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Calcium dependent sucrose uptake links sugar signaling to anthocyanin biosynthesis in Arabidopsis

Dong Ho Shin [a], Myoung-Goo Choi [a], Hyun Kyoung Lee [a], Misuk Cho [a], Sang-Bong Choi [b], Giltsu Choi [c], Youn-Il Park [a,*]

[a] Department of Biological Sciences, Chungnam National University, Daejeon 305-764, Republic of Korea
[b] Division of Bioscience and Bioinformatics, Myongji University, Yongin 449-728, Republic of Korea
[c] Department of Biological Sciences, KAIST, Daejeon 305-701, Republic of Korea

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ABSTRACT

Sugars enhance light signaling-induced anthocyanin accumulation in Arabidopsis seedlings via differential regulation of several positive and negative transcription factors. Ca^{2+} plays a role as a second messenger in sugar signaling in grape and wheat. However, whether anthocyanin pigmentation is modulated by changes in intracellular Ca^{2+} level in Arabidopsis is not known. Here, we used a pharmaceutical approach that Ca^{2+} antagonists strongly interfered with sucrose uptake and anthocyanin accumulation by down-regulating the expression of *sucrose transporter 1* (*SUC1*) and transcriptional regulatory factors, such as *PAP1*. Time course analysis of the effect of Ca^{2+} antagonists showed the early inhibition of sucrose-induced sugar uptake leading to decreased anthocyanin accumulation, indicating that Ca^{2+} signals play a role in sugar uptake rather than in anthocyanin biosynthesis. An early increase in cytosolic Ca^{2+} level in Arabidopsis roots in response to sucrose feeding was significantly inhibited by Ca^{2+} antagonists. Taken together, these results indicate that sucrose-induced sugar uptake in Arabidopsis is modulated by changes in endogenous Ca^{2+} levels, which in turn regulate anthocyanin accumulation.

1. Introduction

Anthocyanins play important roles in many plant physiological processes. They form photoprotective screens in vegetative tissues, act as visual attractors to aid pollination and seed dispersal, and play a role as antimicrobial agents and feeding deterrents in the defense response [1,2]. The expression of anthocyanin biosynthesis pathway structural and regulatory genes is regulated by various internal and external factors including sugar, light, hormones, chilling stress, and nutrient status [3]. Light absorbed by phytochrome and cryptochrome induces anthocyanin production by modulating the HY5-regulated transcriptional activation of anthocyanin biosynthesis genes in *Arabidopsis* [4]. Sugars and hormones, such as ethylene, cytokinin, jasmonic acid, gibberellins, and abscisic acid, positively or negatively modulate light signaling pathways [3,5]. Although the role of the interaction between light and hormones in the regulation of anthocyanin biosynthesis has been well characterized [3], sugar sensing and signaling are poorly understood. In Arabidopsis, glucose repression of photosynthesis-related genes is mediated by the sugar sensor hexokinase 1 (HXK1) [6], whereas sugar-inducible anthocyanin biosynthesis may be regulated by membrane-bound sugar transporters (SUCs) [7]. SUC1 is distributed abundantly in the root and has been implicated in sugar sensing and signaling, as evidenced by reduced anthocyanin accumulation in *suc1*-defective mutants grown in 3% sucrose (Suc) [8]. However, SUC1 appears to be involved in the transport of exogenous Suc to shoots, where anthocyanin accumulates, rather than acting as a Suc sensor [9]. Thus, the mechanisms underlying sugar sensing and signal transduction leading to the expression of anthocyanin biosynthesis genes remain to be elucidated.

Calcium acts as a second messenger in the signal transduction pathways of hormones and environmental stimuli (touch, wind, chilling, light, and elicitors). These stimuli induce a rapid and transient increase in cytosolic Ca^{2+} level [10] that can be mediated by the influx of Ca^{2+} from the apoplast and/or Ca^{2+} release from intracellular stores, including the endoplasmic reticulum, vacuoles, mitochondria, chloroplasts, and nuclei [11]. The mobilization of Ca^{2+} from these different stores is triggered by distinct signals that are transmitted by various Ca^{2+} sensors (Ca^{2+} binding proteins) [12]. Ca^{2+} efflux to the cell exterior and/or sequestration into cellular organelles restores Ca^{2+} levels to those of the resting state [13]. Calcium acts through intracellular protein mediators, such as calmodulin (CaM) and Ca^{2+}-regulated kinases [14]. Increasing evidence indicates that Ca^{2+}-dependent protein kinases (CDPKs) are involved in environmental stress and hormone signaling [15,16].

* Corresponding author. Address: Department of Biological Sciences, Chungnam National University, 99 Daehagro, Youseong, Daejeon 305-764, Republic of Korea. Fax: +82 42 822 9690.
E-mail address: yipark@cnu.ac.kr (Y.-I. Park).

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Sugars increase cytosolic Ca^{2+} through monosaccharide transporters and Suc symporters in Arabidopsis [17]. Suc specific enhancement of intracellular Ca^{2+} concentration was demonstrated in tobacco leaf discs [18] and cell suspensions [19], and in Arabidopsis whole plants [17]. In grape cells, the Ca^{2+} signaling pathway, including its downstream component CaM [20], implicated in Suc-induced anthocyanin accumulation via transcriptional activation of structural genes, such as dihidroflavonol reductase (*DFR*) [21]. Although a similar mechanism of regulation of anthocyanin biosynthesis gene expression by Ca^{2+} signaling is thought to be active in Arabidopsis [6], the specific role of Ca^{2+} in sugar-induced anthocyanin production remains unclear. Analysis of the time-dependency of the induction of anthocyanin biosynthesis by Suc and light in Arabidopsis showed that sugar uptake by root-abundant SUC1 is an early event followed by the accumulation of anthocyanin after 12 h [9]. However, the role of Ca^{2+} signaling in Suc uptake requires further investigation. Here, we show the effect of Ca^{2+} flux antagonists on the inhibition of sugar uptake and anthocyanin pigmentation in Arabidopsis seedlings.

2. Materials and methods

2.1. Plant materials and growth conditions

Arabidopsis thaliana wild-type (Col-0), transgenic (YC3.6-1), and Suc transporter 1 mutant (*suc1-2*) seeds were sterilized by 20% sodium hypochlorite, washed with sterile distilled water [9], and plated onto solidified 1/2-strength Murashige and Skoog (MS) medium supplemented with or without 60 mM (2.16%) sucrose. Seedlings were grown at a light intensity of 140 μmol $m^{-2}s^{-1}$ under an 18 h light/6 h dark photoperiod (22 ± 1 °C). For time course experiments, 9 d old seedlings were transferred to half-strength MS medium supplemented with or without 60 mM Suc and incubated for 24 h under continuous growth light conditions. When required, various concentrations of the calcium antagonists bis (β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), lanthanum nitrate (La^{3+}), and Ruthenium red (RR) were included in the growth medium.

2.2. Measurement of anthocyanin and soluble sugar contents

For anthocyanin extraction, 20 seedlings were boiled in 600 μL of propanol:HCl:H_2O (18:1:81, v/v/v) for 3 min and then incubated for 2 h in the dark at room temperature. After centrifugation, the absorbance of the supernatant was measured at 535 and 650 nm, and the concentration of anthocyanin was calculated using the formula A_{535}–0.3A_{650} [22]. The extraction of soluble sugar and measurement of sugar content in seedlings were conducted as previously described [9]. Mean values were obtained from three or four independent replicates.

2.3. Real-time reverse transcription-qPCR analysis

Total RNA was extracted with the TRI reagent (Molecular Research Center), followed by DNaseI (Takara) treatment. cDNA was synthesized from 1 mg of total RNA using the iScript cDNA Synthesis Kit (Bio-Rad) and qPCR was performed using the CFX96 Real Time System (Bio-Rad), following the manufacturer's instructions. Reactions were performed in triplicate using 5 mL of 23 Dynamo HS Master Mix, 0.5 mM of each primer (Supplementary Table S1), 2 mL of 20-fold-diluted cDNA, and nuclease-free water (Roche Diagnostics) to a final volume of 10 μL. A negative water control was included in each run [9]. Fold changes ($2^{-\Delta\Delta Ct}$) were expressed relative to wild-type control seedlings. Mean values were obtained from three to five biological replicates, each determined in triplicate.

3. Results

3.1. Ca^{2+} antagonists decrease anthocyanin and soluble sugar contents in a concentration dependent manner

To determine whether Ca^{2+} signaling is involved in Suc-induced anthocyanin biosynthesis in Arabidopsis, we tested the effect of Ca^{2+} signaling antagonists on anthocyanin pigmentation in plants stimulated with exogenous Suc. We first assessed the effect of Ca^{2+} flux antagonists on Suc-induced increases in anthocyanin content. Nine-day-old wild-type (Col-0) Arabidopsis seedlings grown without Suc were transferred to filter papers soaked in half-strength MS liquid medium containing 60 mM Suc alone or with various concentrations of Ca^{2+} antagonists, including EGTA, a divalent cation chelating reagent; La^{3+}, a potent Ca^{2+} channel blocker that competes with Ca^{2+} for binding to specific plasma membrane channels; and RR, an endomembrane Ca^{2+} channel blocker [23]. Seedlings were incubated for 48 h under continuous light. The three Ca^{2+} antagonists inhibited anthocyanin accumulation in a dose-dependent manner although to variable extents, with I_{50} (concentration causing 50% inhibition) values of 3 mM, 0.9 mM, and 5 μM, for EGTA, La^{3+}, and RR, respectively (Fig. 1A).

Considering that Ca^{2+} may act downstream of sugar signaling, as shown in other plants, such as grape [20,21], we expected the sugar contents of seedlings to remain unchanged by Ca^{2+} antagonists. However, contrary to our expectations, Ca^{2+} antagonists decreased the total soluble sugar content in parallel with the reduction in anthocyanin pigmentation (Fig. 1B). The decrease in anthocyanin levels was correlated with the downregulation of the expression of Suc-inducible anthocyanin biosynthesis structural (*CHS*, *DFR*, *LDOX*, and *UF3GT*) and regulatory (*PAP1* and *2*) genes (Fig. 1C). Among *SUCs* that are abundantly expressed in vegetative tissues such as *SUC1*, *–2*, *–3*, and *–4*, SUC1-mediated Suc transport is partly responsible for Suc-induced anthocyanin production in Arabidopsis, as demonstrated by the reduction in anthocyanin accumulation in *suc1-1* mutants [8,9]. As shown in Fig. 1C, exogenous Suc increased the transcript levels of *SUC1*, and this increase was significantly inhibited by Ca^{2+} antagonists, albeit to variable extents.

3.2. Ca^{2+} antagonists decrease the soluble sugar content prior to anthocyanin accumulation

In Arabidopsis, the increase in soluble sugar content in response to exogenous Suc occurs before anthocyanin accumulation [9]. Therefore, the decreased accumulation of both anthocyanin and sugars in plants treated with Ca^{2+} antagonists and exposed to exogenous Suc for 24 h in the present study (Fig. 1) suggests that the inhibition of exogenous Suc uptake precedes the decrease in anthocyanin production. To test this hypothesis, we analyzed the kinetics of induction of sugar and anthocyanin in response to exogenous Suc.

Nine-day-old Col-0 seedlings grown without Suc were transferred to liquid medium containing 60 mM Suc and incubated under continuous growth light conditions for up to 24 h. Consistent with a previous report [9], sugar levels increased immediately and reached near saturation at 12 h (Fig. 2A), whereas anthocyanin levels increased 12 h after Suc stimulation concomitant with the highest sugar content of seedlings (Fig. 2B). The Ca^{2+} antagonists EGTA, La^{3+}, and RR significantly inhibited the Suc-induced increase in soluble sugar content, followed by strong inhibition of anthocyanin pigmentation (Fig. 2A and B). In Arabidopsis seedlings, Suc-induced increases in sugar levels in the presence of light are mainly attributable to exogenous Suc uptake rather than photosynthesis-mediated sugar production [9]. Our results indicate that Suc uptake

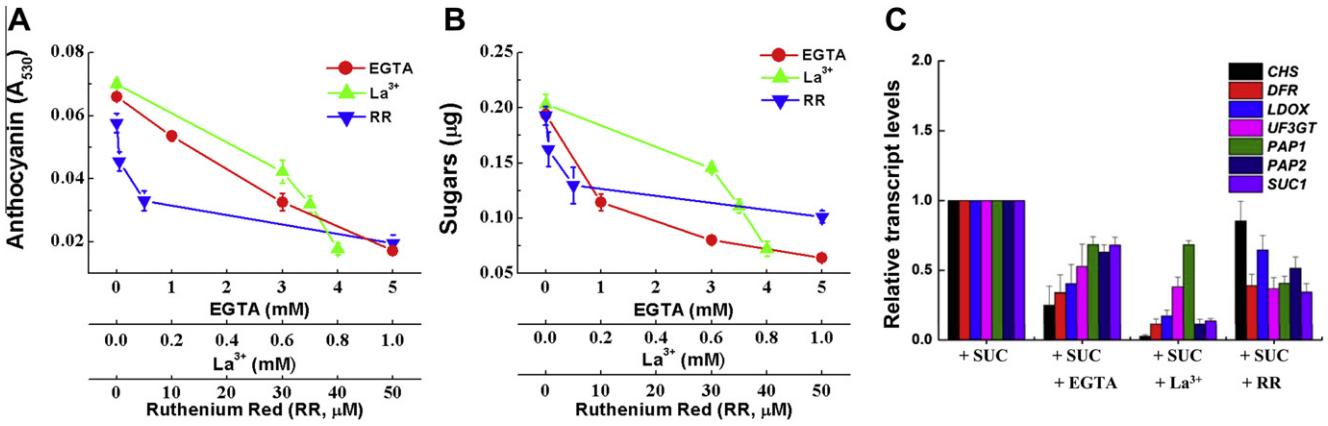

Fig. 1. Effect of Ca^{2+} antagonists on the inhibition of sucrose-induced soluble sugar (A) and anthocyanin (B) accumulation and expression of *SUC1* and anthocyanin biosynthesis-related genes (C). Nine-day-old Arabidopsis seedlings were incubated with 60 mM sucrose supplemented with various (A, B) or fixed (C) concentrations of EGTA (10 mM), La^{3+} (2 mM) and RR (70 μM) for 24 h under continuous white illumination (140 μmol m^{-2} s^{-1}). Error bars represent the SD of the mean of three or five independent replicates.

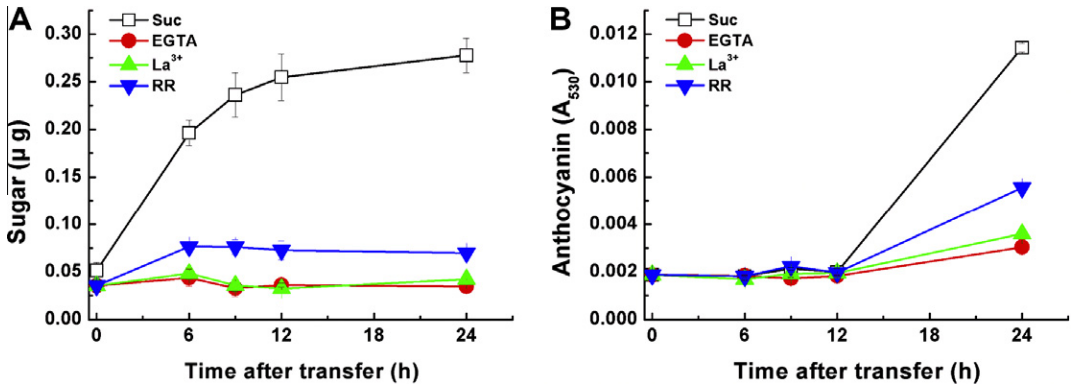

Fig. 2. Effect of Ca^{2+} antagonists on the soluble sugar (A) and anthocyanin (B) contents during the anthocyanin induction period. Nine-day-old Arabidopsis seedlings were incubated with 60 mM sucrose supplemented with 10 mM EGTA, 2 mM La^{3+} and 70 μM RR for the indicated times (0, 6, 9, 12, and 24 h) under continuous white illumination (140 μmol m^{-2} s^{-1}). Error bars represent the SD of the mean of three or five independent replicates.

may be regulated by changes in cytosolic Ca^{2+} levels and that this event occurs earlier than anthocyanin pigmentation.

3.3. SUC1 is expressed earlier than anthocyanin biosynthesis-related genes

Because Suc-induced changes in cytosolic Ca^{2+} levels promote sugar uptake before anthocyanin accumulation, the expression of *SUC1* induced by Suc treatment should occur earlier than that of Suc-responsive anthocyanin biosynthesis genes. As expected, *SUC1* mRNA level increased 30 min after Suc stimulation, reaching peak levels after 3 h followed by a slow decline. Of the Suc-responsive transcription factors tested (*EGL3*, *GL3*, *TT8*, *PAP1*, and *MYBL2*) [9], *PAP1*, a positive regulator of anthocyanin biosynthesis, showed a pattern of induction similar to that of *SUC1* with a 30 min delay. A Suc-responsive late anthocyanin biosynthesis gene, *LDOX*, which is under the regulation of *PAP1* [7,9], showed the slowest response, reaching saturation 12 h after Suc stimulation (Fig. 3). Other Suc-responsive structural genes, such as *CHS*, *DFR*, and *UF3GT* (Fig. S1A), also reached the highest level after 12 h. Transcript levels of Suc-responsive transcription factors, such as *(E)GL3*, *PAP2*, and *TT8* (Fig. S1B), reached the highest levels 3 or 6 h after Suc stimulation, which was earlier than those of structural genes but later than the *SUC1* transcript level. These results indicated that the upregulation of *SUC1* expression is an early response to Suc stimulation that occurs before the induction of anthocyanin biosynthesis.

3.4. Anthocyanin pigmentation in SUC1-defective mutants shows decreased sensitivity to Ca^{2+} antagonists

Our findings showing that the regulation of *SUC1* expression occurs before anthocyanin biosynthesis suggest that anthocyanin pigmentation in response to exogenous Suc treatment in the SUC1 defective mutant (*suc1-1*) should be inhibited compared to that of wild-type plants. Indeed, anthocyanin induction in the *suc1-1* mutants was inhibited by 38.8% when 9-day-old seedlings were transferred to 60 mM Suc-containing liquid growth medium for 24 h under continuous illumination (Fig. 4A), which was consistent with previous findings [8,9]. Ca^{2+} antagonists caused a 70–80% reduction in anthocyanin accumulation in Col-0 plants whereas the *suc1-1* mutant showed a 40–50% decrease. These results indicate that Ca^{2+} signaling-dependent SUC1 expression is required for Suc-induced anthocyanin biosynthesis. Interestingly, anthocyanin accumulation was inhibited in *suc1-1* mutant plants in response to Ca^{2+} antagonist treatment, suggesting the presence of a Ca^{2+} signal-sensitive Suc transport system other than SUC1.

3.5. Sucrose increases intracellular Ca^{2+} concentration in Arabidopsis roots

We next determined in Arabidopsis roots whether Suc increases the cytosolic free Ca^{2+} level in roots under conditions leading to Suc-dependent induction of anthocyanin biosynthesis [9]. We used Arabidopsis transgenic plants expressing a soluble version of the

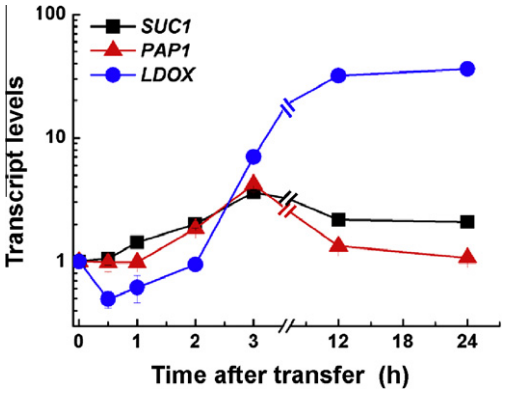

Fig. 3. Effect of Ca^{2+} antagonists on the transcript levels of anthocyanin biosynthesis genes (*LDOX and PAP1*) and *SUC1*. Nine-day-old Arabidopsis seedlings were incubated with (+Suc) 60 mM sucrose supplemented with 10 mM EGTA, 2 mM La^{3+}, and 70 μM RR for the indicated times (0, 6, 9, 12, and 24 h) under continuous white illumination (140 μmol m^{-2} s^{-1}). Error bars represent the SD of the means of three or five independent replicates.

green fluorescence protein-based Ca^{2+} sensor Yellow Cameleon 3 (YC3), driven by the cauliflower mosaic virus 35S promoter [24]. YC3 consists of a calmodulin (CaM) CaM-binding peptide and a yellow version of GFP. Expression of this protein had no detectable effect on anthocyanin pigmentation (data not shown), providing an appropriate approach for the analysis of anthocyanin induction.

Wild-type Arabidopsis seedlings were grown in half-strength MS medium without Suc for 9 d under white light conditions (140 μmol m^{-2} s^{-1}) and then transferred to liquid medium containing 60 mM Suc with or without Ca^{2+} antagonists. As shown in Fig. S2A, YFP fluorescence increased shortly after Suc stimulation and remained at an approximately 2.5-fold higher level than that of the control glucose-fed seedlings, which remained unchanged, indicating a sustained increase in cytosolic free Ca^{2+} for up to 6 h.

To determine the possible mobilization of different Ca^{2+} stores in response to Suc stimulation, various Ca^{2+} antagonists, including EGTA, La^{3+}, and RR, were added to the solution. As shown in Fig. S2B, co-treatment with Ca^{2+} antagonists and 60 mM Suc for 30 min under illumination significantly decreased the fluorescence level to approximately 5–20% of that of the roots treated with Suc alone. These results indicated that Suc increases cytosolic Ca^{2+} concentration by mobilizing Ca^{2+} from intracellular and extracellular stores in Arabidopsis roots.

4. Discussion

The grape anthocyanin biosynthesis gene *DFR* [21] is regulated by Ca^{2+} signaling, as evidenced by the inactivation of Suc-induced anthocyanin biosynthesis caused by Ca^{2+} chelators, and CaM and protein kinase inhibitors in grape cells [20]. However, the regulation of other anthocyanin biosynthesis-related genes, including regulatory genes, by Ca^{2+} flux changes remained unclear in previous results because Suc-induced sugar uptake is an earlier response than anthocyanin accumulation [9]. In the present study, the Suc-induced increase in cytosolic Ca^{2+} levels is primarily associated with sugar uptake and anthocyanin accumulation was shown to be a secondary effect in Arabidopsis. Previous studies showed that sugar uptake is partially regulated by the modulation of SUC1 activity in a dynamic process that occurs at the level of transcription [8,9,25,26]. Consistent with previous studies, our results indicated that Suc-induced *SUC1* transcript levels were modulated by Ca^{2+} antagonists, suggesting that Ca^{2+} flux changes regulate *SUC1* transcription rather than SUC1 activity in Arabidopsis.

A previous study showed that *SUC1* expression could be induced by mono- and di-saccharides and suggested the involvement of an osmotic sensor rather than membrane or intracellular receptors in the regulation of *SUC1* expression [7]. Furthermore, *SUC1* expression in Arabidopsis can be induced effectively by non-metabolizable osmoticums, such as mannitol and palatinose [9], similar to the induction of *CitSUT2* expression [26]. Because osmotic stress, such as that induced by NaCl, is directly correlated

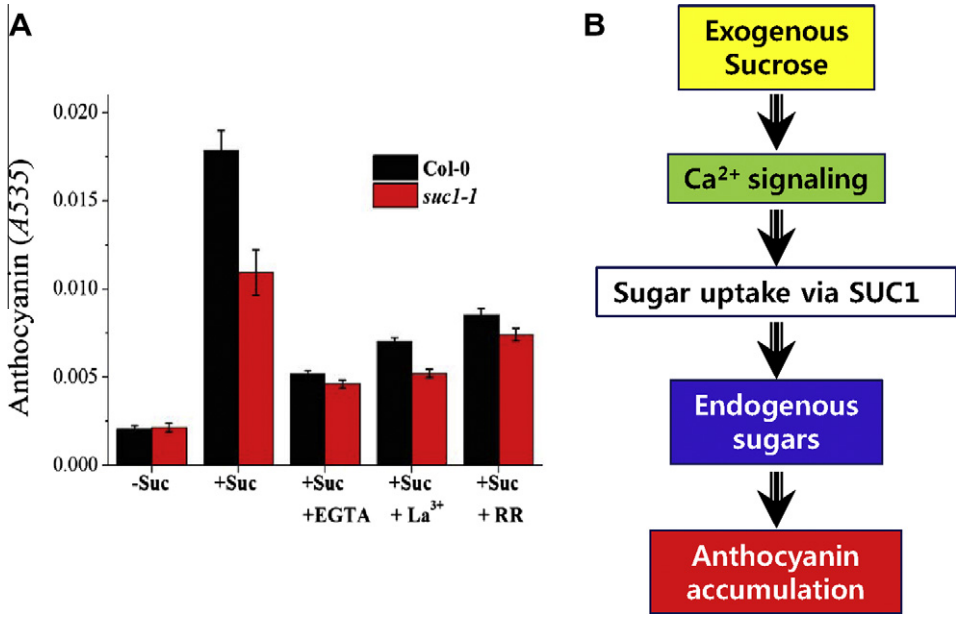

Fig. 4. Sugar uptake via SUC1 is the early target of Ca^{2+} signaling. (A) Suc-induction of anthocyanin pigmentation in wild type (Col-0) and the Suc transporter mutant *suc1-1*. Nine-day-old Arabidopsis seedlings were incubated with (+Suc) 60 mM sucrose supplemented with 10 mM EGTA, 2 mM La^{3+}, and 70 μM RR for 1 d under continuous white illumination (140 μmol m^{-2} s^{-1}). Error bars represent the SD of the means of three or five independent replicates. (B) Ca^{2+} dependent regulation of anthocyanin accumulation in Arabidopsis. Exogenous Suc initiates Ca^{2+} signaling, which leads to Suc uptake via transcriptional regulation of sucrose transporters, including SUC1. Enhanced endogenous sugar levels induce anthocyanin pigmentation via transcriptional activation of several transcription factors related to anthocyanin biosynthesis.

with increased cytosolic Ca^{2+} concentration [27], it is possible that the Suc-mediated induction of *SUC1* expression in Arabidopsis could be mediated by Ca^{2+} signaling.

The mechanism underlying the induction of *SUC1* expression by Ca^{2+} flux changes remains to be elucidated. The involvement of CaM and Ca^{2+}-dependent protein kinases, which were shown to regulate sugar-induced fructan biosynthesis [28] and the expression of β-amylase and sporamin [29], is one possible mechanism. However, experimental evidence of the involvement of CaM and kinases could not be provided in the present study, as plants treated with the CaM antagonists N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7) and chlorpromazine (CPZ), and the kinase inhibitor 6-dimethylaminopurine (DMAP), showed similar sugar and anthocyanin contents and transcript levels of anthocyanin genes and SUC1 to those of control plants (Fig. S3A). On the other hand, the protein phosphatase inhibitors endothall and cantharidin significantly inhibited anthocyanin and sugar contents (Fig. S3A and B), although *SUC1* transcript levels remained unchanged (Fig. S3C). Our results suggest that W7-, CPZ-, and DMAP-sensitive CaM and protein kinases may not play a role in SUC1 activation. However, the effect of phosphatase inhibitors on the levels of anthocyanin suggests the possible involvement of a SUC1 independent Suc signaling pathway regulating the expression of anthocyanin biosynthesis genes. This hypothesis is supported by the sensitivity of *suc1-1* mutants to Ca^{2+} antagonist treatment (Fig. 4A). Further molecular genetics studies are necessary to elucidate the mechanisms underlying the effect of Suc on the modulation of cytosolic Ca^{2+} levels leading to sugar uptake mediated by root SUC1 and other sugar transporters as yet unidentified.

The induction of *SUC1* expression requires the presence of light, but is independent of light signaling components known to be involved in anthocyanin biosynthesis, such as CRY1, PHY B, and HY5 [9]. Instead, photosynthesis-related signaling, and especially the redox state of the plastoquinone (PQ) pools, were suggested to act as a sensor for light- and sugar signaling associated with anthocyanin accumulation [9,30]. This suggests that photosynthesis-derived signals may be involved in the Ca^{2+}-mediated regulation of SUC1 dependent sugar uptake, although the underlying mechanisms are not clear. Nevertheless, our results suggest that sugar transport systems other than SUC1 may be involved in anthocyanin induction, as evidenced by the limited effect of the *SUC1* mutation on photosynthesis-dependent anthocyanin accumulation [9]. The existence of a Ca^{2+}-dependent SUC1-independent Suc transport pathway was supported by our findings showing the sensitivity of *SUC1* mutants to Ca^{2+} antagonists (Fig. 4B). Other than known SUCs, sugar transporter family ERD6-like homologs (At1g08920 and At1g08930), two plastidic Glc transporters (At1g79820 and At5g16150), and four members of the aquaporin gene family (*PIP1:2*, *PIP1:3*, *PIP2:8*, and *SIP1:1*) are abundantly expressed in roots [31], and could be involved in anthocyanin pigmentation in a Ca^{2+} signal-dependent manner.

In conclusion, we demonstrated that Suc-induced Ca^{2+} signaling modulates anthocyanin biosynthesis in *Arabidopsis*. In addition, intracellular Ca^{2+} levels were shown to correlate with *SUC1* transcript levels during Suc induction of anthocyanin accumulation, which can be inhibited by Ca^{2+} blockers. The downstream components of the Ca^{2+} signaling pathway leading to SUC1 expression and the potential SUC1-independent Ca^{2+} signaling pathway contributing to the induction of anthocyanin biosynthesis remain to be characterized.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.11.100.

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