypochlorite (NaOCl), alkyl ether sulfate sodium salt, and sodium hydroxide (NaOH), for 7 min. The seeds were subsequently rinsed five times with sterilized water and then sown on Murashige and Skoog (MS) media containing B5 vitamins, 0.8% (w/v) agar, and 1% (w/v) sucrose (Murashige and Skoog 1962). The MS agar plates were supplemented with 0.1, 1, or 10 mM SrCl_2 . Plates without SrCl_2 were used as controls. Next, to examine the effects of Sr on growth under Ca-deficient conditions, CaCl_2 was omitted from the MS composition. All the cultures were maintained at 23°C under a 16-h light/8-h dark photoperiod and grown for two weeks in an MLR-350 growth chamber (Sanyo, Osaka, Japan). Unless otherwise stated, all the experiments in this study were conducted with *Arabidopsis* seedlings two weeks after sowing.

2.2 Growth assays and determination of Sr and Ca contents under Ca-deficient conditions

Plants grown in the absence of Sr and in the presence of Ca were used as controls (Ca + and Sr-). After two weeks of incubation, the plants were harvested and separated into shoots (A) and roots (B). Fresh weights were measured for each tissue. The Sr and Ca contents of the shoots and roots were determined via atomic absorption spectrophotometry (ContrAA700, Analytic Jena, Germany) after the samples were digested with concentrated nitric acid at 90°C for 1 h (Nagata, 2023).

2.3 Toluidine blue test

The detection of cuticular defects in whole leaves via the toluidine blue test was performed following a previously described method (Tanaka et al. 2004). Seeds were surface sterilized and sown on MS media solidified with 0.4% (w/v) gellan gum (Wako Pure Chemicals Industries Ltd., Osaka, Japan). An aqueous solution of 0.05% (w/v) toluidine blue (TB) (Sigma–Aldrich Japan, Tokyo, Japan), previously filtered through a fiber media filter (pore diameter, 0.2 µm; Sartorius Japan K.K., Tokyo, Japan), was poured directly onto the plates until the plants were fully submerged. After 2 min, the TB solution was removed, and the plates were gently washed with water to remove excess dye from the plants.

2.4 Determination of anthocyanin content

Anthocyanins were extracted from leaves treated with various concentrations of Sr in MeOH:HCl (99:1, v/v) for 48 h in the dark at 4°C. The absorption of the supernatant was measured at 535 nm via a UV/Vis spectrometer (BioSpec Mini, Shimadzu, Japan). The anthocyanin concentration was calculated via an equation that allows the simultaneous determination of anthocyanins in the extract (Abdel et al. 1999).

2.5 Determination of the circular dichroism spectrum

The Sr solution was neutralized with NaOH to obtain a final concentration of 200 mM. This solution was added to 1.5 mg/mL glutathione (reduced form, GSH) (Tokyo Kasei, Tokyo, Japan). Circular dichroism (CD) spectra were recorded via a JASCO J-805 spectropolarimeter (JASCO corp., Tokyo, Japan) at 25°C. Septa-sealed quartz cuvette cells with a 1 mm path length were used for all CD measurements.

2.6 Lignin and suberin lamella analysis

The roots were washed with deionized water, and autofluorescence was detected via a standard GFP filter under a fluorescence microscope (IX83, Olympus, Tokyo, Japan) (Naseer et al. 2012). The excitation and emission wavelengths were 470–495 nm and 510–550 nm, respectively. Additional root samples were stained with 0.1 mg/mL Fluorol Yellow 088 (Setareh Biotech, LLC) for 30 min at 70°C, as previously described (lux 2005). The samples were observed via a microscope equipped with a fluorescence module with the same excitation and emission settings. The ratio of lamellar thickness to total root thickness was quantified via ImageJ. All the images are representative of the staining observed in the roots from ten independent experiments, with microscopic fields randomly selected across the samples.

2.7 Amyloplast staining using Lugol

The effect of Sr on amyloplast formation was assessed via Lugol staining. Roots treated with or without Sr were fixed and dehydrated in an acetic acid and ethanol solution for 1 h. Subsequently, the roots were dehydrated by sequential soaking in an ethanol series (70%/phosphate-buffered saline (PBS) and 30%/PBS) for 5 min each. After this treatment, the solution was replaced with PBS lacking ethanol, and the roots were incubated for an additional 5 min. Whole plants were then stained with Lugol solution (Sigma–Aldrich Japan, Tokyo, Japan) for 10 min. After staining, the roots were washed three times with PBS and immersed in a tissue-clearing reagent, TOMEI (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan). All the images represent the staining patterns detected in ten independent experiments.

2.8 Ion leakage

Next, we tested ion leakage in the root tissues. To do so, roots grown in the presence of Sr were harvested, washed, and immersed in water. After 3 h of incubation, the electrical conductivity was measured to quantify the ions released into the immersion solution. The samples were then heated at 98°C for 1 h, after which the electrical conductivity was measured again. The conductivity measured before heating was divided by that measured after heating, and this ratio was used to evaluate cell membrane damage.

2.9 Superoxide anion radical quantification via MCLA

2-Methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one hydrochloride (MCLA) was used for ROS quantification, particularly for $O_2^{\cdot -}$ and $^1O_2$ (Nakano 1998). ROS measurements were conducted according to Kobayashi et al. (2004). After treatment, each root was washed once with 0.1 mM $CaCl_2$ (pH 4.75) and preincubated with 25 μ M MCLA (Tokyo Kasei, Tokyo, Japan) for 10 min. Subsequently, the roots were washed once with 0.1 mM $CaCl_2$ (pH 4.75) and cut with a razor blade to obtain 10-mm sections from the root tip. One root section was placed in a tube containing 200 μ L of 0.1 mM $CaCl_2$ (pH 4.75), and MCLA-dependent luminescence was measured via a luminometer (model LB9506; EG&G, Berthold, Germany).

2.10 Superoxide anion radical staining using nitro blue tetrazolium

For *in situ* $O_2^{\cdot -}$, samples were stained with nitro blue tetrazolium (NBT) (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) following a previously described method with slight modifications (Fryer et al. 2002). The shoots and roots were vacuum infiltrated for 10 min. Subsequently, the shoots were transferred to a bleaching solution of ethanol:acetic acid:glycerol (3:1:1), whereas the roots were transferred to 5% TOMEI (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) in PBS, and each tissue sample was heated at 98°C for 5 min. After heating, the samples were mounted on glass slides and observed under a microscope. All figures represent staining detected in ten or more independent experiments for each tissue. The staining intensity was quantified via ImageJ (rsb.info.nih.gov/ij).

2.11 Screening and clustering of genes related to Sr phytotoxicity

Arabidopsis seeds were treated with 0.1% ethyl methanesulfonate (EMS; Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) for 16 h and subsequently grown on Sr-containing media. The Sr content in leaves was measured, and candidate plants exhibiting altered Sr accumulation were subcultured to the fourth generation. After harvesting leaves from the fourth generation, genomic DNA was extracted, and base replacement sites were identified via next-generation sequencing. Next-generation sequencing was outsourced to Azenta Japan Corp. (Shinagawa, Tokyo) and performed via strand-specific RNA-Seq via a Novaseq X Plus at 4 Gb per sample. The libraries were prepared via the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (E7760).

The adapter sequences are listed below.

Read1 5'-AGATCGGAAGAGCACACGTCTGAACTCCAGTCA-3'

Read2 5'-AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT-3'

Information on the mutated genes is summarized in the table.

2.12 Predicted protein structures of mutant proteins

The three-dimensional structures of proteins were predicted from mutant genes identified via next-generation sequencing analysis. AlphaFold2 (<https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb>) was used to generate structural predictions and corresponding PDB files. The resulting PDB files were analyzed for 3D disorder via PyMOL 3.1 (<https://www.pymol.org>).

2.13 Statistical analyses

All the experiments were performed with ten independent biological replicates. The data are presented as the means of ten technical replicates. Differences between treatments were statistically assessed via Tukey's test or Dunnett's test. For all the statistical tests, statistical significance was inferred if the *P* value was < 0.05.

3 Results

3.1 Effects of Sr on growth under Ca deficiency

Fresh weights of shoots and roots were measured to evaluate the effects of the presence or absence of Sr and Ca on plant growth. In addition, the Sr and Ca concentrations in the shoots and roots were determined (Fig. 1). Under Ca-deficient conditions, shoot fresh weight was reduced to approximately two-thirds of that of the control, regardless of Sr treatment. The Sr concentration in shoots grown in the

presence of Ca was 1.5-fold greater than that in shoots grown without Ca. Conversely, the Ca concentration in shoots grown in the presence of both Sr and Ca was 50% lower than that in shoots grown in the presence of Sr but without Ca. However, shoots grown in the absence of both Ca and Sr presented Ca concentrations that were 1.5-fold greater than those in shoots grown in the presence of both Sr and Ca.

The fresh weight of roots grown on media supplemented with Sr and Ca was approximately 20% lower than that of the control. Under Ca deficiency conditions, the fresh weight of the roots was reduced to approximately 50% of that of the control, with or without Sr treatment. The Sr concentration in roots grown in the presence of Ca was 1.2-fold greater than that in roots grown in the absence of Ca. Similar to the results observed in shoots, the Ca concentrations in roots grown in the presence of both Ca and Sr were significantly lower than those in the control. Under conditions lacking both Ca and Sr, the Ca concentrations were comparable to those of the control.

3.2 Effects of Sr on shoot physiological functions

First, the effect of Sr on cuticular layer formation was examined. In the control and 0.1 mM Sr treatments, some leaves were stained, whereas most leaves remained unstained (Fig. 2A). In the 1 mM Sr treatment, a greater number of leaves were stained than in the control, and in the 10 mM Sr treatment, staining was observed in most leaves.

Previous studies have reported that, in corn, exogenous Sr treatment significantly increased both the Sr content and the anthocyanin content in kernels (Binhan et al., 2026). Therefore, we measured the anthocyanin content. After a two-week incubation at 25°C, the effects of Sr on anthocyanin concentrations in leaves were assessed. Although no statistically significant differences were detected among the Sr treatments, a decreasing trend in anthocyanin content was observed at 1 and 10 mM Sr (Fig. 2B).

In the absence of Sr, the circular dichroism spectrum of glutathione (GSH) exhibited an absorption minimum between 190 nm and 220 nm (Fig. 2C). In comparison, in the presence of Sr, the spectrum displayed a distinct peak at 210 nm.

3.3 Effects of Sr on the physiological functions of roots

Next, lignin and suberin were observed via fluorescence microscopy to examine the effects of Sr in the mature root zone (Fig. 3A and 3B). Both lignin and suberin were detected in roots exposed to Sr at all the tested concentrations. No ectopic formation of either component was observed. Similarly, no significant differences in lignin or suberin thickness were detected between roots grown in the presence or absence of Sr.

To assess the effects of Sr on the root cap, amyloplast formation in columellar cells was examined by staining and observation of the root tip. Under all the experimental conditions, no differences in amyloplast formation were detected (Fig. 3C).

Root ion leakage was measured to evaluate whether root cell membranes were damaged by ROS, such as superoxide anion radicals. No significant differences in ion leakage were detected between Sr-untreated plants and plants exposed to Sr concentrations less than 1 mM (Fig. 3D). However, the roots grown in the presence of 10 mM Sr presented a slight but significant increase in ion leakage.

3.4 Effects of Sr on ROS products

MCLA was used to quantify superoxide anion radicals ($O_2^{\cdot-}$) in the shoots and roots of *A. thaliana* grown for two weeks on agar media supplemented with various concentrations of Sr. With increasing Sr concentrations, $O_2^{\cdot-}$ in both shoots and roots significantly increased relative to that in the control (Fig. 4A). Luminescence was ~ 2-fold greater in shoots exposed to 10 mM Sr than in the control. In the roots of plants treated with 10 mM Sr, the luminescence increased ~ 4-fold relative to that of the control.

$O_2^{\cdot-}$ can reduce NBT to form a dark blue, insoluble formazan compound. No significant differences in the root meristem were observed between the Sr-untreated plants and the plants exposed to Sr (Fig. 4B).

3.5 Screening of Sr phytotoxicity-related genes

Next, to identify mutations that influence Sr absorption, an EMS-mutagenized population was screened. Cotyledons of ~ 50 seedlings derived from each independent M4 line were first examined for their Sr accumulation capacity, resulting in the identification of one Sr high-accumulator line and one Sr low-accumulator line. The phenotype evaluated in the mutant screening was the Sr accumulation capacity. While the average Sr accumulation concentration in the M4 lines was 15 $\mu\text{mol/g}$, it was 42 $\mu\text{mol/g}$ in the high-Sr-accumulator line and 6 $\mu\text{mol/g}$ in the low-Sr-accumulator line. However, no characteristic observable changes were observed compared with those of the wild-type strain. Leaves from each candidate line were harvested, and genomic DNA was extracted. The sequences obtained via next-generation sequencing were compared with those of the Col-0 wild-type line. In total, 32 mutant genes were identified in the Sr high-accumulator line (Table 1), whereas 27 mutant genes were identified in the Sr low-accumulator line (Table 2). In this study, we focused on genes involved in transport and examined single-nucleotide polymorphisms (SNPs) and insertions of deletions (InDels). As a result, SNP mutations were identified in oligopeptide transporter 1 (OPT6) in the Sr high-accumulator line and in cyclic nucleotide-gated channel 9 (CNGC9) in the Sr low-accumulator line.

Table 1
SNPs and their amino acid variants in Sr-hyperaccumulating EMS mutant strains.

Chromosome	Gene	SNP	Amino acid substitution
1	<i>FAH</i>	904G→A	W247*
1	<i>AT1G33770</i>	1959G→A	E483K
1	<i>PCMP-H68</i>	772G→A	R208K
1	<i>EMB3003</i>	1044C→T	T302I
1	<i>ATNPC1</i>	2206G→A	A999T
1	<i>IRE3</i>	5830G→A	G1150S
1	<i>PEX3</i>	2098C→T	S327L
1	<i>AT1G55090</i>	2345C→T	P701L
2	<i>CLASP</i>	2221G→A	S640N
2	<i>TIC236</i>	10813G→A	G2058E
2	<i>AT2G29060</i>	2029C→T	P568S
2	<i>LTP2</i>	447C→T	P48S
2	<i>AT2G42640</i>	3108G→A	A383T
2	<i>MPK6</i>	622C→T	P131S
2	<i>SDH8</i>	19043068G→A	promoter
3	<i>AT3G20362</i>	119A→G	Q40R
4	<i>NET1C</i>	3278G→A	E980K
4	<i>AT4G03480</i>	1992G→A	C407Y
4	<i>EXO</i>	772C→T	P205S
4	<i>OPT6</i>	2126G→A	A645T
4	<i>scpl29</i>	801C→T	A158V
4	<i>AT4G33600</i>	400G→A	E121K
4	<i>P4H11</i>	131C→T	S44F
4	<i>AT4G38092</i>	735C→T	A92V
5	<i>PAE10</i>	2330C→T	P304S
5	<i>RLP53</i>	450G→A	D127N

Chromosome	Gene	SNP	Amino acid substitution
5	<i>BIP1</i>	1657G→A	A525T
5	<i>BIP1</i>	2618G→A	terminator
5	AT5G33406	260G→A	R87K
5	<i>MSI17.60</i>	287C→T	S52F
5	<i>MG03</i>	852G→A	E256K
5	<i>TUB2</i>	677C→T	S138L

Table 2
SNPs and their amino acid variants in Sr hypoaccumulating EMS mutant strains.

Chromosome	Gene	SNP	Amino acid substitution
1	AT1G11070	1772C→T	P423S
1	AT1G12020	653C→T	R137C
1	AT1G53560	367C→T	P72L
1	<i>TCP8</i>	476C→T	R58W
2	AT2G19680	1589C→T	A94V
2	<i>RPOT3</i>	427G→A	E106K
2	AT2G30710	1209G→A	A305T
2	<i>ATL70</i>	896G→A	A212T
2	<i>PRP38</i>	1134G→A	R311Q
2	<i>CYP86A8</i>	1740G→A	V388M
2	<i>SPA1</i>	939G→A	E78K
4	AT4G03440	2288C→T	P405L
4	<i>PSBQ2</i>	798C→T	A163V
4	AT4G05632	214G→A	A72T
4	<i>MEKK1</i>	1021C→T	P255S
4	<i>MYB41</i>	1353T→A	M281K
4	<i>CNGC9</i>	1381G→A	G187S
4	AT4G36980	2924C→T	L505F
5	AT5G26740	9292260C→T	promoter
5	<i>PAE10</i>	3006G→A	terminator
5	<i>SBT4.12</i>	1479G→A	S244N
5	<i>PCMP-E1</i>	1449G→A	A438T
5	<i>A4</i>	2132C→T	terminator
5	<i>PDCB1</i>	657G→A	G149E
5	<i>TOR1L3</i>	765C→T	S89F
5	AT5G63690	603G→A	G63R

Chromosome	Gene	SNP	Amino acid substitution
5	<i>NPY3</i>	2465G→A	D257N

OPT6 is a transporter with a broad substrate range, including complexes of phytochelatin (PC) and metals (Lubkowitz 2011), and the mutation is located in the 14th transmembrane region (Fig. 5). The amino acid residue at position 645 was mutated from alanine to threonine. Structural prediction indicated that the conformation of the SNP mutant OPT6 did not differ from that of the wild-type OPT6.

CNGC9, a channel known to be involved in Ca^{2+} absorption (Talke et al. 2003), was mutated between transmembrane domains H2 and H3. At position 187, the amino acid residue was substituted from glycine to serine (Fig. 6A). This substitution markedly altered the loop structure. CNGC9 has been proposed to form tetramers, although it remains unclear whether these tetramers are homo or heterotetramers (Jarratt-Barnham et al. 2021). Therefore, homo and heterotetramers were modeled via PyMOL. In the homotetramer, a distinct pore was observed in the wild-type structure, whereas the mutant formed a tetramer lacking an observable pore (Fig. 6B). Among the heterotetramers composed of CNGC9 and CNGC6, pores were observed in the wild type at all the tested ratios (Fig. 6C). In heterotetramers consisting of mutant CNGC9 and CNGC6, pores were observed at 2:2 and 1:3 ratios but not at a 3:1 ratio. Furthermore, in the heterotetramers of CNGC9 and CNGC14, pores were not detected in either the wild-type or mutant forms.

4 Discussion

We have previously shown that Sr induces ROS generation and cell death in Arabidopsis (Nagata 2023). However, the mechanisms underlying Sr phytotoxicity remain unclear. In this study, we focused on Ca, examined its association with toxicity, and sought to identify genes involved in Sr toxicity in randomly mutagenized lines generated with ethyl methanesulfonate (EMS).

This study examined how the presence or absence of Ca influences the growth-inhibitory effects of Sr. In the presence of Ca, Sr toxicity was more pronounced in the roots than in the shoots (Fig. 1). Under Ca deficiency conditions, no significant differences in Sr toxicity were observed between the shoots and the roots. These findings suggest that the growth inhibition caused by Ca deficiency had a greater impact than that caused by Sr toxicity. Under Ca-deficient conditions, Sr did not promote growth in either shoots or roots, indicating that Sr cannot substitute for Ca. Compared with those under Ca-sufficient conditions, the Sr concentrations under Ca-deficient conditions tended to decrease. In contrast, Ca concentrations tended to increase under Sr-present conditions compared with those under Sr-absent conditions. Notably, although Ca-free MS medium was used, residual Ca was likely present due to Ca contamination in the agar. Together, these results suggest that Sr alters Ca accumulation and disrupts Ca-mediated physiological functions.

Among the Ca-dependent physiological processes, we focused on cell wall formation and examined the effects of Sr on cuticular layer development at the cell wall surface. In the plants treated with 10 mM Sr,

most of the leaves presented staining indicative of cuticular defects (Fig. 2A). The inhibition of cell wall formation resulting from Sr-induced Ca deficiency may therefore impair cuticular layer formation. Anthocyanins are known to be antioxidant compounds in plants (Zhang et al. 2015, Xu and Rothstein 2018). In the presence of 1 and 10 mM Sr, the anthocyanin content tended to decrease (Fig. 2B), suggesting that Sr inhibits anthocyanin synthesis in Arabidopsis. In addition, reports indicate that the addition of Sr to other crops, such as wheat, leads to a decrease in biomass and a reduction in the number of leaves and SPAD values, resulting in a deterioration of indicators related to photosynthesis and growth (Ammar et al. 2025). This phenomenon is believed to reflect Sr uptake by the roots, its subsequent transport, and an imbalance in nutrient levels. We next examined the effects of Sr on glutathione (GSH), another key antioxidant, *in vitro*. The circular dichroism spectrum of GSH was altered in the presence of Sr, suggesting the formation of a Sr–GSH complex. It is also possible that such complexes form within plant cells. Finally, since Sr appears to affect shoot morphogenesis and homeostasis, we further investigated its effects on root morphogenesis and homeostasis.

We focused on lignin and suberin formation, which play crucial roles in nutrient absorption. Our results indicate that lignin and suberin deposition were unaffected by Sr treatment (Fig. 3A and 3B), suggesting that their regulatory expression and localization are not disrupted by Sr. Since these structures are formed in the mature root region, the results further imply that Sr absorption in this region may be restricted by the Casparian strip. Columellar cells protect the root tips and contain amyloplasts involved in gravity sensing. Amyloplast formation occurred regardless of the Sr concentration (Fig. 3C). Ion leakage conductivity was also measured to assess root cell membrane integrity. The conductivity increased only in the presence of 10 mM Sr (Fig. 3D), indicating limited membrane damage. These findings suggest that Sr does not inhibit columellar cell formation or substantially damage root cell membranes. Overall, Sr appears to have minimal effects on the mature root region and root tip morphogenesis.

We previously demonstrated that Sr induces cell death in root meristems (Nagata 2023). On the basis of this observation, we hypothesized that cell death is mediated by ROS. To test whether Sr induces ROS production, superoxide anions were quantified via MCLA. The superoxide anion levels increased in a Sr concentration-dependent manner in both the shoots and the roots (Fig. 4A). NADPH oxidase, also referred to as RBOH, is known to catalyze superoxide anion formation (Kaya et al. 2019), suggesting that Sr activates RBOH. Because the spatial distribution of superoxide anions was initially unclear, tissue staining was performed. NBT staining revealed superoxide anion accumulation in the root meristem (Fig. 4B). Notably, staining was detected not only in the root epidermis but also near the central column, suggesting that Sr is absorbed and affects internal plant tissues. Furthermore, the lack of increase in NBT staining, as shown in Fig. 4A, is thought to be due to low sensitivity.

The specific molecular components responsible for Sr uptake into roots remain unidentified. Several genes involved in Sr uptake have been identified in Arabidopsis mutants through QTL analysis (Kanter et al. 2010). In this study, *CYCLIC-NUCLEOTIDE-GATED CHANNEL 9* (*CNGC9*) was reported to be important for Sr responses. In contrast, our analysis revealed SNP mutations in both *OPT6* and *CNGC9*. *OPT6*

encodes a transporter with a broad substrate range, including complexes of phytochelatin (PC) and metals (Lubkowitz 2011), whereas *CNGC9* encodes a channel known to be involved in Ca^{2+} absorption (Talke et al. 2003).

We propose that Sr forms a complex with PC and is taken up although OPT6. The A645 residue is located within the pore through which the PC complex is predicted to pass (Fig. 5). Because threonine is more hydrophilic than alanine is, substitution at this position may facilitate the passage of hydrophilic Sr–PC complexes through the transmembrane domain. Taken together, these observations suggest that OPT6 is the gene responsible for the Sr high-accumulation phenotype observed in the mutant strain.

CNGC proteins are thought to assemble as tetramers (Jarratt-Barnham et al. 2021). In wild-type CNGC9, pore formation was observed in both homotetrameric complexes and heterotetramers formed with CNGC6 (Fig. 6B, C). In contrast, in the CNGC9 mutant, pore formation was not detected in either homo or heterotetramer complexes, except for the 2:2 heterotetramer with CNGC6. The G187 residue of CNGC9 is predicted to contribute to pore formation and influence the transport of substrates such as Ca. Accordingly, CNGC9 is likely the gene responsible for the Sr low-accumulation phenotype in the mutant strain. CNGC9 is thought to be localized to the cell membrane. Therefore, it is expected that Sr will antagonize Ca absorption and that the Sr taken up into the cell will cause various toxic effects.

Taken together, the results shown in Figs. 1 and 6 indicate that Sr may inhibit Ca absorption. In addition, under Ca-deficient conditions, Sr toxicity may exacerbate growth inhibition while simultaneously promoting Sr uptake mediated by CNGCs9.

In conclusion, Sr inhibits plant growth under Ca-deficient conditions and cannot substitute for Ca. In contrast, Sr suppresses growth through ROS generation and peptide denaturation. Furthermore, the identification of Sr hyper and hypoaccumulator lines from EMS mutants indicates that mutations in OPT6 and CNGC9 are likely involved in Sr uptake.

Abbreviations

Sr Strontium

Ca Calcium

GSH Glutathione

ROS Reactive oxygen species

RBOH Respiratory burst oxidase homolog

EMS: ethyl methanesulfonate

OPT6 Oligopeptide transporter 1

CNGC9 Cyclic nucleotide-gated channel 9

Declarations

Conflict of interest

All authors confirm that they have no conflicts of interest.

Ethical Approval

This study did not involve human participants or experimental animals. Therefore, ethical approval was not needed.

Consent to Participate

Not applicable. No human participants were involved in this study.

Consent to Publish

Not applicable. No personal data or identifiable information is included in this manuscript.

Competing Interests

The authors declare that they have no competing interests, either financial or personal, that could have influenced the work reported in this manuscript.

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Author Contributions

Takeshi Nagata conceived the study, designed the experiments, performed the experiments, analyzed the data, and wrote the manuscript.

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Data availability

The datasets generated and analyzed during the current study, including ion concentration measurements, ROS assays, and gene expression data, are available from the corresponding author upon reasonable request.

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Figures

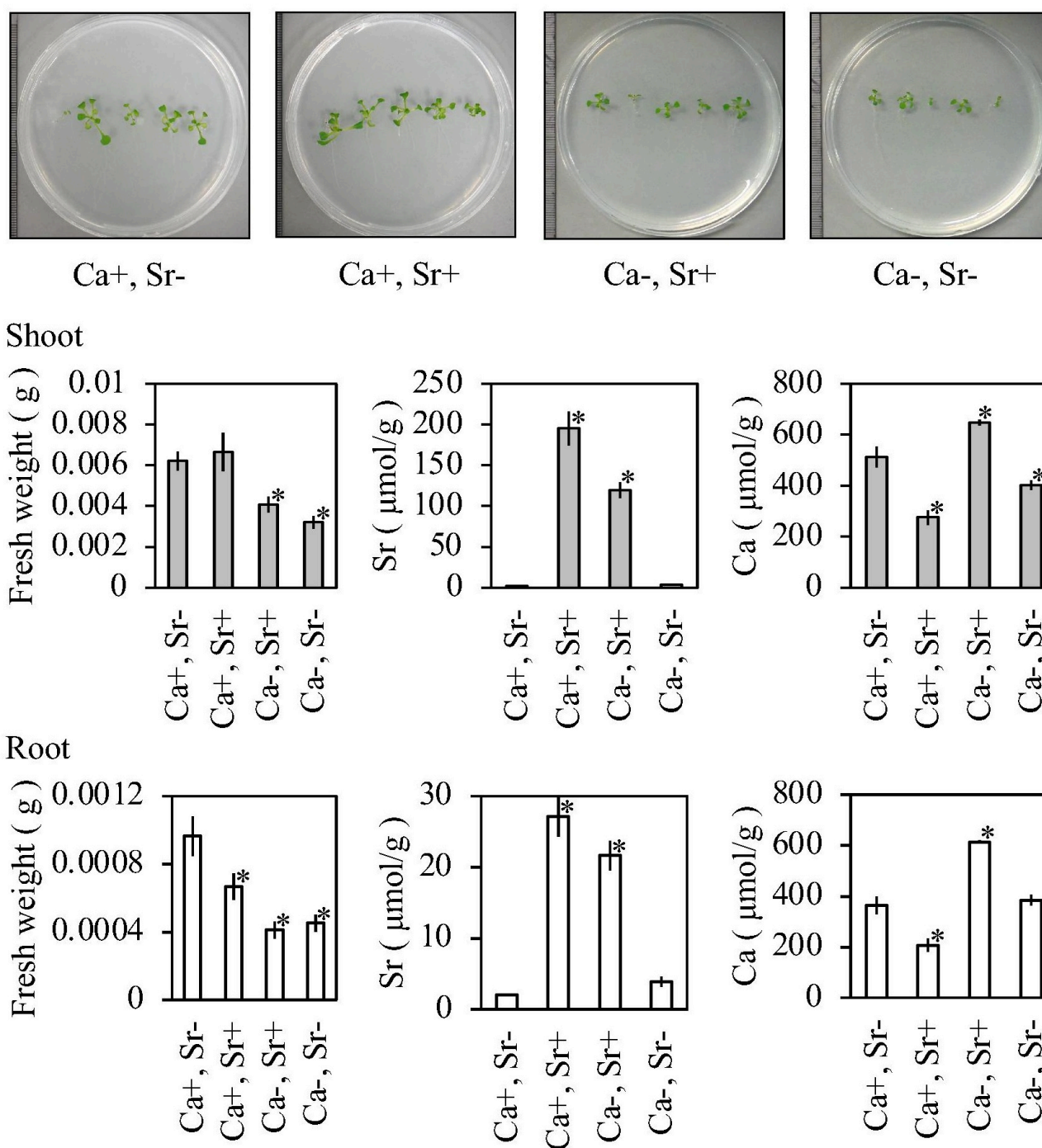


Figure 1

Effect of Sr on growth inhibition under Ca deficiency.

The plants were incubated with or without Ca and Sr in MS agar media as indicated. After 2 weeks of incubation, the effect of Sr on the phenotype was observed. The fresh weights of the shoots and roots and the Sr and Ca concentrations are shown. The data are presented as the means $\pm$ SEs; n = 10–11; $P < 0.05$

0.05, one-way ANOVA and post hoc Tukey test. Asterisks indicate a significant difference from control plants(Ca+, Sr-).

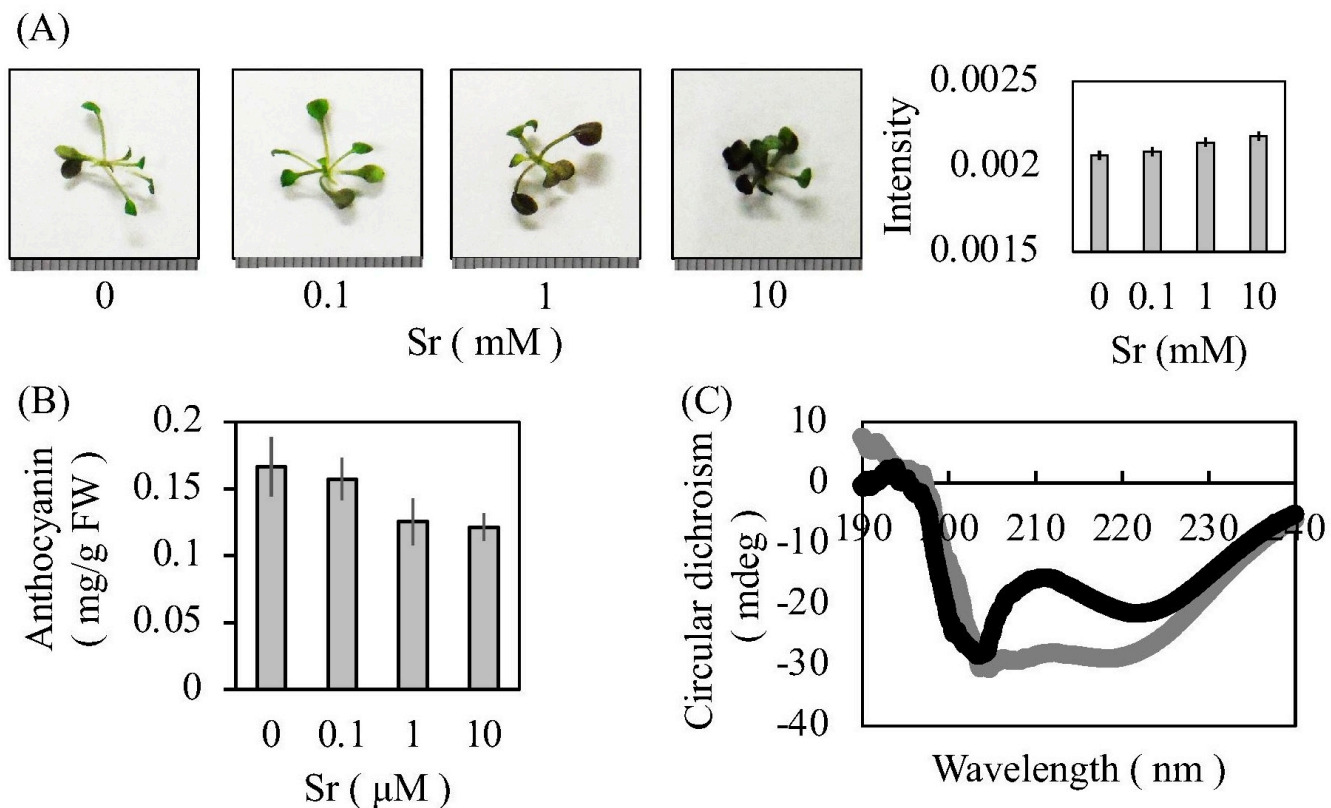


Figure 2

Phytotoxicity of Sr to shoots in Arabidopsis.

(A) Cuticular formation was detected by the toluidine blue test in whole leaves. Areas where cuticles did not form were stained blue purple. The experiment was performed at least ten times. (B) The anthocyanin concentration in the shoots was determined. The data are presented as the means $\pm$ SEs; $n= 8-10$, $P < 0.05$; one-way ANOVA and post hoc Dunnet test were used. (C) CD spectra of glutathione (reduced form, GSH) with or without Sr are shown. Sr concentrations: 0 mM (gray line) and 200 mM (black line).

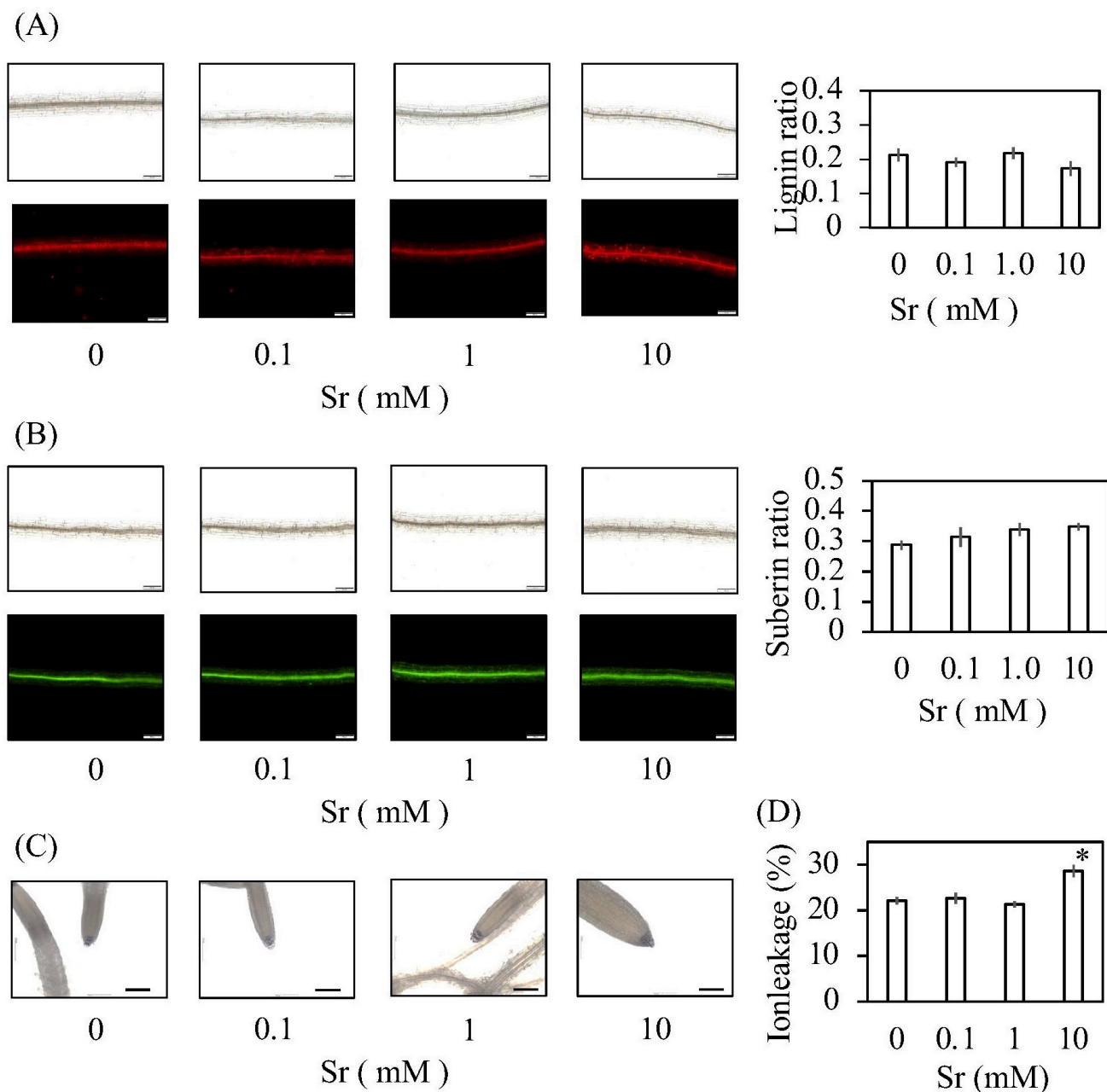


Figure 3

Phytotoxicity of Sr to roots in Arabidopsis.

(A) To examine lignin formation in the mature region of the root, the autofluorescence of lignin was observed via fluorescence microscopy. The scale bar represents 100 μm . The ratio of the thickness of the lignin layer to the thickness of the root was calculated. The experiments were performed at least ten times. (B) To examine suberin formation in the mature region of the root, suberin was observed via

fluorescence microscopy with a Fluoro Yellow 066. The scale bar represents 100 μm . The ratio of the thickness of the suberin layer to the thickness of the root was calculated. The experiments were performed at least ten times. (C) Lugol staining was used to evaluate the effect of Sr on gravity sensing. Amyloplasts in the root cap were stained purple. The scale bar represents 100 μm . The experiments were performed at least ten times. (D) Electrical levels were measured, and damage to the roots was assessed by ion leakage. The data are presented as the means $\pm$ SEs; $n= 10$, $P < 0.05$; one-way ANOVA and post hoc Dunnet test were used. Asterisks indicate a significant difference between the Sr-treated plants and the control plants.

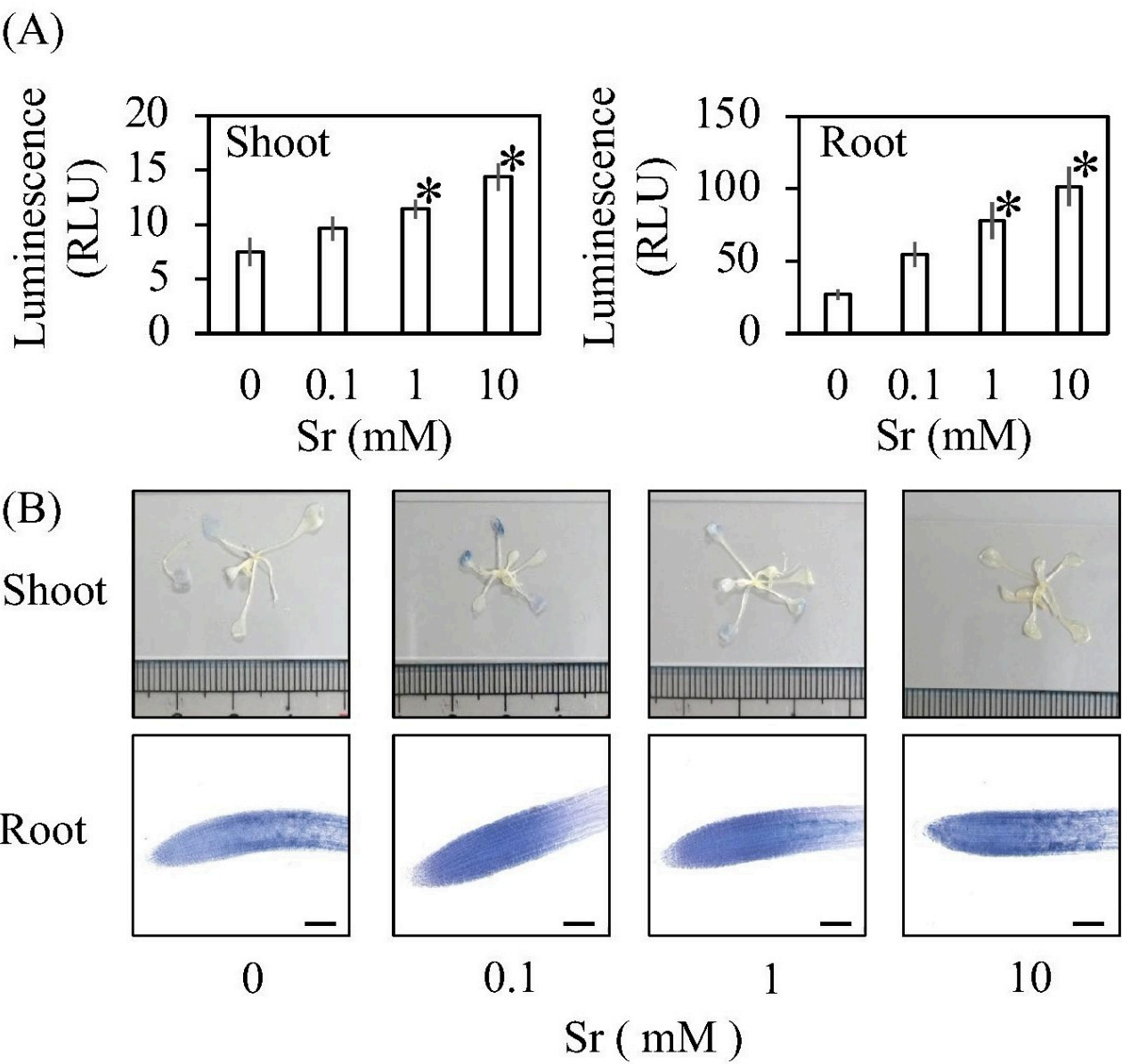


Figure 4

Involvement of superoxide anion radicals in Sr toxicity.

(A) Superoxide anion radicals and singlet oxygen produced by Sr were measured via MCLA. The data are presented as the means $\pm$ SEs; $n=10$, $P < 0.05$; one-way ANOVA and post hoc Dunnet test were used. Asterisks indicate a significant difference between the Sr-treated plants and the control plants. (B) NBT staining for superoxide anion radicals and singlet oxygen produced by Sr at the root tips. The scale bar represents 100 μm . The experiment was performed at least ten times.

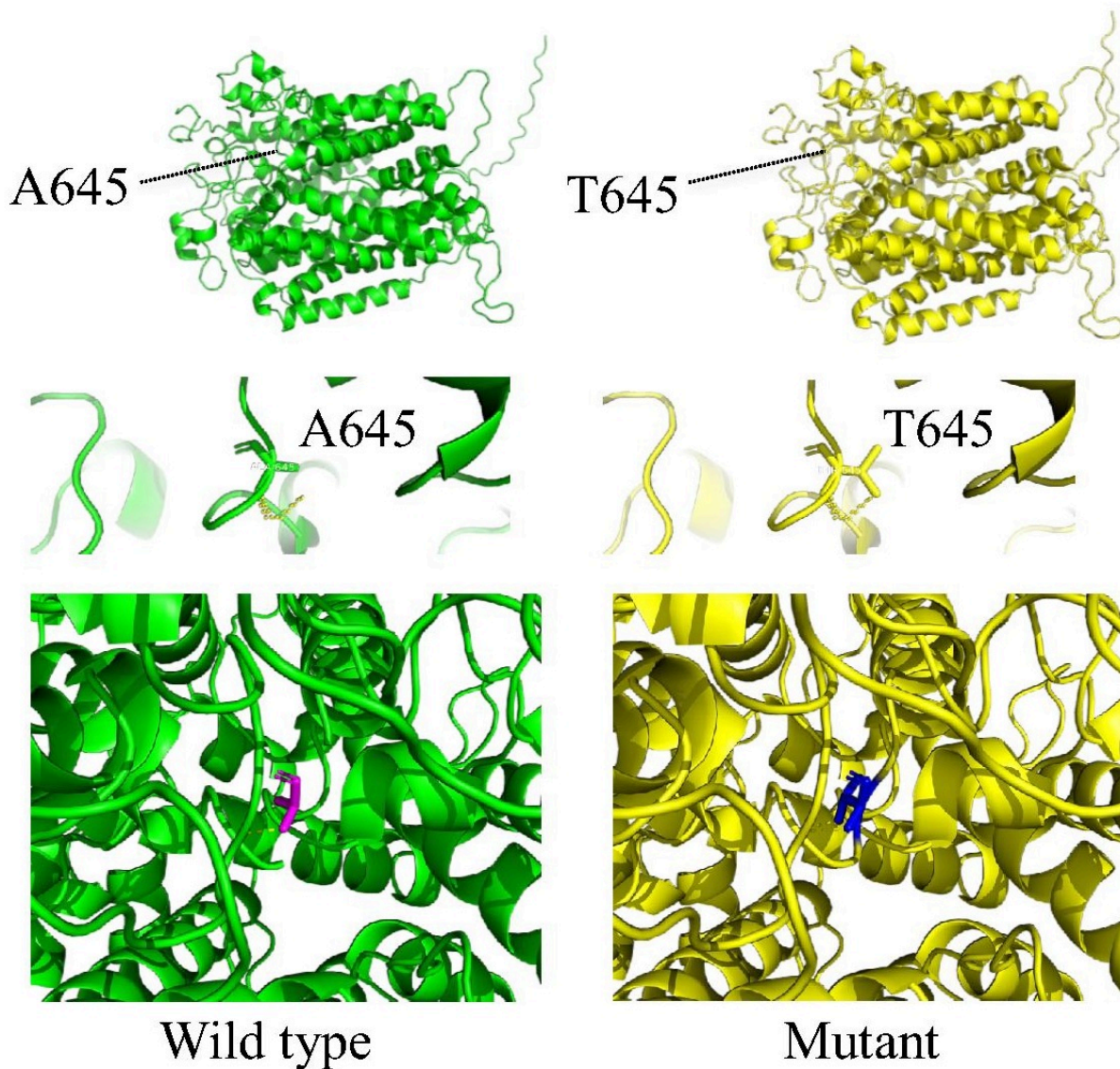


Figure 5

Structural prediction of the OPT6 protein induced by SNP mutation.

The structure of OPT6 found in Sr hyperaccumulator mutants was estimated. Three-dimensional structure estimation was performed via PyMOL. In the upper section, the structures of the wild-type and mutant forms of OPT6 are shown. In the middle section, the structures are enlarged near the mutant amino acid. In the lower section, amino acid side chains are indicated in pink (wild-type OPT6) and blue (mutant OPT6).

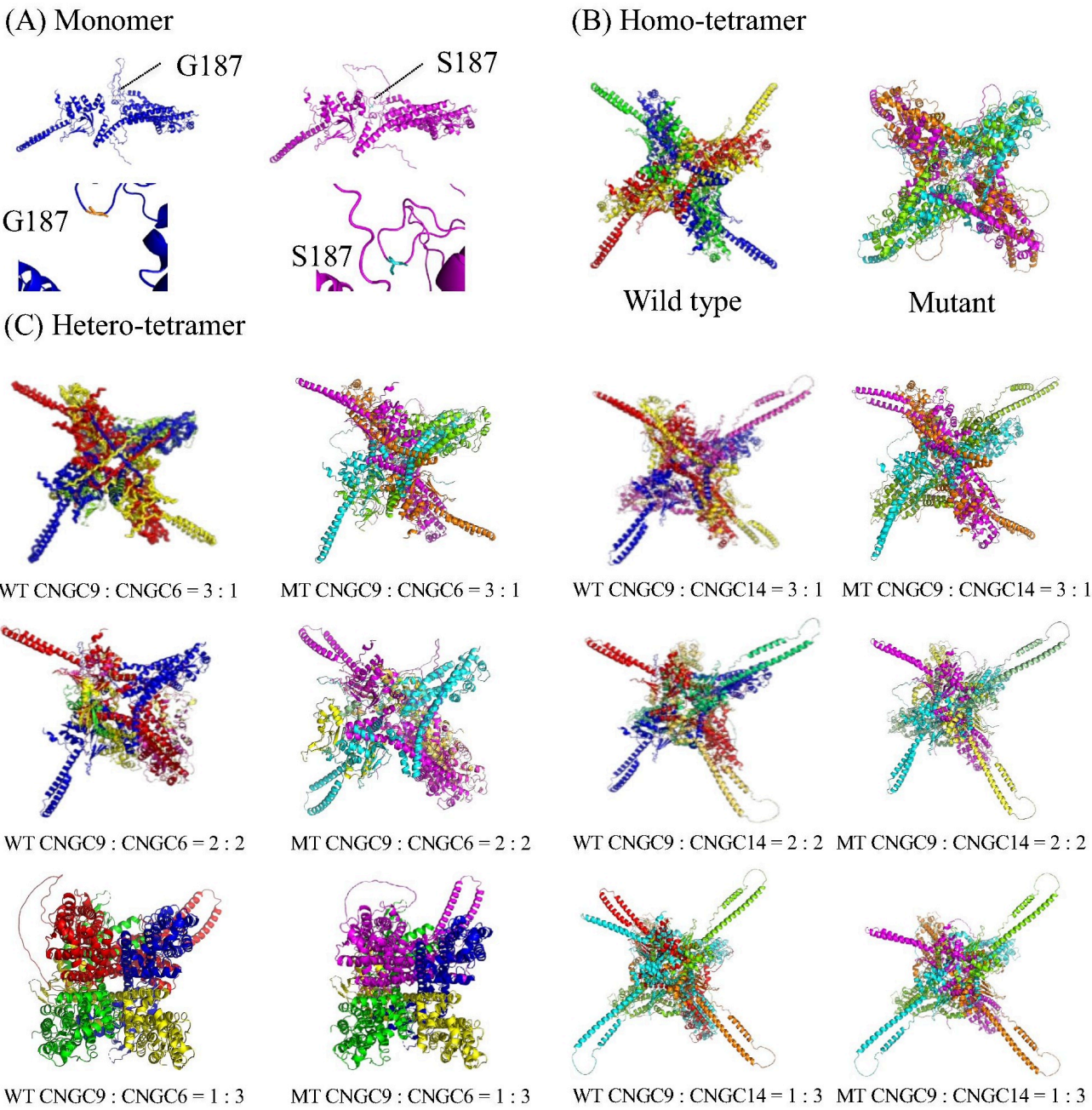


Figure 6

Structural estimation of the CNGC9 protein and its multimers caused by SNP mutations.

The structure of CNGC9 found in Sr hypoaccumulator mutants was estimated. Three-dimensional structure estimation was performed via PyMOL. (A) The structures of the wild-type and mutant monomers of CNGC9 are shown, with enlarged views of the regions near the mutated amino acid. (B) The structures of the homotetramer are shown. (C) The structures of the hetero tetramers are shown. Combination ratios with CNGC6, 9, and 14 are also indicated.