Polyamine Biosynthesis Regulated by StARD Expression Plays an Important Role in Potato Wound Periderm Formation

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An acireductone dioxygenase (ARD) gene of potatoes was isolated from the expressed sequence tags (ESTs) of potato post-suberization cDNA libraries. The highest expression levels of the StARD gene and the protein appeared 36 h after suberization. An approximate 9-fold increase in ARD activity was detected at 36 h after wounding. Real-time reverse transcription–PCR (RT–PCR) analysis and immunolocalization studies revealed that StARD transcripts increase at the wound surface of potato tubers. The polyamine (PA) contents increased significantly after wounding at the wound surface. The increased PA content and ARD activity may play an important role in wound periderm formation.

Keywords: ARD — Methionine salvage — Polyamine — Potato — Suberization.

Abbreviations: ACO, aminocyclopropane-1-carboxylic acid (ACC) oxidase; ACS, ACC synthase; ARD, acireductone dioxygenase; ARGD, arginine decarboxylase; BSA, bovine serum albumin; EST, expressed sequence tag; GST, glutathione S-transferase; HCAA, hydroxycinnamic acid amide; MTA, 5′-methylthioadenosine; PDOP, 1-phosphonoxy-2,2-dihydroxy-3-oxo-3-phenylpropane; Put, putrescine; PVDF, polyvinylidine fluoride; RT–PCR, reverse transcription–PCR; SAM, S-adenosyl-L-methionine; SAMDC, SAM decarboxylase Spd, spermidine; SPDS, spermidine synthase; Spm, spermine.

The nucleotide sequence reported in this paper has been submitted to the GenBank database under accession number EU127480.

Plants form a protective layer, called suberin, next to the wound surface to prevent dehydration and potential attack by pathogens. This physical barrier results from specific cell wall modifications characterized by both a polyaromatic domain and a wax-embedded polyaliphatic domain. Most compositional and structural studies of suberin and associated materials have been performed using potato wound-induced periderm in potato tubers (Kolattukudy 1974, Stark and Garbow 1992), because the suberization that occurs in response to wounding is quick and uniform (Bernards et al. 1995, Bernards and Lewis 1998).

The polyamines (PAs), putrescine (Put), spermidine (Spd) and spermine (Spm), are low molecular weight polyamions, which are found in all organisms. They have been implicated in a wide range of biological processes, including plant growth, development and stress responses (Walden et al. 1997, Bouchereau et al. 1999, Shen et al. 2000).

The methionine (Met) salvage pathway in plants has been described at the biochemical level (Wang et al. 1982, Miyazaki and Yang 1987). In recent years, the genes encoding acireductone dioxygenase (ARD) and S-methyl-5′-thioadenosine (MTA) nucleosidase of the Met salvage pathway have been identified and characterized in rice (Sauter et al. 2005, Rzewuski et al. 2007). In our previous published report, we found that S-adenosyl-L-methionine (SAM) plays important roles in potato tuber suberization, not only as a methyl group donor but also as a substrate for the syntheses of ethylene and PA during the entire stage of the suberization process (Kim et al. 2007). Thus, the purpose of the present study was to determine the relationship between PA synthesis, ARD activity and the Met salvage pathway during suberization in potato tubers.

An in silico screening of expressed sequence tags (ESTs) from potato post-suberization cDNA libraries was performed to obtain a full-length cDNA of StARD. The full-length cDNA of StARD (GenBank accession No. EU127480) contains a coding domain sequence of 902 bp that encodes a polypeptide of 200 amino acids with a calculated molecular mass of 23.3 kDa. BLAST analysis of the homology of the deduced amino acid sequence revealed high homologies with Arabidopsis thaliana (GenBank accession No. NM117556), Plantago major (AM404079), Oryza sativa (AF068332), Homo sapiens (AF087863) and...
Klebsiella pneumoniae (AF102514)—identities of 83.9, 83.5, 74.9, 59.8 and 16.8%, respectively (Supplementary Fig. 1). As shown in Fig. 1A, Southern blot analyses were performed. There are at least three copies of the StARD gene in the potato genome, indicating that StARD is a member of a small gene family. Northern blot analysis (Fig. 1B) using six different potato organs showed that StARD was expressed at higher levels in the flower, while it was expressed at lower levels in the other organs. In addition, the expression of StARD increased 6 h after wounding, reaching the maximal level at 36 h and remaining elevated for 120 h (Fig. 1C).

Using polyclonal StARD antibodies, we performed Western blot analysis. The sizes of detected proteins were approximately 23.7 kDa for the potato ARD protein, and the recombinant ARD protein contained five additional amino acids (Gly–Ser–Pro–Glu–Phe) at the N-terminus, which was a slight increase (Fig. 2A). There is no problem detecting the StARD protein, even if there are a few non-specific bands in the Western blot analysis. StARD protein levels in the partially purified proteins of the tuber slices were markedly increased 36–72 h after wounding (Fig. 2A). Enzyme assays and kinetic studies were performed on the partially purified proteins by varying the concentration of a model substrate. A significant increase (about 9-fold) in ARD activity was detected at 36 h after wounding (Fig. 2B). The pattern of increased ARD activity during the wound healing process paralleled the elevated StARD transcript and protein levels. Therefore, ARD activity was regulated at the transcriptional level. Velocity vs. substrate plots was prepared by varying the concentration of 1-phosphonoxy-2,2-dihydroxy-3-oxo-3-phenylpropane (PDOP). The Michaelis constant, $K_m$, for the model ARD substrate was 39.2 $\mu$M, with a maximal reaction velocity, $V_{max}$, of 1.02 $\mu$mol min$^{-1}$ (Fig. 2C).

Put was synthesized directly from ornithine decarboxylase and indirectly from arginine by arginine decarboxylase (ARGDC). Put was then converted into Spd and Spm by the addition of propylamino groups from decarboxylated SAM, which is itself produced from SAM by the action of SAM decarboxylase (SAMDC) (Walden et al. 1997). Put and Spd contents increased significantly after wounding, whereas the level of Spm was unchanged. Interestingly, there was no increase in the Spd content after wounding plus submergence; however, the concentration of Spm increased (Fig. 3A). The large increase in Put concentration may be due to the large amount of Put required in suberized tissues. PAs are present in plant cells as free molecular bases and conjugated forms, such as hydroxycinnamic acid amides (HCAAs). The increased accumulation of HCAAs in potato tubers may reflect the antimicrobial barrier function of suberized tissues.

The induction of the ARGDC, SAMDC and spermidine synthase (SPDS) genes for 36 h after wounding was

![Fig. 1](https://academic.oup.com/pcp/article-abstract/49/10/1627/1889825)
Fig. 2  Western blot analysis and activity assay for ARD protein during the wound healing process. (A) Proteins extracted from samples at different incubation times after wounding were partially purified and separated using SDS–PAGE and then were electroblotted onto PVDF membranes. The blots were probed using polyclonal StARD antibodies, and protein was detected using chemiluminescence. PC, recombinant proteins; M, size marker. (B) Assays were performed using 50 μg of total protein for 50 min. Results are the average of four measurements. (C) The double reciprocal (1/v vs. 1/[S]) Lineweaver–Burk plot was prepared by varying the concentration of PDOP, while maintaining the oxygen concentration.

Fig. 3  Changes in free polyamine content and in expression of genes related to the Met salvage pathway during the potato wound healing process. Total RNA was isolated from tubers about 0 and 36 h after wounding, and after submergence in water, followed by analysis using Northern blotting.
greater than the induction of the aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO) genes (Fig. 3B). This result indicates that the PA content of potato tubers was increased by up-regulation of the ARGDC, SAMDC and SPDS genes. In our previous report (Kim et al. 2007), the expression of ACS and ACO was increased approximately 6 h after wounding and decreased thereafter. Ethylene and PA synthesis may not occur simultaneously during the wound healing process. The expression data obtained in the present study suggest that biosynthesis of PAs, and not of ethylene, is closely related to the expression of the StARD gene and protein.

It is well known that submergence (or flooding) causes plants to produce more ethylene. In order to see whether or not StARD is associated with submergence-induced ethylene production, we determined the expression levels of StARD and several genes related in ethylene and PA biosynthesis under submergence conditions (Fig. 3B). The expression of the StARD, ARGDC, SAMDC and SPDS genes significantly decreased, but that of SPMS, ACS and ACO genes did not. These results indicate that the regulation of these genes with respect to specific PA biosynthesis and StARD during wounding and submergence conditions might be coupled to PA rather than ethylene biosynthesis. In rice, two highly homologous ARD genes have been identified. The levels of OsARD1 transcripts showed a rapid and early increase (within 2 h) upon submergence and after ethephon treatment. OsARD2 expression was not changed by submergence or by ethephon treatment (Sauter et al. 2005). OsARD1 appeared to be involved in the ethylene signaling pathway soon after submergence. Thus, the biological role of StARD is probably different from that of OsARD1.

We further analyzed the levels of StARD protein and the transcript, as well as free and conjugated PAs in wounded potato tubers (Fig. 4). StARD protein was mainly...
detected in the phellogen layer just below the suberized cells. StARD transcripts increased predominantly at the wound surface of the potato tuber. The levels of free and conjugated PAs (e.g. Put and Spd) at the wound surface were much higher than at other sites of wounded potatoes (Fig. 4D, E). These data implicate StARD in PA synthesis and suggest that a specific PA is required throughout the entire wound healing process.

When potato tubers are wounded, they form a protective suberin layer. However, we found that there was no wound periderm formation and no increase in StARD transcripts and Spd content under submersion conditions (Figs. 3, 4B). These data indicate that a specific PA may play an important role in potato wound periderm formation. Unlike animals, plants cannot mobilize specialized cells devoted to wound healing. Although PAs play an essential role in wound healing responses in animals—primarily regulation of genes encoding cytoskeletal proteins (Kamińska et al. 1992) and activation of macrophages (Messina et al. 1992)—in plants, the role of PAs in the wound response remains largely unknown. However, the results of the present study suggest that specific PAs are associated with plant wound responses as evidenced by increased Put and Spd concentrations.

Because PAs have been implicated in a variety of plant growth and developmental processes, including cell proliferation and differentiation, morphogenesis, dormancy and germination, embryogenesis and response to external stress (Walden et al. 1997, Bouchereau et al. 1999), it is plausible that PAs produced after wounding induce cell differentiation required for the formation of a new wound periderm. There is abundant evidence that PAs play a role in controlling gene expression and, more specifically, in controlling cell division. Increased PA biosynthesis during the transition from the G1 to the S phase of the cell cycle, preceding the onset of DNA synthesis in dividing cells, appears to be a universal phenomenon in animals and plants (Lin et al. 1984, Fuller et al. 1997). During PA biosynthesis, MTA, a by-product of the spermidine and spermine synthase reactions, is released. The increased PA content suggested that large amounts of MTA are produced in the wounded layer. Since MTA is a strong inhibitor of PA biosynthesis, the concentration of MTA should be tightly regulated through the Met salvage pathway, where MTA is recycled back to L-methionine. Thus, PAs can potentially be produced at high rates without interfering with Met or SAM homeostasis (Wang et al. 1982, Miyazaki and Yang 1987). Therefore, the up-regulation of StARD, a branch point in the Met salvage pathway, is probably required during PA biosynthesis. This is the first study demonstrating that specific PAs are involved in the formation of wound-healed periderm and that suberization is regulated via StARD in the Met salvage pathway.

### Materials and Methods

Potato tubers (Solanum tuberosum L. cv. Desiree) harvested from greenhouse cultivation were used. For the suberization tests, tubers were cut with a knife into sections approximately 1 cm thick. The sections were incubated at room temperature in the dark for 0, 6, 12, 24, 36, 72 and 120 h after wounding. For the wounded combined with submergence treatment, the above tuber sections were submerged in gently running tap water for 36 or 48 h. To obtain a full-length cDNA of StARD, in silico screening of ESTs from potato post-suberization cDNA libraries was performed (Kim et al. 2007). Southern and Northern blot analyses were performed based on the methods described by Kim et al. (2007). The probes for ACS, ACO, StARD, ARGDC, SAMDC, SPDS and SPMS of potatoes were generated using PCR with specific primers designed from the EST sequences according to a previously described method (Kim et al. 2007).

StARD antibody was prepared as follows. The isolated full-length cDNA was cloned into the pgEX-4T-1 expression vector (Pharmacia Biotech). The overexpressed glutathione S-transferase (GST)-StARD fusion protein was purified by glutathione–Sepharose 4B affinity chromatography and cleaved using a Trombin CleanCleave™ kit (Sigma, St. Louis, MO, USA). Polyclonal rabbit antisera were raised against the recombinant StARD protein (LabFrontier Co., Ltd., Korea). A 100 µg dose of the purified recombinant ARD protein was used for immunization.

Partial purification of the potato tuber ARD protein was carried out according to the method of Wray and Abeles (Wray, 1993). Extracted proteins were separated using SDS-PAGE and electroblotted onto polyvinylidene fluoride (PVDF) membranes. The blots were probed using StARD antibodies (dilution 1:3,000) and horseradish peroxidase-conjugated anti-rabbit IgGs; detection was achieved using chemiluminescence.

Enzyme assays were performed using a model substrate. E1 enolase-phosphatase and the phenyl E1 substrate (PDOP) were used to synthesize the ARD model substrate according to Zhang et al. (2004). A 5 µl aliquot of E1 enzyme were added to an assay mixture comprised of 980 µl of E1 reaction buffer, 10 µl of catalase solution (1 mg ml⁻¹) and 5 µl of the E1 enolase-phosphatase substrate, PDOP, in a 1 ml septum-sealed cuvette. The reaction was monitored by changes in absorbance at 318 nm. After addition of 5 µl of 20 mM NADH and 60 µl of bovine heart lactate dehydrogenase (Sigma), consumption of NADH was monitored by the change in absorbance at 340 nm.

Extraction and quantification of free PAs were performed based on the method described by Flores and Galston (1982). The amount of dansyl-PA in each sample was determined by calculating the integrated optical density of the bands compared with those of the appropriate dilution of the dansylated standard PAs. For the determination of conjugated PAs, an equal volume of homogenate and 12 M HCl were mixed into a vial and heated at 100°C for 20 h. After that, it was filtered, dried and redisolved in 5% HClO₄, and dansylated.

For immunolocalization of StARD, samples were fixed, dehydrated and infiltrated with paraffin. The 14 µm thick paraffin sections were incubated with TBST (10 mM Tris, 250 mM NaCl, 0.3% Tween-20, pH 7.2) +1% bovine serum albumin (BSA) at room temperature for 1 h and replaced with antibody diluted with the same solution (1:50). Then, the samples were incubated at room temperature in a humid chamber for 4 h. The samples were covered with 1:100 diluted anti-rabbit IgG–alkaline phosphatase antibody (Sigma No. A025). The samples were washed twice...
with TBST + BSA, followed by washing twice with TBST. Color was developed using TBST containing NBT/BCIP (Roche).

For real-time reverse transcription–PCR (RT–PCR) analysis of StARD, the wound surface of potatoes was sectioned in 0.5 mm thick slices and collected separately for total RNA extraction. Real-time PCR was performed using the Greenstar PCR master mix (Bioneer, Daejeon, Korea) on a machine (Bio-Rad, Hercules, CA, USA). As an internal control for the correct PCR conditions and RNA amount in each sample, cDNA fragments of the constitutively expressed 18S rRNA (GenBank accession No. X62738) were amplified using the following primers: 5'-GAGCCTTTATCATGACAGG-3' and 5'-CGGTTCTTGA TTAATGAAAACATCCT-3'. Results were expressed as fold change relative to control.

Supplementary material

Supplementary material are available at PCP Online.

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References


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