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1 **Possible inhibition of Arabidopsis VIP1-mediated mechanosensory signaling by**  
2 **streptomycin**

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13 **Key words:** *Arabidopsis thaliana*; bZIP transcription factor; calcium signaling; hypo-osmotic  
14 stress; mechanical stress; root tropisms

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18 **Abstract**

19 VIP1 (VIRE2-INTERACTING PROTEIN 1) and its close homologues are *Arabidopsis*  
20 *thaliana* bZIP proteins regulating stress responses and root tropisms. They are present in the  
21 cytoplasm under steady conditions, but transiently accumulate in the nucleus when cells are  
22 exposed to mechanical stress such as hypo-osmotic stress and touch. This pattern of changes  
23 in subcellular localization is unique to VIP1 and its close homologues, and can be useful to  
24 further characterize mechanical stress signaling in plants. A recent study showed that calcium  
25 signaling regulates this pattern of subcellular localization. Here, we show that a possible  
26 calcium channel inhibitor, streptomycin, also inhibits the nuclear accumulation of VIP1.  
27 Candidates for the specific regulators of the mechanosensitive calcium signaling are further  
28 discussed.

29

30 VIP1 (VIRE2-INTERACTING PROTEIN 1) and its close homologues are *Arabidopsis*  
31 *thaliana* bZIP proteins. They are members of the plant bZIP protein group I,<sup>1</sup> and are  
32 involved in regulating responses to biotic and abiotic stresses.<sup>2-15</sup> Such stresses include  
33 hypo-osmotic stress and touch.<sup>6,9-11</sup> Hypo-osmotic stress and touch are not identical, but both  
34 impose mechanical stress on cells and induce partially common cellular responses.<sup>16</sup> When  
35 either VIP1 or its close homologue bZIP29 is expressed as a repression domain-fused form in  
36 *Arabidopsis*, touch-induced root bending is enhanced.<sup>10-12</sup> At least six *Arabidopsis* group I  
37 bZIP proteins (VIP1, PosF21/bZIP59, bZIP69, bZIP29, bZIP30 and bZIP52) are present in  
38 the cytoplasm under steady conditions, but transiently accumulate in the nucleus when cells  
39 are exposed to hypo-osmotic stress.<sup>6,9,10</sup> The transient nuclear accumulation of VIP1 can also be  
40 induced by touch.<sup>11</sup> No other plant proteins with similar subcellular localization patterns have  
41 been identified thus far. The group I bZIP proteins can therefore be a clue to more deeply  
42 understanding mechanical stress signaling.

43         Phosphorylation is a factor regulating the nuclear-cytoplasmic shuttling of VIP1 and  
44 its close homologues. Under steady conditions, VIP1 is largely phosphorylated and bound by  
45 14-3-3 proteins, which are thought to retain VIP1 in the cytoplasm. Hypo-osmotic stress  
46 causes dephosphorylation of VIP1, and this dephosphorylation can be needed for the nuclear  
47 accumulation of VIP1.<sup>14</sup> Calcium signaling has recently been found to be another factor  
48 regulating the nuclear-cytoplasmic shuttling of VIP1 and its close homologues. These

49 proteins physically interact with the calmodulin calcium-binding proteins, and both the  
50 nuclear accumulation and the cytoplasmic accumulation of VIP1, bZIP59 and bZIP29 are  
51 inhibited by either a calcium chelator (EDTA or EGTA) or a calmodulin inhibitor  
52 (chlorpromazine).<sup>15</sup> Calcium-dependent protein kinases and B'' subunits of the protein  
53 phosphatase 2A complex may regulate phosphorylation states of group I bZIP proteins in  
54 calcium-dependent manners,<sup>12,17</sup> although neither of them was co-immunoprecipitated with  
55 VIP1 in a previous study (i.e., they may not interact with VIP1 very strongly).<sup>15</sup> The same  
56 study also showed that the nuclear-cytoplasmic shuttling of VIP1 is not affected either by the  
57 double knockout of two calcium channels, MCA1 and MCA2, or by GdCl<sub>3</sub> or LaCl<sub>3</sub>, which  
58 can inhibit other types of mechanosensitive calcium channels.<sup>15,18,19</sup>

59         Which factor initiates the calcium signaling that regulates functions of group I bZIP  
60 proteins, then? In our recent experiments, 1 mM streptomycin, which can inhibit  
61 mechanosensitive calcium channels in animals,<sup>20,21</sup> was found to inhibit the hypo-osmotic  
62 stress-induced nuclear accumulation of VIP1 (Fig. 1, middle panel). This effect of  
63 streptomycin was not observed when 20 mM CaCl<sub>2</sub> was present (Fig. 1, right panel). Land  
64 plants have no homologues of transient receptor potential (TRP) channels,<sup>22</sup> which are  
65 putative targets of streptomycin in animals,<sup>20,21</sup> and the major target of streptomycin is  
66 thought to be plastid 70S ribosome-mediated protein synthesis in plants.<sup>23</sup> However, it would  
67 be still possible that the streptomycin-sensitive proteins or processes are initiators of the

68 mechanosensory signaling mediated by VIP1 and its close homologues. In a similar  
69 experiment, cycloheximide, which binds to 60S ribosomes and thereby inhibits cytoplasmic  
70 protein synthesis, did not affect the nuclear-cytoplasmic shuttling of VIP1, raising the  
71 possibility that cytoplasmic protein synthesis is not required for either the calcium signaling  
72 or downstream events regulating the subcellular localization of VIP1. Two calcium  
73 ionophores, ionomycin and A23187, did not affect it, either, raising the possibility that a  
74 calcium gradient does not primarily control the nuclear cytoplasmic shuttling of VIP1. Effects  
75 of inhibitors of calcium signaling and protein synthesis on the subcellular localization of  
76 VIP1 are summarized in Table 1. These results, again, support the idea that the calcium  
77 signaling regulating the subcellular localization of VIP1 and its close homologs is generated  
78 by specific factors. It should be important to dissect specificity in cellular and physiological  
79 functions of possible regulators of calcium signaling.

80

#### 81 **Disclosure of interest**

82 The authors report no conflict of interest

83

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87

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149

150 Table 1. Summary of effects of inhibitors of calcium signaling and/or protein synthesis on the  
 151 subcellular localization of VIP1.

| Chemical          | Known role  | Conc. used | Effects on the VIP1 subcellular localization   |
|-------------------|---|------------|--|
| EDTA              | Divalent cation chelator  | 2 mM       | Inhibitory on both the nuclear accumulation and the cytoplasmic accumulation <sup>15</sup> |
| EGTA              | Divalent cation chelator  | 2 mM       | Inhibitory on both the nuclear accumulation and the cytoplasmic accumulation <sup>15</sup> |
| Chlorpromazine    | Calmodulin inhibitor  | 0.5 mM     | Inhibitory on both the nuclear accumulation and the cytoplasmic accumulation <sup>15</sup> |
| GdCl <sub>3</sub> | Inhibitor of mechanosensitive calcium channels                  | 1 mM       | Not detected <sup>15</sup>   |
| LaCl <sub>3</sub> | Potential replacement of calcium in the cell wall               | 1 mM       | Not detected <sup>15</sup>   |
| Streptomycin      | Inhibitor of 70S ribosomes and mechanosensitive calcium channel | 1 mM       | Inhibitory on the nuclear accumulation of VIP1 (this study)                                |
| Cycloheximide     | Inhibitor of 60S ribosomes                                      | 0.5 mM     | Not detected (this study)  |
| A23187            | Calcium ionophore   | 0.1 mM     | Not detected (this study)  |
| Ionomycin         | Calcium ionophore   | 0.05 mM    | Not detected (this study)  |

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153

154 **Figure legend**

155 Figure 1. Streptomycin inhibits the hypo-osmotic stress-induced nuclear accumulation of VIP1.  
156 Arabidopsis plants overexpressing GFP-tagged VIP1<sup>6</sup> were grown for 10 days on half strength  
157 MS medium containing 1 % (w/v) sucrose and 0.8 % (w/v) agar, incubated for 0 or 10 minutes in  
158 a hypotonic solution (20 mM Tris-HCl, pH 7.5) containing 0 or 1 mM streptomycin and 0 or 20  
159 mM CaCl<sub>2</sub>, and subjected to epifluorescence microscopy to detect GFP signals. The presence of  
160 streptomycin and CaCl<sub>2</sub> in the solution is indicated as “+ Streptomycin” and “+ CaCl<sub>2</sub>”. For each  
161 treatment, more than five individual plants were observed, and representative results are  
162 presented. Scale bars = 0.1 mm.

163

