Pak. J. Bot., 42(1): 353-359, 2010.

SENSITIVITY TO ABSCISIC ACID REGULATES STOMATAL OSCILLATION AND CLOSURE IN *ARABIDOPSIS THALIANA*

QIU MU-QING[1*] AND ZHANG HAO[2]

[1]*Institution of Life Science, Shaoxing University, Shaoxing, 312000, P.R. China*
[2]*Institution of Digital Agricultural Research, Zhejiang Academy of Agricultural Sciences, Hangzhou, 310021 P. R. China*
**Correspondence author e-mail: qiumuqing@126.com*

Abstract

Stomatal oscillation has been described as an efficient mechanism to prevent water from loss, decrease the transpiration rate and to improve water use efficiency under severe drought conditions. Former researches demonstrated that ABA-induced H_2O_2 production and H_2O_2-activated Ca^{2+} channels were important mechanism for ABA-induced stomatal oscillation. In this study, the sensitivity to abscisic acid (ABA) for regulation of stomatal oscillation was investigated in *Arabidopsis thaliana* mutants (*abi1-1*, insensitive to ABA and *era1-2*, hypersensitive to ABA) and wild type. The results showed that (1) hypersensitive to ABA strengthen stomatal oscillation and closure induced by ABA; (2) insensitive to ABA abolish stomatal oscillation and closure induced by ABA; and (3) there is positive relationship between stomatal oscillation, closure and sensitivity to abscisic acid. These data indicate that sensitivity to ABA may regulate stomatal oscillation and closure in *Arabidopsis thaliana*.

Introduction

Stomata function as the gates between the plant and the atmospheric environment. Stomatal oscillation, as a special rhythmic stomatal movement, can keep CO_2 absorption at a sufficient level and reduce water loss at the same time, suggesting a potential improvement in water use efficiency (Upadhyaya, 1988; Wang *et al*., 2001; Yang *et al*., 2003). It also has been confirmed by continuous microscopic observation *in situ* (Pei *et al*., 2000; Kaiser & Kappen, 2001). In recent years, researches have been concentrated on the mechanism of stomatal oscillation induced by environmental stress (Allen *et al*., 2000).

Growing evidences suggest that calcium oscillations in guard cell may play a vital role in stomatal oscillation (Yang *et al*., 2003). McAnish *et al*., (1995, 2000) showed the calcium-permeable channels were involved in the generation of calcium oscillations in guard cell. Evidence also indicates that abscisic acid (ABA) increase the probability for the opening of calcium-permeable channels (Allen *et al*., 1999), suggesting that it may be involved in the generation of calcium oscillations that frequently accompany ABA-induced stomatal oscillation (Staxén *et al*., 1999). Although the role of calcium and ABA in controlling stomatal oscillation is becoming clearer with the application of new molecular genetics and imaging techniques (Sanders *et al*., 1999), the relationship between ABA sensing and stomatal oscillation remains unclear.

In *Arabidopsis*, the ABI1 gene encodes for a member of the 2C class of protein serine/threonine phosphatases (PP2C) and the *abi1-1* mutation markedly reduces ABA responsiveness (Françoise *et al*, 1999). ERA1 gene encodes for farnesyltransferase, which mediate membrane targeting of specific soluble signaling proteins by attachment of a hydrophobic farnesyl group to the C terminal target sequences. The *era1-2* farnesyltransferase β-subunit is a negative regulator of ABA signal transduction in seeds

and guard cell (Pei *et al*., 1997; 1998). Therefore, protein phosphatase 2C (PP2C) and farnesyltransferase are negative and positive regulators of ABA signaling respectively (Françoise *et al*., 1999).

We have already shown that calcium oscillations can induce stomatal oscillations, small in magnitude, after inducing a rapid decline in aperture by calcium and ABA treatments (Yang *et al*., 2003). Here, we hypothesize that sensitivity to ABA in guard cell regulates stomatal oscillation and closure. If this hypothesis is true, we can predict that stomatal oscillation and closure in dose- and time-dependent way may change in response to high ABA buffer for sensitivity to abscisic acid in guard cell. In this study, *Arabidopsis thaliana* mutants (*abi1-1*, insensitive to ABA and *era1-2*, hypersensitive to ABA) and wild type were used to study whether guard cell sensitivity to ABA affect stomatal oscillation and closure.

Materials and Methods

Plant material and growth conditions: *Arabidopsis thaliana* mutants (*abi1-1*, insensitive to ABA and *era1-2*, hypersensitive to ABA) and wild type were grown in a 12-h light/12-h dark cycle at photon fluency rate of 100 μmol m^{-2} s^{-1} at 22ºC for 6-8 weeks. The lower fully expanded leaves of 4-week-old plants were harvested. The epidermis was carefully peeled from the abaxial surface and used for the following measurements (Yang et al., 2003; 2006).

Steady treatments with extracellular $Ca^{2+}$and ABA: Leaf abaxial epidermal strips were incubated in the light (0.12-0.16 mmol m^{-2} s^{-1} at 20-22ºC) for 2 h in buffer contained 50mM KCl and 10mM MES-Tris pH 6.15 (MES-KCl) to induce stomatal open. Epidermis was then transfer to buffer containing 50 mM KCl, 50 mM mannitol and 10 mM Tris-MES pH 8.3 in the presence of $CaCl_2$, or ABA respectively. Optimal concentration of $CaCl_2$ and ABA were determined from dose-dependent curves.

Rapid exchange treatments with extracellular Ca^{2+}, and ABA: After incubation in the light (0.12-0.16 mmol m^{-2} s^{-1} at 20-22ºC) for 2 h in MES-KCl buffer to open stomata, epidermal strips were transferred to buffers containing 100 mM KCl, 0 $CaCl_2$ and 10 mM Tris-MES pH 6.15 (depolarizing buffer) and 0.1 mM KCl, 10 mM $CaCl_2$ and 10 mM Tris-MES pH 6.15 (hyperpolarizing buffer). Similar, protocols were carried out in the presence of ABA instead of $CaCl_2$ at 50μm.

Stomatal aperture measurements: Individual stomatal pores were measured 1-2 minute after treatment using a digital microscope (Nikon, Japan). Stomatal movement in 15-30 cells was measured for each treatment. The stomatal aperture was calculated as pore width/length. Mean stomatal apertures was described as A_t. Data were presented as mean ± S.D. of mean.

Analyses of data: All experiments were repeated at least three times and representative data are presented. Data were analyzed by One-Way ANOVA, using SPSS for windows 10 (America, SPSS Company, Chicago) and taking $p<0.05$(LSD) as significant.

Results and Discussion

Stomatal aperture declined with time and pore width deceased by 35% and 42% after 60 mins., of treatment with ABA in wild type and *era1-2* respectively, while stoma reduced the pore width was inhibited in *abi1-1* (Fig. 1, Table 1). Figure 2 and Table 1 showed that, for ABA treatments, stomatal apertures in wild type decreased by 35% at 10 μM and closer to 40% at 50 μM, less than that in *era1-2* at 50 μM. While for *abi1-1*, the decrease in stomatal aperture in dose-independent way was shown to be abolished after 60 mins (Fig. 2, Table 1).

Stomatal oscillation frequency of three genotypes *Arabidopsis* also was influenced by sensitivity to ABA. Under control conditions, stomatal oscillation did occur (no ABA or Ca^{2+}) and the frequency was low (0.12~0.16). After treatments with ABA, the frequency of oscillation increased (0.47~0.52), especially when rapid exchange treatments were used (0.79~0.82) with wild type and *era1-2*. However, ABA treatment did not cause *abi1-1* to high frequency (Table 2). Under steady ABA treatment, stomatal oscillation occurred after a sharp decline in stomatal aperture (Fig. 3). No obvious oscillation occurred under ABA treatment in *abi1-1* (Fig. 3). Under hyperpolarizing/depolarizing buffer conditions, stomatal oscillation could be induced by ABA treatment for all three *Arabidopsis* except *abi-1* (Fig. 4).

Meanwhile, all data indicated that sensitivity to ABA affect stomatal aperture and oscillations. Stomatal aperture of three *Arabidopsis* decreased significantly in time- and dose-dependent way (Fig. 1 and Fig. 2, Table 1). Also, Table 2 showed that frequency of stomatal oscillation increased from 0.14 to 0.81 after treatment Ca^{2+} in three genotypes *Arabidopsis*. Fig. 3 and Fig. 4 indicated that stomatal oscillations induced by Ca^{2+} treatment were free charge of sensitivity to ABA in three genotypes *Arabidopsis*.

Therefore, these data suggest that sensitivity to abscisic acid may regulate stomatal oscillation. Previous reports have shown that ERA1 and ABI1 may acts upstream of calcium influx (Pei *et al*., 1997). For ERA1, cytosolic calcium increase occur in guard cell protoplast pre-exposed to ABA and resulted in the ABA-hypersensitive activation of anion channels, even though the calcium was buffered subsequently to 280-nM during patch clamp experiments (Pei *et al*., 1998). Equally, anion channel activation may occur via a calcium-independent pathway, as proposed by Allen *et al*., (2000) and Li *et al*., (2000). This pathway may also be hypersensitive to guard cells ABA in *era1-2*. Besides, a previous study with the *abi1-1* and *abi2-1* PP2C mutants demonstrated that the addition of external calcium resulted in stomatal closure in both the wild type and PP2C mutants by activating processes downstream of increases cytosolic calcium (Allen *et al*., 2000). This showed that mutations *abi1-1* and *abi2-1* can be bypassed in the stomatal closing pathway. Furthermore, because mutations *abi1-1* and *abi2-1* disrupt the ABA activation of plasma membrane Ca^{2+} channels, the ability to impose cytosolic calcium increases by adding external Ca^{2+} suggests that the external Ca^{2+}-induced Ca^{2+} oscillation pathway differs from the ABA-induced Ca^{2+} oscillation pathway (Allen *et al*., 2000). This hypothesis is strengthened by the finding that the *det3* mutant affects the external Ca^{2+} pathway, but not the ABA signaling pathway (Allen *et al*., 2000), and proved indirectly by our data.

Briefly, this study showed that (1) hypersensitive to ABA strengthen stomatal oscillation and closure induced by ABA; (2) insensitive to ABA abolish stomatal oscillation and closure induced by ABA; and (3) there is positive relationship between stomatal oscillation and closure and sensitivity to abscisic acid. These data indicate that sensitivity to ABA may regulate stomatal oscillation and closure in *Arabidopsis thaliana*.

Table 1. Stomatal concentration-dependence by steady extracellular Ca^{2+}, ABA treatments in 60mins at different concentrations.

Treatments		Ca^{2+}				ABA		
		wild type	*abi1-1*	*era1-2*		wild type	*abi1-1*	*era1-2*
Time	0 min	0.220 ± 0.009a	0.216 ± 0.008a	0.222 ± 0.009a	15mins	0.224 ± 0.008a	0.221 ± 0.008a	0.223±0.009a
	15mins	0.176 ± 0.007b	0.172 ± 0.007b	0.173 ± 0.007b	15mins	0.197 ± 0.006b	0.210 ± 0.007a	0.178 ± 0.007c
	30mins	0.158 ± 0.005c	0.154 ± 0.005c	0.155 ± 0.005c	30mins	0.177 ± 0.005c	0.209 ± 0.005a	0.154 ± 0.005d
	60mins	0.139 ± 0.004d	0.136 ± 0.004d	0.136 ± 0.004d	60mins	0.150 ± 0.004d	0.210 ± 0.004a	0.130 ± 0.005e
Concentration	0 mM	0.212 ± 0.007a	0.210 ± 0.008a	0.209 ±0.007a	0μM	0.209 ± 0.007a	0.208 ± 0.007a	0.212 ± 0.007a
	0.8mM	0.188 ± 0.005b	0.182 ± 0.006b	0.180 ± 0.004b	5μM	0.172 ± 0.006b	0.207 ± 0.006a	0.171 ± 0.005b
	1.5mM	0.172 ± 0.005c	0.174 ± 0.006c	0.165 ± 0.005c	8μM	0.155 ± 0.005c	0.206 ± 0.005a	0.153 ± 0.005c
	3 mM	0.155 ± 0.005d	0.158 ± 0.005d	0.143 ± 0.004e	10μM	0.146 ± 0.002d	0.205 ± 0.005a	0.142 ± 0.002d
	6 mM	0.142 ± 0.003e	0.149 ± 0.003d	0.134 ± 0.004f	15μM	0.140 ± 0.004d	0.206 ± 0.003a	0.132 ± 0.003e
	10 mM	0.136 ± 0.002f	0.143 ± 0.003e	0.125 ± 0.003g	50μM	0.129 ± 0.003e	0.205 ± 0.003a	0.120 ± 0.003f

Values of aperture (unit: *μM*) are means of six independent experiments; error bars indicate S.D. of mean (n=6 leaves comprising 60 stoma.). Significant differences at $p<0.05$ are indicated by different letters for different treatments and genotypes.

Table 2. The Frequency of stomatal oscillation in the strips of *Arabidopsis thaliana* leaves under control (no treatment), (presence of $CaCl_2$ or ABA) and rapid exchange treatments (hyperpolarizing/depolarizing buffer) conditions.

Treatments	Ca^{2+}			ABA		
	wild type	*abi1-1*	*era1-2*	wild type	*abi1-1*	*era1-2*
Control	0.15 ± 0.02a	0.14 ± 0.02a	0.16 ± 0.02a	0.16 ± 0.02a	0.12 ± 0.02a	0.13 ± 0.02a
Steady treatments	0.50 ± 0.05b	0.52 ± 0.05b	0.49 ± 0.04b	0.48 ± 0.04b	0.11 ± 0.02a	0.47 ± 0.04b
Rapid exchange treatments	0.81 ± 0.10c	0.80 ±0.09c	0.80 ± 0.10c	0.79 ± 0.10c	0.12 ± 0.02a	0.82 ± 0.10c

Values are means of six independent experiments (mean ± S.D. of mean) and 60 stoma were observed. Significant differences at $p<0.05$ are indicated by different letters for different treatments and genotypes.

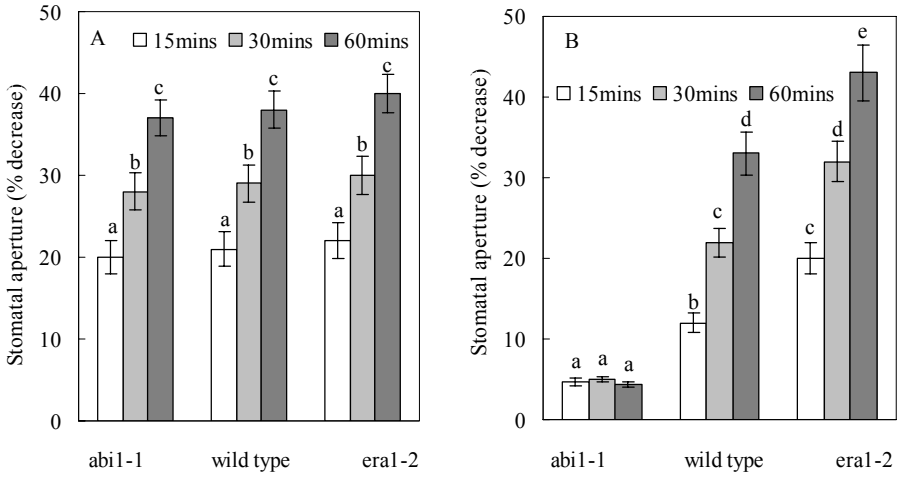

Fig. 1. Stomatal apertures measured after 15, 30 and 60 mins treatments with (A) 10 mM Ca^{2+} and (B) 50 μm ABA. Values are means of six independent experiments; error bars indicate S.D.of mean (n=6 leaves comprising 60 stoma.). Significant differences at $p<0.05$ are indicated by different letters for different treatments and genotypes.

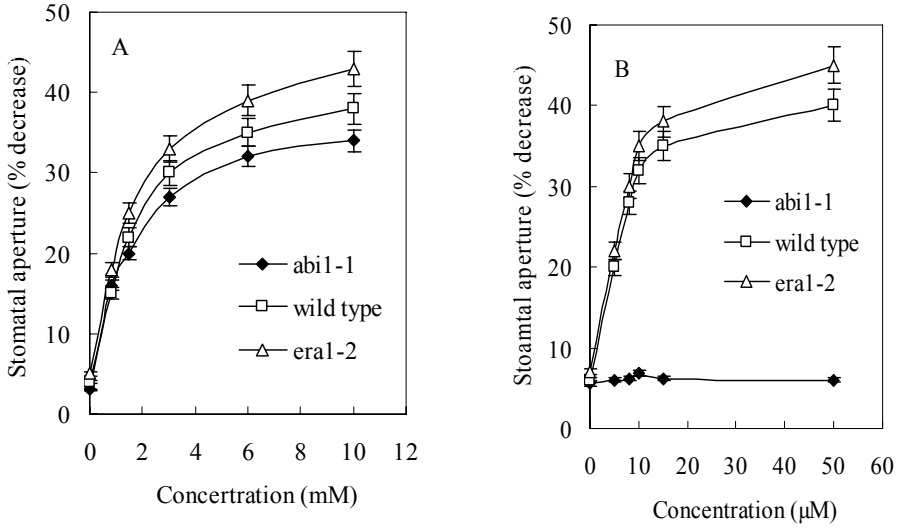

Fig. 2. Stomatal apertures 60mins after steady extracellular Ca^{2+}, ABA treatments at different concentrations: (A) Ca^{2+}; (B) ABA. Values are means of six independent experiments; error bars indicate S.D. of mean (n=6 leaves comprising 60 stoma).

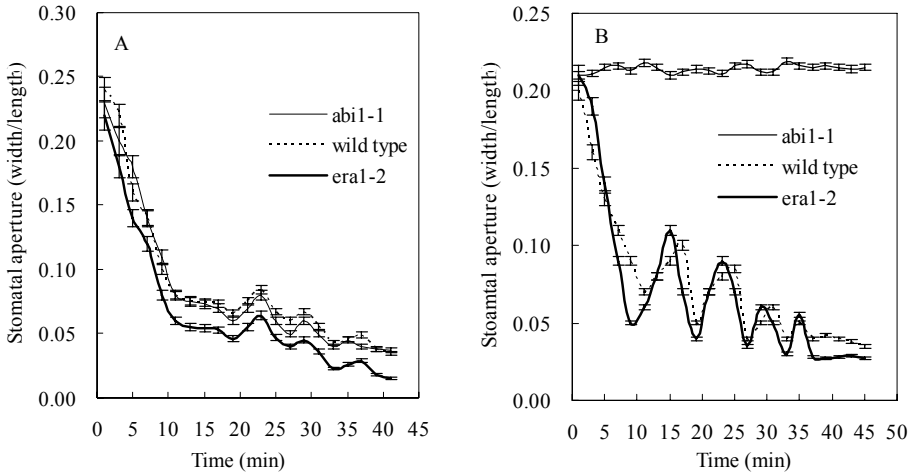

Fig. 3. Stomatal apertures after steady of extracellular (A)10 mM Ca^{2+} and (B) 50μM ABA treatments. At least five oscillation stomata were measured and average five here for each treatment. Error bars indicate S.D. of mean.

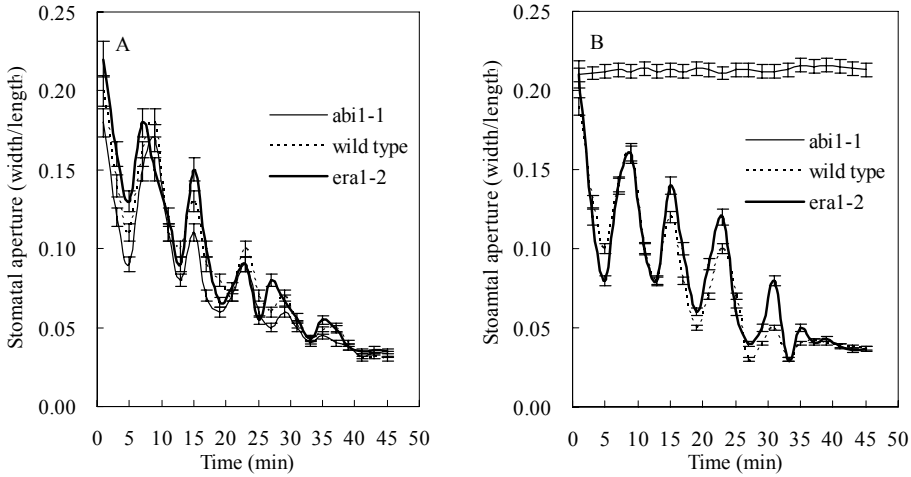

Fig. 4. Stomatal apertures after six times of rapid exchanges treatments of extracellular (A)10 mM Ca^{2+} and (B) 50μM ABA treatments. At least five oscillation stomata were measured and average five here for each treatment. Error bars indicate S.D. of mean.

Acknowledgements

We thank comments from Dr Charles S Whitehead and two anonymous reviewers have helped to improve the manuscript. The study was supported by the National Science Foundation of China (No. 30800126 and 30730020) and partially funded by Hi-tech Research and Development Program of China (No. 2006AA100202).

References

Allen, G.J., K. Kuchitsu, S.P. Chu, Y. Murata and J.I. Schroeder. 1999. Arabidopsis *abi1-1* and *abi2-1* phosphatase mutations reduce abscisic acid-induced cytoplasmic calcium rises in guard cells. *Plant Cell*, 11:1785-1798.

Allen, G.J., S.P. Chu, K. Schumacher, C.T. Shimazaki, D. Vafeados, A. Kemper, S.D. Hawke, G. Tallman, R.Y. Tsien, J.F. Harper, J. Chory and J.I. Schroeder. 2000. Alteration of stimulus-specific guard cell calcium oscillations and stomatal closing in Arabidopsis *det3* mutant. Science, 289: 2338-2342.

Anjum, P., A. Rubina and F. Rabab. 2007. Stomtal types of some dicots within flora of Karachi, Pakistan. *Pak. J. Bot*., 39(4): 1017-1023.

Christina, E., Reynolds-Hennea, L. Anita, M. Jan, S. Nicole, Z. Anita and F. Urs. 2010. Interactions between temperature, drought and stomatal opening in legumes. *Environmental and Experimental Botany*, 68(1): 37-43.

Françoise, G., B. Nathalie, S. Carine, A.R. Alex, N.V. Webb and G. Jérôme. 1999. ABI1 protein phosphatase 2C is a negative regulator of abscisic acid Signaling. *Plant Cell*, 11: 1897-1909.

Kaiser, H. and L. Kappen. 2001. Stomatal oscillation at small apertures: indications for a fundamental insufficiency of atomatal feedback-control inherent in the stomatal turgor mechanism. *Journal of Experimental Botany*, 52: 1303-1313.

Ishfaq, H., H. Farrukh and D. Ghulam. 2008. Stomatal studies of some selected medicinal plants of polygonaceae. *Pak. J. Bot*., 40(6): 2273-2280.

Li, J., X.Q. Wang., M.B. Watson and S.M. Assmann. 2000. Regulation of abscisic acid-induced stomatal closure and anion channels by guard cell AAPK kinase. *Science*, 287: 300-303.

McAinsh, M.R., A.A.R. Webb, J.E. Taylor and A.M. Hetherinngton. 1995. Stimulus-induced oscillation in guard cell cytosolic free calcium. *Plant Cell*, 7: 1207-1219.

McAinsh, M.R., J.E. Gray, A.M. Hetherington, C.P. Leckie and C. Ng. 2000. Calcium signalling in stomatal guard cells. *Biochem. Soc. Trans*., 28: 476-481.

Pei, Z.M., M. Ghassemian, C.M. Kwak, P.M. Court and J.I. Schroeder. 1998. Role of farnesyktransferase in ABA regulation of guard cell anion channel and plant water loss. *Sicence*, 282: 287-290.

Pei, Z.M., K. Kuchitsu, J.M. Ward, M. Schwarz and J.I. Schroeder. 1997. Differential abscisic acid regulation of guard cell slow anion channels in Arabidopsis wild-type and abi1 and abi2 mutants. *Plant Cell*, 9: 409-423.

Pei, Z.M., Y. Mutrata, G. Benning, S. Thomine, B. Kiusener, G.J. Allen, E. Brill and J.I. Schroeder. 2000. Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cell. *Nature*, 406: 731-734.

Sanders, D., C. Brownlee and J.F. Harper. 1999. Communicating with calcium. *Plant Cell*., 11:691-706.

Staxén, I., C. Pical, L. Montgomery, J. Gray, A.M. Hentherington and M.R. McAinsh. 1999. Abscisic acid induces oscillations in guard cell cytosolic free calcium that involve phosphoinositide-specific phospholipase C. *Proc. Natl. Acad. Sci. USA*., 96: 1779-1784.

Upadhyaya, S.K 1988. Role of stomatal oscillation on transpiration, assimilation and water use efficiency of plant. *Eco. Model*, 41: 27-40.

Wang, G. X., J. Zhang, J.X. Liao and J.L. Wang. 2001. Hydropassive evidence and effective factors in stomatal oscillations of Glycyrrhiza inflata under desert conditions. *Plant Science*, 160: 1007-1013.

Yang, H M., X.Y. Zhang, G.X. Wang, X.P., Y. Li and X.P. Wei. 2003. Cytosolic calcium oscillation may induce stomatal oscillation in *Vicia faba*. *Plant Science*, 165: 1117-1122.

Yang, H.M., X.Y. Zhang, G.X. Wang and J.H. Zhang. 2006. Water channels are involved in stomatal oscillations encoded by parameter-specific cytosolic calcium oscillations. *Journal of Integrative Plant Biology*, 48: 790-799.

(Received for publication 9 September 2008)