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1 ***Running title: DMSO accelerating Hypo-OS-induced Ca²⁺ elevation***

2

3 **Enhanced elevations of hypo-osmotic shock-induced cytosolic and nucleic calcium**
4 **concentrations in tobacco cells by pretreatment with dimethyl sulfoxide**

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1 **Abstract**

2 Dimethyl sulfoxide (DMSO) is a dipolar aprotic solvent widely used in biological assays.

3 Here, we observed that DMSO enhanced the hypo-osmotically induced increases in the

4 concentration of Ca^{2+} in cytosolic and nucleic compartments in the transgenic cell-lines of

5 tobacco (BY-2) expressing aequorin.

6

7 **Key words:** cytosolic calcium concentration; dimethyl sulfoxide; hypo-osmotic shock;

8 nucleic calcium concentration

9

10

1 Dimethyl sulfoxide (DMSO) is a dipolar aprotic solvent that can solubilize a variety of
2 polar and nonpolar molecules [1]. As a powerful solvent, DMSO has widely been used for
3 biological applications, particularly for pharmacological researches [2]. In biological assays,
4 numerous chemicals with low water solubility need to be dissolved in organic solvents prior
5 to addition to living systems [2]. The unique characteristic of DMSO is that it permeates into
6 living cells without causing significant damage, thus, making DMSO as one of the most used
7 solvents [3]. However, recent studies started to unveil a series of hidden and unexpected
8 actions of DMSO such as removal of hydroxyl radical at high concentration [4], and
9 cyto-toxicity at relatively low concentration in several biological models [1,2], including
10 plants [5], possibly due to the nature of DMSO to be readily associated with water, proteins,
11 carbohydrates, nucleic acid, ionic substances and other constituents in living systems [3].

12 A study by Gurtovenko and Anwar (2007) [6] had elucidated the molecular mechanism
13 for DMSO interacting with synthetic phospholipid membranes, in which DMSO induces
14 membrane thinning at low concentration (< 10 %) and induces water pores at higher
15 concentration (10 - 20 %). Apparently, DMSO (even at low concentration) firstly acts on the
16 plasma membrane in living cells leading to the increased fluidity of water into the
17 intracellular spaces, therefore, implying the possibility that the cells are sensitized towards
18 hypo-osmotic shock (Hypo-OS).

19 Interestingly, Hypo-OS which is a model stimulus for studying the mechanical stretching

1 of plasma membrane in plant cells [7], reportedly stimulates the elevations of free Ca^{2+}
2 concentration in the cytosol and/or nuclei through the actions of putative mechano-sensitive
3 calcium channels in tobacco and rice cells [8-10]. Among the cations, Ca^{2+} plays a pivotal
4 role as a second messenger in plants [11-13], thus, the elucidation of Ca^{2+} signaling profile
5 upon Hypo-OS is of great importance. Here, we attempted to evaluate the effect of DMSO on
6 the hypo-osmotically induced elevations of $[\text{Ca}^{2+}]_{\text{C}}$ and $[\text{Ca}^{2+}]_{\text{N}}$ in plant cells by using two
7 transgenic lines of tobacco (*Nicotiana tabacum* L.; cell-line, BY-2) expressing a Ca^{2+} -sensing
8 protein (aequorin) in either cytosol [8] or nucleus [14].

9 In the present study, we followed the recently proposed protocols, in which native and
10 fluorine(F)-substituted coelenterazines are used for reconstitution of cytosolically localized
11 native aequorin and nucleically localized F-aequorin in two transgenic lines of tobacco BY-2
12 cells (7-day-old culture) [15]. The aequorin luminescence reflecting the changes in
13 Hypo-OS-induced $[\text{Ca}^{2+}]_{\text{C}}$ and $[\text{Ca}^{2+}]_{\text{N}}$ was monitored by using a luminometer and expressed
14 as relative luminescence units (RLU) [15]. Hypo-OS was applied by injecting a known
15 volume of pure water onto the cell suspension (100 μL) [15]. The extent of given Hypo-OS
16 was set at 112 mOsm (mmol kg^{-1}).

17 To study the impacts of DMSO on the Hypo-OS-induced $[\text{Ca}^{2+}]_{\text{C}}$ and $[\text{Ca}^{2+}]_{\text{N}}$, the cells
18 were pre-incubated with different concentrations of DMSO for 5 min, which was sufficiently
19 long enough for stabilization of cells prior to addition of Hypo-OS (Fig. S1). Low (0.1 %;

1 v/v), moderate (0.5 %) and high doses (1 %) of DMSO were used and the results are shown
2 in Fig. 1(a-g). The typical traces of Hypo-OS-induced $[Ca^{2+}]_C$ and $[Ca^{2+}]_N$ showed that
3 Hypo-OS caused the biphasic increases in both $[Ca^{2+}]_C$ and $[Ca^{2+}]_N$, consisted of the first
4 transient minor increases (1st peak; observed within initial 30 s) and the second major
5 increases (2nd peak; peaking time, *ca.* 85 ± 15 s) (Fig. 1a,b). Hypo-OS-triggered the 1st and
6 2nd peaks in $[Ca^{2+}]_C$ and $[Ca^{2+}]_N$ were not altered by low dose of DMSO. Upon application of
7 moderate or high dose of DMSO, significant increases in the 1st and 2nd peaks in $[Ca^{2+}]_C$ and
8 $[Ca^{2+}]_N$ were observed (Fig. 1e,f). The most evident actions of DMSO (at high dose) was to
9 enhance the 1st peaks of hypo-osmotically induced $[Ca^{2+}]_C$ and $[Ca^{2+}]_N$, by 450 % and 250 %,
10 respectively (Fig. 1g). These results suggested that DMSO can enhance the actions of
11 Hypo-OS which is reflected by the increases in both $[Ca^{2+}]_C$ and $[Ca^{2+}]_N$.

12 As the concentrations of DMSO applied in this study were much lower than the reported
13 concentration (10 - 20 %) causing the formation of pores [6], the pore formation on the
14 membranes is not likely to be induced. Instead, cells could be sensitized by DMSO's
15 membrane thinning effect [6], therefore, we have hypothesized that upon combination of
16 DMSO and Hypo-OS, transient infusion of water into the cells would be induced, and if this
17 is the case, any bulk movement of water may non-selectively accompany the movement of
18 solutes including extracellular Ca^{2+} . In Fig. 1 (c and d), two sets of log-log plots emphasizing
19 the early events within few seconds following the Hypo-OS are shown, effectively

1 visualizing the impact of 1% DMSO on the Hypo-OS-induced immediate spikes prior to the
2 naturally induced 1st peak. Here, we view that such immediate supply of Ca²⁺ may act for
3 potentiating the ion channel-mediated natural phases of [Ca²⁺]_C and [Ca²⁺]_N elevations.

4 Since the extents of DMSO-dependent enhancement observed in the 1st and 2nd phases
5 ([Ca²⁺]_C and [Ca²⁺]_N) were not proportional (Fig. 1g), it is tempting to speculate that the
6 regulatory mechanisms for the 1st and 2nd [Ca²⁺] peaks may be independent from each other,
7 possibly involving different Ca²⁺ sources. The Hypo-OS-induced increases in [Ca²⁺]_C and
8 [Ca²⁺]_N are reportedly sensitive to Gd³⁺, a known inhibitor of stretch-activated Ca²⁺ channels,
9 suggesting that extracellular Ca²⁺ is required for elevations of [Ca²⁺]_C and [Ca²⁺]_N in response
10 to Hypo-OS [9,16]. In addition, internal Ca²⁺ stores, such as endoplasmic reticulum (ER), are
11 also considered to take part in intracellular [Ca²⁺] elevations [9,15]. Recently, Wang et al.
12 (2017) [17] conducted a model assay on the side effect of DMSO used as a cryoprotectant in
13 bovine oocytes and observed that DMSO induces Ca²⁺ release from ER allowing the Ca²⁺
14 elevations in both cytosol and mitochondria during the vitrification of bovine oocytes. By
15 analogy, DMSO might enhance the release of Ca²⁺ from internal Ca²⁺ stores in plant cells
16 under Hypo-OS.

17 Concerning the physiological significance of the use of DMSO, our stance is clear that
18 the use of DMSO in plant cellular models is far from naturally functioning physiological
19 condition. Our data clearly suggested that the use of DMSO which is highly favored in a

1 variety of biological models should be avoided in order to study the truly physiological
2 cellular responses to a variety of stimuli such as Hypo-OS. In addition, more examples of the
3 induced physiological distortion by DMSO are gradually accumulated in various models such
4 as hydroponically grown rice seedlings [5] uncovering the actions of DMSO (up to 0.1 %,
5 w/v; 72 h) inducing the growth inhibition, cell death, alteration of protein contents,
6 accumulation of H₂O₂, and alteration of the antioxidant enzyme activities; and *Candida* yeast
7 [18] showing the alteration of catalase activity and expression of cell wall proteins by
8 non-lethal concentration (< 1 %, w/v) of DMSO.

9 In conclusion, DMSO may work as a factor to accelerate the hypo-osmotically induced
10 increases in [Ca²⁺]_C and [Ca²⁺]_N in plant cells. The synchronized responses in [Ca²⁺]_C and
11 [Ca²⁺]_N in the presence of different concentrations of DMSO supported the view that [Ca²⁺]_C
12 and [Ca²⁺]_N may share the same regulatory mechanisms in response to Hypo-OS.

13

14 **Author contributions**

15 HTHN collected and analyzed the data. FB and CM prepared two tobacco BY-2 cell-lines and
16 discussed the results. MK synthesized the coelenterazines using in this study. TK supervised
17 this study and designed the experiments. HTHN and TK wrote the manuscript. All authors
18 read and approved this paper.

19

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5

6 **Conflict of interest**

7 The authors declare no conflict of interest.

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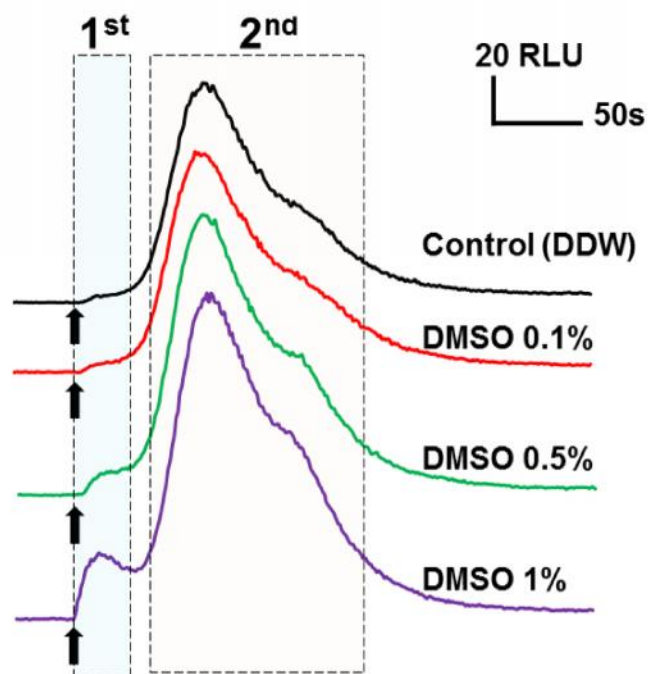
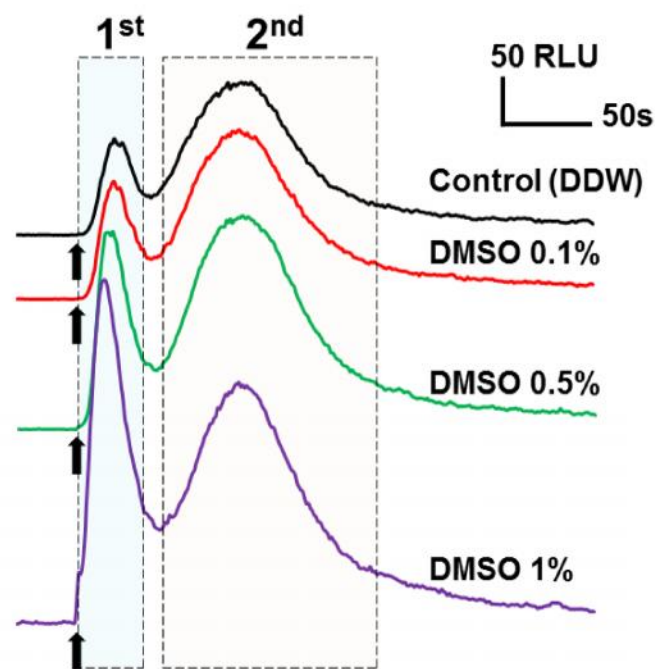
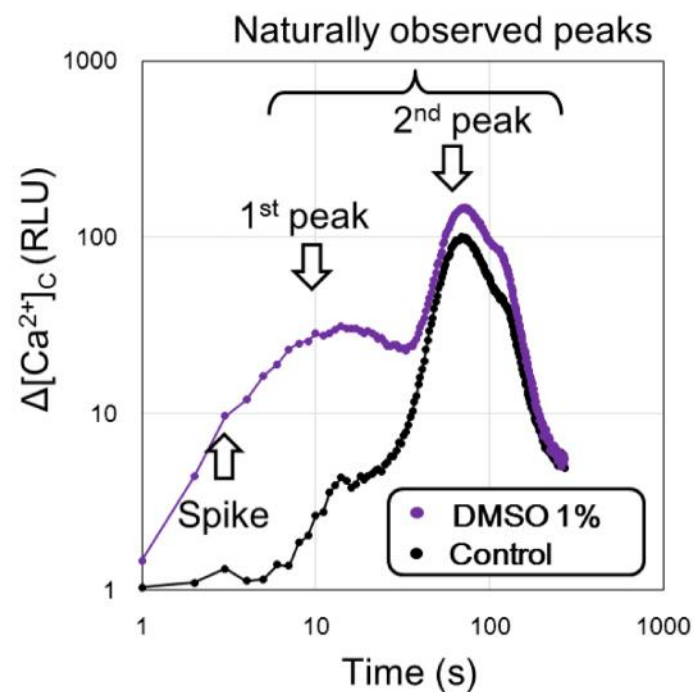
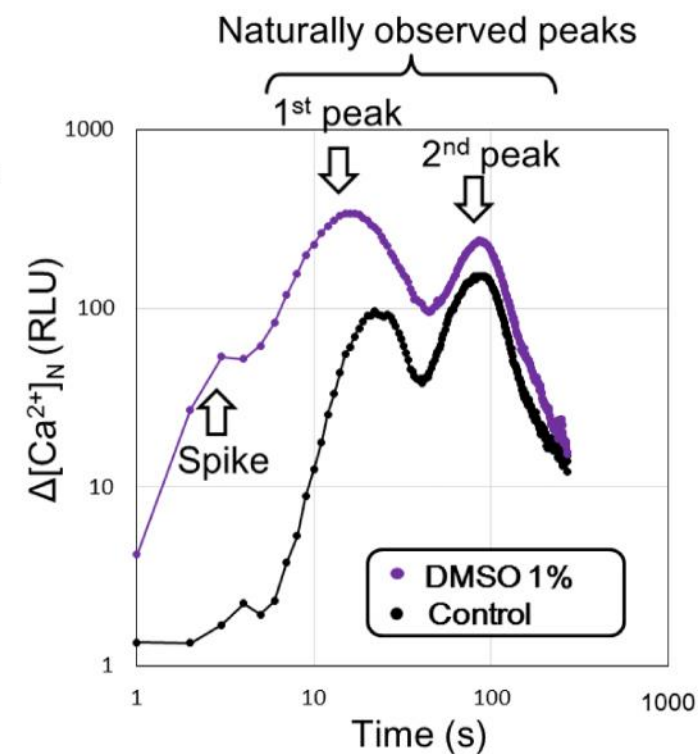
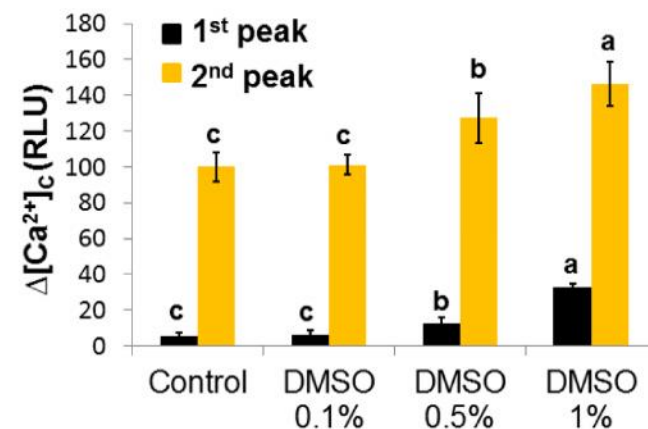
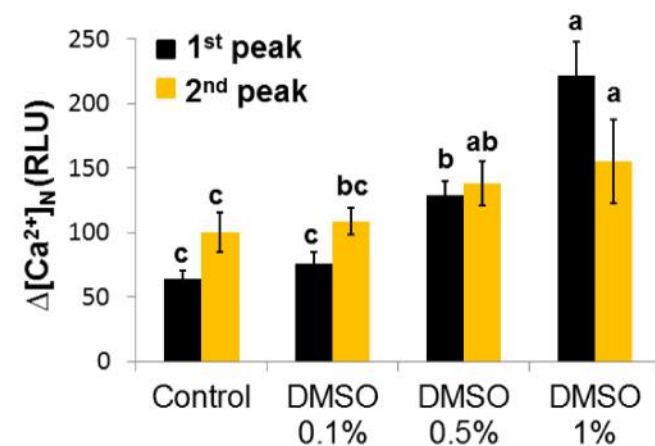
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1 **Figure legends**

2 **Figure 1.** Effect of DMSO on the Hypo-OS-induced $[Ca^{2+}]_C$ and $[Ca^{2+}]_N$. Aequorin signals
3 reflecting the changes in $[Ca^{2+}]_C$ and $[Ca^{2+}]_N$ were monitored after the cells were treated with
4 DMSO for 5 min prior to Hypo-OS (112 mOsm). Mean traces of Hypo-OS-induced $[Ca^{2+}]_C$
5 (a) and $[Ca^{2+}]_N$ (b) monitored for 5 min in the presence of different concentration of DMSO.
6 Log-log plots of aequorin signals and time course after addition of Hypo-OS visualizing the
7 DMSO-sensitized and Hypo-OS-induced immediate spikes of $[Ca^{2+}]_C$ (c) and $[Ca^{2+}]_N$ (d).
8 Peak-height of Hypo-OS-induced increase in $[Ca^{2+}]_C$ (e) and $[Ca^{2+}]_N$ (f) are compared.
9 Extents of stimulation by DMSO in the 1st and 2nd phases in $[Ca^{2+}]_C$ and $[Ca^{2+}]_N$ are
10 compared (g). The traces and values are means of 5 replications. Error bars in (e) and (f)
11 represent S.D. (n=5). Statistical significance ($P < 0.05$) labelled with different symbols in (e)
12 and (f) were calculated with one-way ANOVA and Tukey's test.

13

(a) $[Ca^{2+}]_c$ **(b) $[Ca^{2+}]_N$** **(c) $[Ca^{2+}]_c$** **(d) $[Ca^{2+}]_N$** **(e)****(f)****(g)**