Arabidopsis mutant plants with diverse defects in polyamine metabolism show unequal sensitivity to exogenous cadaverine probably based on their spermine content

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Abstract *Arabidopsis* plants do not synthesize the polyamine cadaverine, a five carbon-chain diamine and structural analog of putrescine. Mutants defective in polyamine metabolic genes were exposed to exogenous cadaverine. Spermine-deficient *spms* mutant grew well while a T-DNA insertion mutant (*pao4-1*) of polyamine oxidase (PAO) 4 was severely inhibited in root growth compared to wild type (WT) or other *pao* loss-of-function mutants. To understand the molecular basis of this phenomenon, polyamine contents of WT, *spms* and *pao4-1* plants treated with cadaverine were analyzed. Putrescine contents increased in all the three plants, and spermidine contents decreased in WT and *pao4-1* but not in *spms*. Spermine contents increased in WT and *pao4-1*. As there were good correlations between putrescine (or spermine) contents and the degree of root growth inhibition, effects of exogenously added putrescine and spermine were examined. Spermine mimicked the original phenomenon, whereas high levels of putrescine evenly inhibited root growth, suggesting that cadaverine-induced spermine accumulation may explain the phenomenon. We also tested growth response of

cadaverine-treated WT and *pao4-1* plants to NaCl and found that spermine-accumulated *pao4-1* plant was not NaCl tolerant. Based on the results, the effect of cadaverine on *Arabidopsis* growth and the role of PAO during NaCl stress are discussed.

Keywords *Arabidopsis* · Cadaverine · NaCl response · Polyamine oxidase · Putrescine · Spermidine · Spermine

Abbreviations

ACL5	T-Spm synthase
ADC	Arginine decarboxylase
Cad	Cadaverine
PAO	Polyamine oxidase
PAs	Polyamines
Put	Putrescine
SAMDC	S-adenosylmethionine decarboxylase
Spd	Spermidine
SPDS	Spd synthase
Spm	Spermine
SPMS	Spm synthase
T-Spm	Thermospermine

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Introduction

Polyamines (PAs) are small aliphatic amines found in almost all living organisms. Putrescine (Put), spermidine (Spd) and spermine (Spm) are the most common PAs in plants (Tabor and Tabor 1984; Cohen 1998). Plant PAs exert important roles in a wide range of physiological processes such as embryogenesis, cell division, organogenesis, flowering and senescence, and also in the responses to abiotic and biotic stresses (Alcázar et al. 2010; Groppa and Benavides 2007; Kusano et al. 2008). The presence of another tetraamine,

thermospermine (T-Spm), has been proved in plants by genetic and biochemical approaches (Knott et al. 2007; Kakehi et al. 2008; Naka et al. 2010; Takano et al. 2012). The other diamine, cadaverine (Cad), is found in legumes but not in *Arabidopsis* (Fig. 1). Therefore, functional analyses on this diamine are very much limited (Gamarnik and Frydman 1991; Shevyakova et al. 2000; Bunsupa et al. 2012).

The plant PA biosynthetic pathway has been well elucidated (Kusano et al. 2008; Bagni and Tassoni 2001; Fuell et al. 2010). PA biosynthesis starts from arginine (Arg) in *Arabidopsis* and all the enzymes were assigned in this organism. Arg is converted to Put via agmatine by three sequential reactions catalyzed by arginine decarboxylase (ADC), agmatine iminohydrolase and *N*-carbamoylputrescine amidohydrolase. Put is converted to Spd by Spd synthase (SPDS). An aminopropyl group is transferred from the decarboxylated *S*-adenosylmethionine (dcSAM), which is synthesized from methionine in two sequential reactions of methionine adenosyltransferase and *S*-adenosylmethionine decarboxylase (SAMDC), respectively. Triamine Spd is further converted to two tetraamine isomers, Spm and T-Spm, and the reactions are catalyzed by Spm synthase (SPMS) and T-Spm synthase (ACAULIS5 or ACL5), respectively (Fig. 1).

In contrast to PA biosynthesis, its catabolism is not fully unveiled. Diamine oxidase (DAO) and polyamine oxidase (PAO) are the enzymes involved in PA catabolism (Bagni and Tassoni 2001; Cona et al. 2006). The former enzyme requires copper as a cofactor and catalyzes oxidation of Put to 4-aminobutanal with concurrent production of NH_3 and H_2O_2 , and the resulting aldehyde is further metabolized to γ -aminobutyric acid via Δ^1 -pyrroline (Bagni and Tassoni 2001; Cona et al. 2006). PAO is a flavin adenine dinucleotide (FAD)-dependent enzyme. It catalyzes the oxidative deamination of PAs at the secondary amino-group (Cona

et al. 2006). In plants, it is elucidated that PAO oxidizes Spd and Spm and produces 4-aminobutanal and *N*-(3-aminopropyl)-4-aminobutanal, respectively, along with 1,3-diaminopropane and H_2O_2 (Cona et al. 2006; Federico et al. 1990), indicating that plant PAOs are involved in the terminal catabolism. Initially characterized maize and barley PAOs showed such activity (Tavladoraki et al. 1998; Binda et al. 1999; Cona et al. 2005; Cervelli et al. 2006). On the other hand, mammalian PAOs are involved in a PA back-conversion pathway (Vujcic et al. 2003; Wu et al. 2003). In addition, mammalian Spm oxidase converts Spm to Spd along with the production of 3-aminopropanal and H_2O_2 without the acetylation process (Wang et al. 2001; Vujcic et al. 2002; Cervelli et al. 2003; Seiler 2004). Even in plant, PA back-conversion activity was reported for AtPAO1, an isoform of five *Arabidopsis* PAOs (Tavladoraki et al. 2006). More recently, it was evidenced that AtPAO3 and AtPAO4 catalyze a PA back-conversion reaction; AtPAO3 catalyzes the oxidation of Spm to Put via Spd, while AtPAO4 converts Spm to Spd, but not to Put (Moschou et al. 2008; Kamada-Nobusada et al. 2008). Furthermore, knowledge on Plant PAOs have started to be accumulated (Moschou et al. 2008; Kamada-Nobusada et al. 2008; Takahashi et al. 2010; Fincato et al. 2011; Ono et al. 2012).

In this study, we address how Cad affects the growth of *Arabidopsis* mutants with defects in PA metabolism, and find that *pao4-1* is hypersensitive to Cad whereas Spm-deficient mutant (*spms*) is rather Cad tolerant compared to WT. To find the underlying mechanism, the respective PA contents of the tested plants are analyzed. Based on the results, we propose that differential sensitivity to Cad of the mutants with defects in PA metabolism may be explained by Spm contents in the respective plants.

Fig. 1 The simplified PA metabolism in *A. thaliana* and structure of Cad. *Black and white arrows* indicate biosynthetic and catabolic paths, respectively, and the enzymes which catalyze the corresponding reactions are also indicated. Cad is highlighted by a dotted-line square. *ACL5* T-Spm synthase; *PAO* polyamine oxidase; *SPDS* Spd synthase; *SPMS* Spm synthase. In the PA structures, *black dot* indicates carbon atom

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia-0 (Col-0) served as the wild type (WT) in this study. Seeds of Spm-deficient mutant (*spms*) and T-Spm-deficient mutant (*acl5*) were provided by Prof. Taku Takahashi (Okayama University). The T-DNA insertion lines for *AtPAO1* (SAIL_882_A11), *AtPAO2* (SALK_046281), *AtPAO3* (GK-209F07), *AtPAO4* (SALK_133599, Figure S1) and *AtPAO5* (SALK_053110) were obtained from the Arabidopsis Biological Resource Center (Ohio State University, USA) and their homozygous lines were selected. All seeds were surface-sterilized by treatment of 70 % ethanol for 1 min, then in a solution of 1 % sodium hypochlorite and 0.1 % Tween-20 for 15 min, followed by extensive washing with sterile distilled water. Sterilized seeds were placed onto vermiculite or on half-strength Murashige-Skoog (MS)-1.5 % agar plates (pH 5.6) containing 1 % sucrose and B5 vitamin. Growth conditions were 22 °C with a 14 h light/10 h dark photoperiod.

Chemicals

Put, Spd, Spm and Cad were purchased from Nacalai-Tesque Ltd. (Kyoto, Japan). T-Spm, aminopropyl Cad (ApCad) and diaminopropyl Cad (DiApCad) were chemically synthesized (Samejima et al. 1984; Niitsu and Samejima 1986). All other chemicals were obtained at the analytical grade from Sigma-Aldrich Corp (St. Louis, MO, USA), Wako Pure Chemical Industries Ltd. (Osaka, Japan) and Nacalai-Tesque Ltd.

Polyamine treatment

Half-strength MS agar media containing PAs [Put (5 mM), Spm (0.3 mM), Cad (0.5 mM, 1 mM, 2 mM)] were prepared in rectangle plates (50 mm in height × 90 mm in width). The sterilized seeds were placed onto the respective PA-contained media. The plates were held at vertical position with 85° and incubated in a plant growth room under 22 °C with a 14 h light/10 h dark photoperiod.

Analysis of PA contents in plants by high-performance liquid chromatography (HPLC)

PA contents were analyzed by the procedure described in Naka et al. (2010). To extract polyamines, plant samples (about 0.1–0.2 g fresh weight per sample) were homogenized in five volumes (1 ml per 0.2 g plant sample) of 5 % (w/v) cold perchloric acid (PCA). The mixtures were transferred into tubes and kept on ice for 1 h. After centrifugation at 15,000×g for 30 min at 4 °C, the supernatants were combined and filtered using a filter syringe (pore size, 0.2 µm). One ml

of 2N NaOH was added to 600–800 µl of plant extract, mixed, then 10 µl of benzoyl chloride was added, mixed and incubated at room temperature for 20 min. After addition of 2 ml saturated sodium chloride and 2 ml of diethyl ether followed by strong mixing, phase separation was performed by centrifugation at 3,000×g for 10 min at 4 °C. Aliquots of the organic solvent phase (1.5 ml each) were evaporated and the residue was resuspended in 100 µl of methanol. The benzoylated PAs were analyzed with a programmable Hewlett Packard series 1100 liquid chromatograph using reverse-phase column (4.6 × 250 mm, TSK-GEL ODS-80Ts, TOSO, Tokyo, Japan) and detected at 254 nm. One cycle of the run resume is consisting of 60 min at a flow rate of 1 ml/min at 30 °C; i.e., 42 % acetonitrile for 25 min for PA separation, increased up to 100 % acetonitrile during 3 min, 100 % acetonitrile for 20 min for washing, decreased down to 42 % acetonitrile during 3 min, then 42 % acetonitrile for 9 min.

Real-time RT-PCR analysis

Total RNA was extracted from the ground parts of 2-week-old *Arabidopsis* seedlings grown on agar using Sepasol-RNA I Super (Nacalai Tesque, Kyoto, Japan). First-strand cDNA was synthesized with ReverTra Ace (Toyobo Co. Ltd., Osaka, Japan) and oligo-dT primers. Quantitative real-time RT-PCR was performed with Fast-Start Universal SYBR Green Master (ROX) (Roche Applied Science, Mannheim, Germany) on a StepOne real-time polymerase chain reaction (PCR) system (Life Technologies Japan, Tokyo, Japan). The primers were designed by Primer Express Software Version 3.0 (Applied Biosystems) and are listed in Table S1. They were used for the two-step real-time RT-PCR that was performed with the following program: one cycle of 94 °C for 10 min, followed by 40 cycles of 94 °C for 15 s and 60 °C for 60 s. Melting curves were generated after 40 cycles by heating the sample up to 95 °C for 15 s followed by cooling down to 60 °C for 1 min and heating the samples to 95 °C for 15 s. *Arabidopsis CBP20* was used as an internal control for the assays.

NaCl sensitivity assay

For assaying NaCl sensitivity, seedlings grown for 10 days on 1/2MS agar media with or without 1 mM Cad were carefully detached from agar plates and transferred to 1/2 MS agar plates containing 150 mM NaCl, then further incubated for a week.

H₂O₂ detection by the DAB-uptake method

Two-week-old *Arabidopsis* seedlings were placed in 1 mg/ml 3,3'-diaminobenzidine (DAB, Sigma, MO, USA # D-8001)-HCl acidic (pH 3.8) solution, vacuumed and incubated in the growth chamber overnight (Thordal-Christensen et al. 1997; Fryer et al. 2003).

Results

Growth response of WT, Spm-deficient, T-Spm-deficient plants and *pao4-1* mutant to exogenous cadaverine

We tested the effect of Cad on the growth of *Arabidopsis* mutants with defects in the genes involved in polyamine metabolism. Here we used *spms* (Spm-deficient) mutant, *acl5* (T-Spm-deficient) mutant, knockout mutants of 5 polyamine oxidase (PAO) genes. In 2 mM Cad-contained media, *spms* mutant grew well but, in contrast, *pao4-1* was severely inhibited compared to that of WT and the other *pao* mutants, especially in root growth (data not shown). The growth phenotype of WT, *spms*, *pao4-1* and *acl5* in presence of 2 mM Cad was reconfirmed (Fig. 2). The degree of root growth inhibition of *acl5* by 2 mM Cad was pronounced compared to that of WT while that of *pao4-1* was more significant. In contrast, the root growth of *spms* was only slightly inhibited by Cad treatment.

PA contents in WT, *spms* and *pao4-1* seedlings treated with Cad

To understand the molecular basis of this growth phenotype, we examined the PA contents of WT, *spms* and *pao4-1* plants grown in the absence or presence of 0.5 mM or 1 mM Cad. In all the three plants, Put contents increased when the host plants were treated with Cad (Fig. 3). Spd contents of WT and *pao4-1* plants decreased whereas that of *spms* plant did not change by Cad treatment. We could not detect any ApCad and DiApCad in Cad-treated *Arabidopsis* plants, suggesting that *Arabidopsis* SPDS does not recognize Cad as its substrate. Spm contents of WT and *pao4-1* increased drastically in a dose-dependent manner of Cad (Fig. 3).

Effect of Cad on the expression of PA biosynthetic genes

In Cad-treated *Arabidopsis* WT seedlings, PA contents were profoundly modulated (Fig. 3). Thus we examined if this modulation is associated with transcriptional levels of corresponding genes involved in PA metabolism. Both *arginine decarboxylase* (*ADC1* and *ADC2*) transcripts became less abundant after Cad treatment. *Spd synthase 1* (*SPDS1*) transcripts were more accumulated in 0.5 mM Cad-treated plants but stayed at similar levels in 1 mM Cad-treated plants, while *SPDS2* transcripts of both 0.5 mM and 1 mM Cad-treated seedlings stayed at constant levels (Fig. 4). The levels of *Spm synthase* (*SPMS*) transcripts increased more in seedlings treated with higher Cad concentration (Fig. 4). Spm accumulation in Cad-treated seedlings may be explained by the increment of *SPMS* transcripts. However, changes of Put and Spd contents in the Cad-treated seedlings may not be explained by the levels of the *ADC* and *SPDS* transcripts, suggesting the regulation occurs at different level such as enzymatic activity.

Growth response of WT, *spms*, *pao4-1* and *acl5* to Put or Spm

Upon exposure to Cad, contents of Put and Spm increased drastically in WT and *pao4-1* (Fig. 3). Next we addressed whether exogenously applied high levels of Put and Spm induce similar growth responses in WT, *spms*, *pao4-1* and *acl5*. At 5 mM concentration, Put did not cause any differential growth inhibition to the plants tested (Fig. 5). Spm of 0.3 mM concentration disturbed root growth of all the plants tested, while root growth inhibition of *spms* was alleviated compared to that observed in WT, *pao4-1* and *acl5* (Fig. 6). Therefore, the difference in Spm accumulation triggered by Cad treatment may explain the degree of the respective root growth inhibition.

Fig. 2 Growth phenotypes of WT, *spms*, *pao4-1* along with *acl5* in the absence or presence of Cad. Seeds of WT, *spms*, *pao4-1* and *acl5* mutants were germinated and grown for 2 weeks on vertical agar plates with 1/2 MS media (*left*, control) and 2 mM Cad-contained media (*right*)

Fig. 3 Change of PA contents in WT (Col-0), *spms* and *pao4-1* grown for 2 weeks on agar plates with or without the addition of 0.5 mM or 1 mM Cad. The above-ground parts of the seedlings were collected. Free PAs were extracted, derivatized, and analyzed by HPLC. Mean values $\pm$ SD were shown

Response of Cad-treated WT, *spms* and *pao4-1* plants to high salt

As it was shown that Spm has a defensive role against high salinity in *Arabidopsis*, we tested whether Cad-treated WT plants and *pao4-1* mutant plants show better salt tolerance due to presence of higher levels of Spm in

them (Fig. 3). The respective seeds were placed onto either 1 mM Cad-contained media or normal MS media in rectangular plates that were placed in vertical position with 85° angle. After 10-day-incubation in the growth room under the described condition, the seedlings were transferred onto either the same media or the media containing 150 mM NaCl. *spms* was hypersensitive to NaCl compared to WT, while *pao4-1* unexpectedly showed NaCl hypersensitivity. Furthermore, Cad-treated WT and *pao4-1*, that contained higher levels of Spm, became more sensitive to NaCl (Fig. 7).

Fig. 4 qRT-PCR analysis of PA biosynthetic genes in Cad-treated *Arabidopsis* plants. Total RNAs were extracted from the above ground parts of 2-week-old seedlings grown on 0 mM (Control), 0.5 mM and 1 mM Cad-contained 1/2 strength MS media. After converting to cDNAs, quantitative PCRs were performed. The primer pairs used for qRT-PCR were listed in Table S1. White column, control plant; gray column, 0.5 mM Cad-treated plant; black column, 1 mM Cad-treated plant. AGI codes of the genes: *ADC1* (At2g16500), *ADC2* (At4g34710), *SPDS1* (At1g23820), *SPDS2* (At1g70310), *SPMS* (At5g53120)

The root growth inhibition correlates with H₂O₂ production

To explain the NaCl sensitivity seen in Fig. 7, we hypothesized that Cad-treated plant roots, especially *pao4-1*, became sensitive to NaCl. To find a clue, we grew the plants with or without 2 mM Cad treatment and subjected them to DAB staining. With 2 mM Cad treatment, root growth of *pao4-1* and *acl5* was severely inhibited (Fig. 8a). Intriguingly those roots, especially their root tip portions were heavily stained by DAB solution (Fig. 8b e, g, f and h), indicating that those roots produced higher levels of H₂O₂. Even in WT and *spms* plants, their roots were stained by DAB solution, while their brown colour intensity showed some but not significant change by Cad treatment (Fig. 8b a, b, c and d). The degree of root growth inhibition may be explained by the amounts of Spm and/or H₂O₂ production.

Fig. 5 Growth phenotypes of WT, *spms*, *pao4-1* and *acl5* in the absence or presence of Put. Seeds of WT, *pao4-1*, *spms* and *acl5* mutants were germinated and grown for 2 weeks on vertical agar plates with 1/2 MS media (left, control) and 2 mM Put-contained media (right)

Discussion

Exogenously applied Cad may inhibit SPDS and activate SPMS in *Arabidopsis*

Lysine (Lys) decarboxylase (LDC) and ornithine (Orn) decarboxylase are key enzymes involved in the formation of Cad and Put by the decarboxylation of Lys and Orn, respectively. Recently, it has reported that LDCs isolated from leguminosae plants, which are involved in alkaloid biosynthesis, recognize both Lys and Orn (Bunsupa et al. 2012), indicating that some LDC recognizes C5 (Lys) and C4 (Orn) as a substrate. In the analysis of PA contents in *Arabidopsis* plants after Cad treatment a peak for Cad but not for ApCad and DiApCad was detected, indicating that *Arabidopsis* SPDS does not recognize a C5 diamine, Cad, as a substrate. The result also indicates that the observed difference in root growth of WT, *spms*, *pao4-1* and *acl5* is not in consequence of Cad metabolites. Instead, Put and Spm were heavily accumulated in Cad-treated WT and *pao4-1* plants, while Spd was decreased in those plants, suggesting that Cad controls SPDS negatively and SPMS positively. qRT-PCR analysis showed that *ADC1* and *ADC2* are down-regulated and that *SPDS1* and *SPDS2* remain constant in Cad-treated plants. In contrast, *SPMS* was upregulated in Cad-treated WT plants. It is likely that Cad acts

as a substrate analog for SPDS and inhibits its activity. Meanwhile, SPMS may be activated by Cad either at transcriptional level or at enzymatic activity level or both.

Difference in root growth of Cad-treated WT, *spms* and *pao4-1* may be explained by Spm contents

Severity of root growth inhibition in Cad-treated plants was in order of *pao4-1* > WT > *spms*. Put contents increased in all the Cad-treated plants in the order of *pao4-1* > WT > *spms* and Spm contents also increased in order of *pao4-1* > WT > *spms* = 0 in Cad-treated plants (Fig. 3). However, Spd contents decreased in WT and *pao4-1*, and remained constant in *spms* plants. Thus, both Put and Spm contents have good correlation with the degree of root growth inhibition by Cad treatment. We, therefore, tested the effect of high levels of Put and Spm on root growth of WT, *spms* and *pao4-1*. Judged from the phenotypes (Figs. 5 and 6), the difference in Spm contents may explain the observed root growth in WT, *spms* and *pao4-1*. The previous works showed that *Arabidopsis* PAO4 specifically back-converts Spm to Spd (Kamada-Nobusada et al. 2008; Takahashi et al. 2010; Fincato et al. 2011). Furthermore, in *pao4* mutant, Spm basal contents became high compared to that of WT plant due to the lack of Spm back conversion activity (Fig. 3; Kamada-Nobusada et al.

Fig. 6 Growth phenotypes of WT, *spms*, *pao4-1* and *acl5* in the absence or presence of Spm. Seeds of WT, *pao4-1*, *spms* and *acl5* mutants were germinated and grown for 2 weeks on vertical agar plates with 1/2 MS medium (left, control) and 0.3 mM Spm-contained media (right)

Fig. 7 Growth response of WT, *spms* and *pao4-1* mutant plants treated with or without 1 mM Cad on 150 mM NaCl-contained media. WT, *spms* and *pao4-1* mutant plants were grown on MS agar media with or without 1 mM Cad for 10 days, then transferred onto MS agar media or MS agar media containing 150 mM NaCl and further incubated for a week

Fig. 8 DAB staining of WT, *spms*, *pao4-1* and *acl5* seedlings' roots with or without 2 mM Cad treatment. **a** Phenotypes of WT, *spms*, *pao4-1* and *acl5* grown for 2 weeks on 1/2 strength MS agar plate (left 4 seedlings) or on 1/2 strength MS agar plate containing 2 mM Cad (right 4 seedlings). **b** DAB staining of WT, *spms*, *pao4-1* and *acl5* seedlings' roots with or without 2 mM Cad treatment. The 2-week-old seedlings shown in **a** were stained in acidic solution of 0.1 mg/ml DAB overnight. **a, b**: WT; **c, d**: *spms*; **e, f**: *pao4-1*; **g, h**: *acl5*. **a, c, e, g**: Control; **b, d, f, h**: 2 mM Cad-treated seedlings. Bar=0.5 mm

2008). Even in *acl5*, basal levels of Spm contents are twice compared to that of WT with unknown mechanism (Naka et al. 2010). This explains why *acl5*'s root growth was more severely inhibited in relative to WT in the presence of Cad.

Relationship between Spm contents and NaCl sensitivity

In common ice plant, NaCl-induced Cad accumulation in leaves was reported (Kuznetsov et al. 2007). Kuznetsov and his colleagues proposed that the resulting Cad has a protective role in the adaptation to salt stress through acting as a free radical scavenger (Kuznetsov et al. 2007). For exogenous Cad, we could not see any protective effect on *Arabidopsis* plant growth in presence of NaCl (Fig. 7).

We previously showed that *spms*·*acl5* double mutant became hypersensitive to NaCl, and that exogenously added Spm recovered its hypersensitive phenotype, suggesting that Spm has a defensive role to NaCl stress (Yamaguchi et al. 2006). In this work, we showed that *spms* mutant, which cannot synthesize Spm, was hypersensitive to NaCl compared to WT (Fig. 7), being consistent with the previous result (Yamaguchi et al. 2006). We also showed that Spm contents are increased by Cad-treatment in WT and *pao4-1* mutants. With the above background, we tested the response of Cad-treated WT and *pao4-1* to NaCl. The result showed that neither Cad-treated WT nor Cad-treated *pao4-1* were NaCl tolerant (Fig. 7). Kamada-Nobusada et al. (2008) reported that *pao4* mutant is not drought tolerant, even though many drought-responsive genes were upregulated in that *pao4* mutant. The result indicates that high Spm contents do not simply contribute to NaCl tolerance. The fact indirectly suggests that catabolic flux of Spm is required to exert NaCl-, probably also drought-stress tolerance.

Increased H₂O₂ production may sensitize host plants to NaCl stress

To find the reason why Cad-treated *pao4-1* mutant becomes hypersensitive to NaCl, DAB staining was performed to compare the H₂O₂ levels. *pao4-1* produced a significantly high level of H₂O₂ in the root tip region when the plant was treated with Cad (Fig. 8b). *acl5* plant also produced higher levels of H₂O₂. Exogenous Cad-induced H₂O₂ production was reported in the common ice plant (Kuznetsov et al. 2009). Its production was diminished when Cad was applied along with an inhibitor for DAO, aminoguanidine, suggesting that H₂O₂ is produced by DAO-catalyzed Cad oxidation. In our case, difference in H₂O₂ production is not simply explained by DAO-catalyzed Cad oxidation because the Cad contents in WT and *pao4-1* were almost same.

In summary, we propose that Cad treatment activates SPMS and induced Spm accumulation. With the different genetic background, the maximal Spm contents changed;

i.e., in *spms* mutant, endogenous Spm synthesis is totally blocked, whereas, in *pao4-1*, PAO4 enzyme which functions in back-conversion in Spm-specific manner malfunctions. The change in Spm levels in the respective mutants may explain the different root growth in presence of Cad. Concomitantly Cad-triggered high Spm contents may induce H₂O₂ production, especially in root tip portion, which may sensitize the host plant to NaCl stress. To get the supporting evidences, further work is required in the future.

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