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# WRKY71 Acts Antagonistically Against Salt-Delayed Flowering in *Arabidopsis thaliana*

Yanchong Yu[1,2], Long Wang[1], Jiacai Chen[2], Zhenhua Liu[1], Chung-Mo Park[3] and Fengning Xiang[1,*]

[1]The Key Laboratory of Plant Cell Engineering and Germplasm Innovation, School of Life Sciences, Shandong University, Jinan 250100, China
[2]Shandong Key Laboratory of Plant Biotechnology, College of Life Sciences, Qingdao Agricultural University, Qingdao 266109, China
[3]Molecular Signaling Laboratory, Department of Chemistry, Seoul National University, Seoul 151-742, Korea
*Corresponding author: E-mail, xfn0990@sdu.edu.cn; Fax: +86-531-8856-5610
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**Soil salinity affects various aspects of plant growth and development including flowering. Usually, plants show a delayed flowering phenotype under high salinity conditions, whereas some plants will risk their life to continue to grow, thereby escaping serious salt stress to achieve reproductive success. However, the molecular mechanisms of the escape strategies are not clear yet. In this work, we report that the transcription factor WRKY71 helps escape salt stress in Arabidopsis. The expression of the *WRKY71* wild-type (WT) allele was salinity inducible. Compared with Col-0, high salt stress caused only a marginal delay in the flowering time of the activation-tagged mutant *WRKY71-1D*. However, flowering in the RNA interference (RNAi)-based multiple *WRKY* knock-out mutant (*w71w8 + 28RNAi*) was dramatically later than in the WT under high salinity conditions. Meanwhile, expression of *FLOWERING LOCUS T* (*FT*) and *LEAFY* (*LFY*) was greater in *WRKY71-1D* than in the WT, and lower in *w71w8 + 28RNAi* under salinity-stressed conditions. The suggestion is that WRKY71 activity hastens flowering, thereby providing a means for the plant to complete its life cycle in the presence of salt stress.**

**Keywords:** *Arabidopsis thaliana* • Escape • Flowering • High salinity • WRKY71.

**Abbreviations:** *BFT*, *BROTHER OF FT AND TFL1*; BL, brassinolide; DAG, days after germination; FM, flower meristem; *FT*, *FLOWERING LOCUS T*; GUS, β-glucuronidaase; LD, long day; *LFY*, *LEAFY*; MS, Murashige and Skoog; NPA, *N*-1-naphthylphthalamic acid; NTL, NTM1-LIKE 8; RNAi, RNA interference; RT–qPCR, quantitative reverse transcription–PCR; SA, salicylic acid; WT, wild type.

## Introduction

As sessile organisms, plants have to encounter various stress conditions during their life cycles, while flowering is the most crucial process for reproductive success. Therefore, to survive hostile environmental stress and ensure successful propagation, plants have evolved a spectrum of mechanisms against external harmful cues during the evolutionary processes (Levy and Dean 1998, Meyre et al. 2001). One of these is flowering at the appropriate time under stress conditions. Several studies have demonstrated that some stress factors, such as pathogen infection, drought, extreme temperatures and a high level of radiation, are able to accelerate flowering (Levy and Dean 1998, Korves and Bergelson 2003, Martinez et al. 2004, Kumar et al. 2012, Riboni et al. 2013, Stief et al. 2014, Xu et al. 2014). The relevant mechanisms of the above stress-induced flowering have been demonstrated in wild-type (WT) Arabidopsis. For instance, the increasing temperature promotes the basic helix–loop–helix (bHLH) transcription factor PHYTOCHROME INTERACTING FACTOR4 (PIF4) to activate *FLOWERING LOCUS T* (*FT*) directly (Kumar et al. 2012).

Although other stresses could promote flowering, salt stress is considered as a negative factor for the flowering of most plants (Apse et al. 1999). A number of the molecular mechanisms underlying salinity-delayed flowering have been described. A recent study demonstrated that the delay was caused by a DELLA-dependent pathway (Achard et al. 2006). Salinity elevates the stability of DELLA proteins, and then the latter restrain plant growth. Therefore, compared with control plants, in the quadruple-DELLA mutant, salt stress does not extend the vegetative stage and thus does not delay flowering time; simultaneously, expression of the flowering integrator *LEAFY* (*LFY*) in this mutant is not suppressed by the imposition of high salinity (Achard et al. 2006).

In addition, the membrane-bound transcription factor NTM1-LIKE 8 (NTL8) is also showed to be involved in salt-responsive flowering (Kim et al. 2007). The *NTL8* gene is salinity inducible. The overexpression of active NTL8 protein inhibits the expression of *FT*, thereby resulting in a late flowering phenotype. However, compared with Col-0, in the *ntl8-1* mutant, salinity no longer represses *FT* expression (Kim et al. 2007). The suggestion is that NTL8 mediates salinity-delayed flowering initiation via supressing *FT*.

Other examples with respect to salinity-delayed flowering include the function of the *BROTHER OF FT AND TFL1* (*BFT*), which encodes a member of the FT/TERMINAL FLOWER 1 (TFL1) family (Ryu et al. 2011, Ryu et al. 2014). The *BFT* gene is induced by high salinity. *BFT* overexpression in transgenic Arabidopsis shows a late flowering phenotype compared with the WT whether under normal or salinity conditions. However, flowering of *bft-1* mutants is insensitive to high salinity (Ryu et al. 2011). A further study showed that BFT competes with FT for FD binding, thereby suppressing *AP1* expression (Ryu et al. 2014).

Although the growth of most plants is slowed or even stopped by mild salt stress, some plants pursue a riskier strategy

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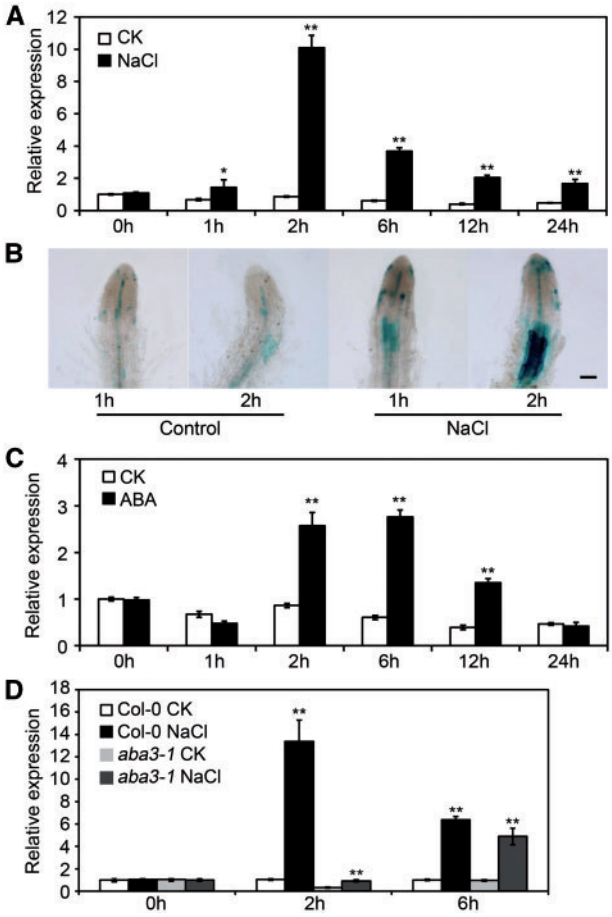


**Fig. 1** WRKY71 expression profiles in response to high salinity and ABA. (A) The effect of salt stress on *WRKY71* expression. h, hours elapsed since the imposition of the treatment; CK, non-stressed plants; NaCl, NaCl-stressed plants. (B) GUS activity in roots exposed to 200 mM NaCl. Scale bar = 50 μm. (C) The effect of ABA on *WRKY71* expression. CK, control plants; ABA, treated plants. (D) Transcription level of *WRKY71* in Col-0 and *aba3-1* exposed to 200 mM NaCl. CK, non-stressed plants; NaCl, NaCl-stressed plants. Error bars in (A), (B) and (D) represented the SEM. Statistical significance was evaluated by Student's *t*-test (**$P < 0.01$).

by continuing to grow even when the level of stress is serious, presumably in an attempt to reproduce before dying (Zhu 2001). The implication is that there are salinity-induced early flowering pathways in Arabidopsis. However, the molecular basis of accelerated flowering has not yet been demonstrated.

The *WRKY* genes encode a large family of transcription factors in plants. Members of this family contain one or two conserved DNA-binding regions, designated the WRKY domain, typically having a conserved WRKYGQK motif at the N-terminus, and followed by a $C_2H_2$ or $C_2HC$ zinc-finger motif at the C-terminis. More than 20 years have passed since the first WRKY protein (SPF1) was identified (Ishiguro and Nakamura 1994). A large number of molecular and genetic investigations revealed that WRKY transcription factors play crucial roles in a wide range of biotic/abiotic stress responses and some developmental and physiological processes, such as seed and trichome development, secondary metabolism, leaf senescence and embryo morphogenesis (Eulgem et al. 2000, Rushton et al. 2010, Chen et al. 2012). For instance, AtWRKY8, AtWRKY18, AtWRKY33, AtWRKY40 and AtWRKY60 have been shown to be involved in pathogen- and salicylic acid (SA)-mediated defense (Xu et al. 2006, Lai et al. 2011, Chen et al. 2013, Liao et al. 2016). AtWRKY8, AtWRKY25, AtWRKY33 and AtWRKY63 are known to participate in abiotic stress (Jiang and Deyholos 2009, Ren et al. 2010, Li et al. 2011, Hu et al. 2013). AtWRKY6, AtWRKY44, AtWRKY57 and AtWRKY71 are reported to be involved in the developmental process (Robatzek and Somssich 2002, Rushton et al. 2010, Wang et al. 2010, Guo et al. 2015). Several WRKYs, AtWRKY6, AtWRKY12, AtWRKY13, AtWRKY53, GsWRKY20, MlWRKY12 and OsWRKY11, are implicated to play a role in regulating flowering (Robatzek and Somssich 2002, Miao et al. 2004, Luo et al. 2013, Yu et al. 2013, Cai et al. 2014, Li et al. 2016). Our recent studies clarified the molecular mechanism of WRKY in flowering regulation. WRKY71 binds to the promoters of *FT* and *LFY*, and upregulates their expression, thereby accelerating floral transition in Arabidopsis (Yu et al. 2016). Another study shows that *WRKY71* is rapidly induced by $H_2O_2$, ABA and mannitol, suggesting that WRKY71 participates in the regulation of plant responses to abiotic stresses (Guo and Qin 2016). In this study, we report that WRKY71 speeds up the life cycle of Arabidopsis in response to high salinity. We indicate that overexpression of WRKY71 makes flowering of Arabidopsis insensitive to high salinity, while the deletion or decline of WRKY71 and its homologs, WRKY8 and WRKY28, makes flowering of Arabidopsis more sensitive. Meanwhile, transcript levels of *FT* and *LFY*, targets of WRKY71, are greater in *WRKY71-1D* than in the WT, and lower in *3w + 28RNAi* under salinity-stressed conditions. The suggestion is that WRKY71 acts antagonistically against salt-delayed flowering in Arabidopsis, and it is able to speed up the life cycle of Arabidopsis in response to high salinity.

## Results

### *WRKY71* was induced by high salinity, drought and ABA

In our previous study, WRKY71 was revealed to play a positive role in Arabidopsis flowering (Yu et al. 2016). Multiple studies have demonstrated that the WRKY transcription factors tend to respond to several abiotic stress factors (Chen et al. 2012). In order to investigate whether WRKY71 mediates stress signaling to affect flowering, we analyzed its expression profiles in response to a variety of stress factors and hormones. *WRKY71* transcription was induced by drought, but not by low temperature (**Supplementary Fig. S1**). In response to salt stress, transcript abundance peaked after a 2 h exposure (**Fig. 1A**); When approximately 3 kb of the *WRKY71* promoter sequence was fused to *GUS* (β-*glucuronidase*) and introduced into Col-0, GUS activity was enhanced in the seedling root at both 1 and 2 h after the addition of 200 mM NaCl to the growth medium (**Fig. 1B**). In addition, WRKY71 was induced by ABA treatment, and the peak abundance occurred after 6 h (**Fig. 1C**). WRKY71 was slightly inhibited by *N*-1-naphthylphthalamic acid (NPA), while treatment with other phytohormones, IAA, brassinolide

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(BL), gibberellic acid and SA), had no effect on *WRKY71* transcription (**Supplementary Fig. S1**). When expressed in the ABA-deficient mutant *aba3-1*, *WRKY71* remained inducible by salt stress, but to a lesser extent than in the WT (**Fig. 1C**). Thus, the salt stress induction of *WRKY71* appeared to be dependent on ABA to some extent. Taken together, we hypothesized that WRKY71 may be involved in salt-responsive early flowering.

## Flowering of *WRKY71-1D* was relatively insensitive to salt stress

To determine the function of WRKY71 in salt-responsive early flowering, we first examined the flowering phenotype of Col-0, *WRKY71-1D* (an activation tagging line) and *wrky71-1* under high salinity conditions. The flowering time of *WRKY71-1D* was rarely affected by high salt stress (**Fig. 2A, B**). Unfortunately, as under non-stressed conditions (Yu et al. 2016), *wrky71-1* displayed a phenotype indistinguishable from that of the WT under NaCl-stressed conditions (**Fig. 2A, B**). Next, we checked the floral meristem (FM) formation rate of Col-0, *WRKY71-1D* and *wrky71-1* under high salinity conditions. In plants exposed to salinity, all of the *WRKY71-1D* plants had formed their FM by 11 days after germination (DAG), only 1 d later than under non-stressed conditions (Yu et al. 2016), while only 21.3 ± 2.6% of the *wrky71-1* mutants had done so by 14 DAG (28.8 ± 2.8% by 15 DAG). The WT proportions were 35.5 ± 3.2% (14 DAG) and 43.5 ± 3.6% (15 DAG) (**Fig. 2C, D**). Thus, FM formation of the *WRKY71-1D* line was also scarcely influenced by high salt stress, while that of *wrky71-1* was quite severely affected compared with the WT. Taken together, flowering of *WRKY71-1D* was relatively insensitive to salt stress.

In order to obtain insight into the response of *WRKY71-1D* to salt stress, we investigated its other development processes, including the salt stress phenotype, germination rate and growth rate of roots. When grown under NaCl-stressed conditions, *WRKY71-1D* was not tolerant to high salinity; it died earlier than Col-0 and *wrky71-1* (**Supplementary Fig. S2A**). The germination rate of Col-0, *WRKY71-1D* and *wrky71-1* was similar under non-stressed conditions, while under high salinity conditions, at the early stage (48 h), *WRKY71-1D* germinated remarkably later than Col-0 and *wrky71-1* (**Supplementary Fig. S2B**). The root growth rate of Col-0, *WRKY71-1D* and *wrky71-1* showed no significant difference under NaCl-stressed or non-stressed conditions (**Supplementary Fig. S2C–E**). In conclusion, although *WRKY71-1D* flowered insensitively to salt stress, it was not tolerant to high salinity. Thus, we suggest that WRKY71 could help Arabidopsis complete its life cycle quickly to escape salt stress but not render it tolerant to it.

## Redundant function of WRKY8, WRKY28 and WRKY71 in salt-responsive flowering

In the regulation of flowering, redundancy among WRKY8, WRKY28 and WRKY71 has been demonstrated (Yu et al. 2016). To find out if WRKY8 and WRKY28 were also involved in salt-responsive flowering, we tested the response of both genes to high salinity. As shown in **Fig. 3A and B**, the transcription of both *WRKY8* and *WRKY28* in WT plants was induced by salt stress, but to a lesser extent than that of *WRKY71* (**Fig. 1A**). Further study indicated that like *WRKY71-1D*, the flowering of two transgenic lines, *35S::WRKY8* and *35S::WRKY28*, was also insensitive to salt stress (**Fig. 3C**; **Supplementary Fig. S3**). Molecular analysis showed that *FT* and *LFY* were promoted in *35S::WRKY8* and *35S::WRKY28* (**Supplementary Fig. S4**), which is similar to that in *WRKY71-1D* (Yu et al. 2016). However, like *wrky71-1*, none of the single or multiple mutants, *wrky8*, *wrky28* (named *SALK_007497* in our previous study), *w71 w8*, *w71 w28*, *w8 w28*, and *w71 w8 w28* had a non-WT flowering phenotype when the plants were subjected to salt stress (**Fig. 3D**; **Supplementary Fig. S5**). These results suggested that the redundancy among WRKY8, WRKY28 and WRKY71 exists in the regulation of salt-responsive flowering.

## Flowering of the *w71w8 + 28RNAi* was more sensitive to salt stress

Due to the redundancy descibed above, we were prompted to analyze the other two lines which we generated previously, *w71w8 + 28RNAi-8* and *W71-SRDX* (Yu et al. 2016). Under NaCl-stressed conditions, *WRKY71-1D* flowered earlier than the WT, while *w71w8 + 28RNAi-8* and *W71-SRDX* flowered later (**Fig. 4–C**), similar to the case under non-stressed conditions (Yu et al. 2016). To quantify the effect of salinity stress on flowering, percentage change in the number of leaves and the time taken to reach flowering were measured. In Col-0, the increase with respect to the former parameter was 17.6 ± 2.9% and to the latter it was 20.0 ± 1.5%; in contrast, the respective increases in *WRKY71-1D*, *w71w8 + 28RNAi-8* and *W71-SRDX* were 5.7 ± 2.7% and 10.9 ± 3.1%, 27.6 ± 2.6% and 36.5 ± 5.9%, and 12.9 ± 2.8% and 16.3 ± 2.4% (**Fig. 4D, E**). Thus, flowering in the *w71w8 + 28RNAi-8* line was the most sensitive to salinity stress, while *WRKY71-1D* was insensitive.

## *FT* and *LFY* were involved in WRKY71-mediated salt-responsive flowering

Our previous study showed that *FT* and *LFY* were the direct targets of WRKY71; *APETALA1* (*AP1*), *CAULIFLOWER* (*CAL*) and *FRUITFULL* (*FUL*) were also up-regulated by WRKY71 (Yu et al. 2016). Therefore, to provide insight into the mechanism of WRKY71 in salt-responsive early flowering, we examined their expression profiles in Col-0, *WRKY71-1D*, *w71w8 + 28RNAi-8* and *W71-SRDX* plants exposed to high salinity for 6 d. Transcript levels of *FT* and *LFY* in the Col-0 plants were significantly decreased by salt stress, and that of *FUL* seemed to be not affected (**Fig. 5**). In contrast, the *FT*, *LFY* and *FUL* genes were more greatly decreased in *w71w8 + 28RNAi-8* by salt treatment (**Fig. 5**), which is consistent with the salt sensitivity of flowering in this line (**Fig. 4A–D**); the three genes were slightly suppressed in *WRKY71-1D* with salt treatment (**Fig. 5**), in accordance with the line's flowering behavior under salt stress (**Figs. 2A, B; 4A–E**). For *AP1* and *CAL*, the most suppression appeared in Col-0 (**Fig. 5**). The overall conclusion was that WRKY71, WRKY8 and WRKY28 participated in salt-responsive flowering via regulating *FT* and *LFY*.



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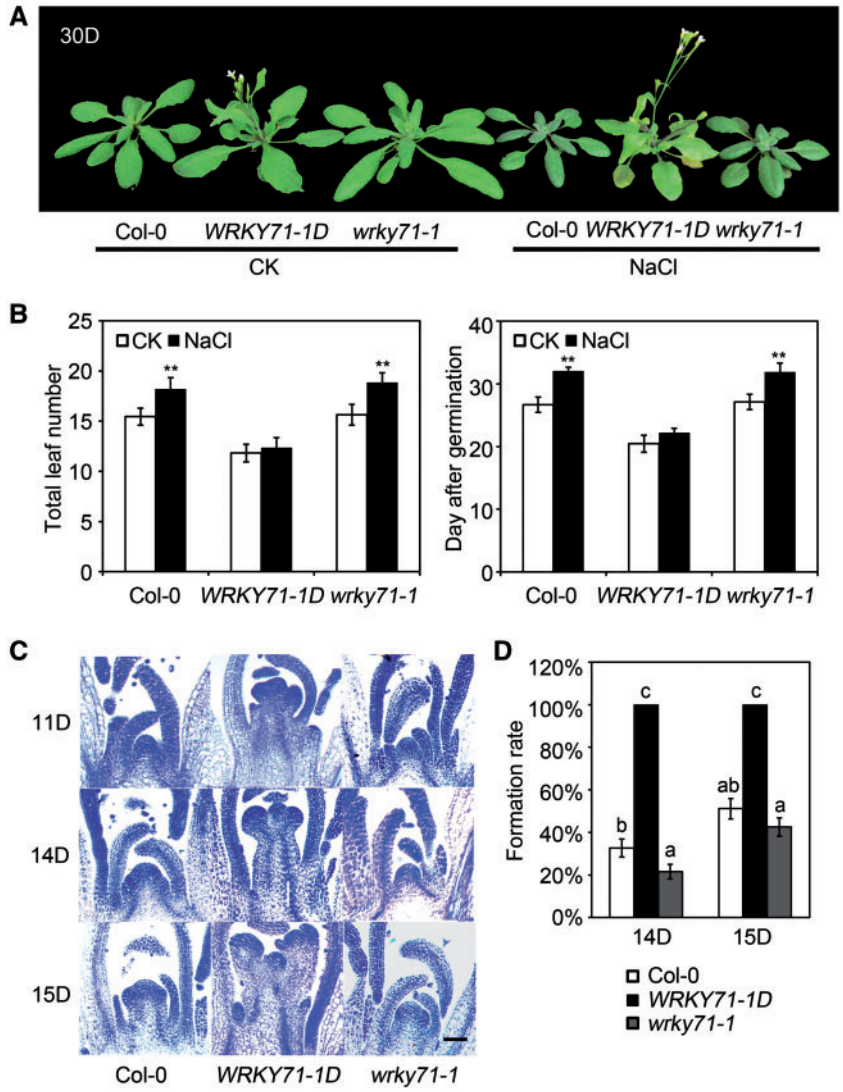


**Fig. 2** The effect of high salinity on the flowering phenotype of Col-0, *WRKY71-1D* and *wrky71-1* plants. (A and B) The flowering phenotype (A) and flowering time (B) of Col-0, *WRKY71-1D* and *wrky71-1* plants exposed to 200 mM NaCl. CK, non-stressed plants; NaCl, NaCl-stressed plants. Error bars represented the SEM ($n = 3$). Statistical significance was evaluated by Student's *t*-test (**$P < 0.01$). (C) Shoot apical meristem sections of salinity-stressed Col-0, *WRKY71-1D* and *wrky71-1* plants. (D) The rate of FM formation. Error bars represent the SEM ($n = 3$). Different lower case letters above columns represent significant differences in each group (one-way ANOVA, $P < 0.01$). The upper case D in (A), (C) and (D) means day after germination.

## Discussion

During their entire life cycle, plants experience a variety of environmental stresses which affect plant growth and developmental processes. Of particular note is the influence of abiotic stresses on flowering time, since it is a limiting factor for multiplication. Floral transition is highly plastic. To cope with changes in environmental conditions, plants will alter their flowering time (early or late) to ensure production success (Xu et al. 2014). A number of stresses have been found to accelerate flowering in Arabidopsis (Korves and Bergelson 2003, Martinez et al. 2004, Schroeder and Kuhn 2006, Kumar et al. 2012, Riboni et al. 2013, Stief et al. 2014, Xu et al. 2014). However, a common plant response to salt stress is growth retardation and delayed flowering (Kim et al. 2007).

In Arabidopsis, a number of genes have been identified as being responsible for salinity-induced delays to flowering. The quadruple-DELLA mutant is insensitive to salt stress, and its flowering is unaffected by salt stress, due to the up-regulation of *LFY* (Achard et al. 2006). NTL8 also mediates the salinity-induced delay in flowering, acting through *FT*; the overexpression of *NTL8* represses *FT*, thereby resulting in a late flowering phenotype, while the *nlt8* mutant has a phenotype indistinguishable from that of the WT (Kim et al. 2007). The overexpression of *BFT* generates a delayed flowering phenotype, but the flowering time of the *bft* mutants is similar to that of the WT; however, like the quadruple-DELLA mutants, the flowering time of *bft* in the presence of salt stress is earlier than that of the WT. Transcription analysis has shown that *AP1* is not down-regulated in *bft* plants exposed to NaCl, which has been



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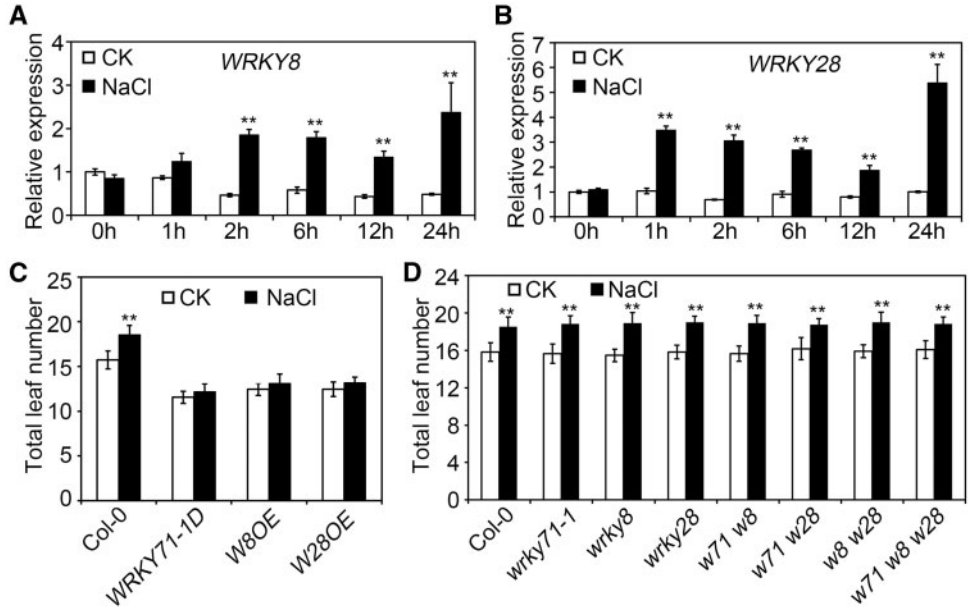


**Fig. 3** WRKY8 and WRKY28 were involved in salt-responsive flowering. (A and B) The effect of salt stress on *WRKY8* and *WRKY28* transcripts. h, hours elapsed since high salinity treatment. (C and D) The flowering time of different plants exposed to 200 mM NaCl. CK, non-stressed plants; NaCl, NaCl-stressed plants. Error bars represent the SEM ($n$ = 3). Statistical significance was evaluated by Student's $t$-test (**$P < 0.01$).

interpreted to imply that *BFT* mediates salinity-induced delay in the flowering of Arabidopsis via *AP1* (Ryu et al. 2011). BFT has been shown to delay flowering in plants exposed to salt stress by competing with FT for binding to the FD (Ryu et al. 2014). Li et al. proved that *CONSTANS* (*CO*), *FT*, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS* (*SOC1*) and *LFY* are all down-regulated by salinity stress (Li et al. 2007).

Although the fact that salt stress inhibits plant flowering is acknowledged, some species, specifically the halophytes, will run the risk of dying by continuing to grow in the presence of salinity (Zhu 2001); in *Mesembryanthemum crystallinum*, salt stress accelerates flowering (Adams et al. 1998). Accordingly, the hypothesis was that salinity-induced early flowering pathways would exist in higher plants, including Arabidopsis. As is widely known, salinity retards the flowering of WT Arabidopsis and, to date, all molecular evidence indicates that salinity will repress the expression of flowering-promoting genes (Achard et al. 2006, Kim et al. 2007, Ryu et al. 2011, Ryu et al. 2014). However, we cannot deny that during evolution, Arabidopsis has developed a related mechanism to antagonize/escape this negative effect of salinity, although this antagonism/escape cannot completely eliminate the negative effect of salinity on its flowering. In the present study, we confirmed the antagonism/escape assumption; a distinct pathway in which WRKY71 mediated salinity-induced early flowering did exist in Arabidopsis. A large number of WRKY family members have been revealed to be involved in abiotic stress, and the induced accumulation of WRKY transcript is typically rapid and transient (Rushton et al. 2010, Chen et al. 2012). WRKY71 is no exception; a recent study showed that it is induced by $H_2O_2$, ABA and mannitol (Guo and Qin 2016), and our precise examination indicated that WRKY71 was induced by drought, and especially by high salinity, and the induction was rapid and transient (**Fig. 1A, B**). This is consistent with the publicly available AtGenExpress data (AtGenExpress Visualization Tool; http://jsp.weigelworld.org/expviz/expviz.jsp). Ectopic expression of WRKY71 has been shown to result in an early flowering phenotype (Yu et al. 2016). Therefore, the suggestion was that high salinity was able to activate the transcription of WRKY71, then the latter promoted flowering in Arabidopsis.

Maintaining a high level of *WRKY71* transcription could convert Arabidopsis into a precociously flowering species under salt stress (**Fig. 2A, B**). In the presence of salt stress, *WRKY71-1D* flowered earlier than the WT, even earlier than the non-stressed WT (**Fig. 2A, B**). However, the elimination or decrease of *WRKY71*, *-8* and *-28* in *w71w8 + 28RNAi-8* caused a significant late flowering phenotype of Arabidopsis exposed to high salinity (**Fig. 4A, B**). Previous studies have demonstrated that *FT* and *LFY* transcription is suppressed by salinity-induced cues, such as NTL8, BFT and DELLAs (Achard et al. 2006, Kim et al. 2007, Ryu et al. 2011, Ryu et al. 2014). In contrast, our data indicated that *FT* and *LFY* transcription was up-regulated by salt-inducible *WRKY71*, *-8* and *-28* (**Fig. 5**; **Supplementary Fig. S2**). Therefore, in the WT exposed to NaCl stress, *FT* and *LFY* transcription was influenced by two aspects, one was the suppression by NTL8, BFT and DELLAs, and the other was the activation by WRKY71, -8 and -28; since the former were dominant, *FT* and *LFY* transcription in the WT was finally inhibited under salinity conditions (**Fig. 5**), with the result that flowering of the WT Arabidopsis tends to be delayed by high salinity (**Figs. 2A, B**, **6**). In contrast, the loss of the elevation of *FT* and *LFY* expression by *WRKY71*, *-8* and *-28* in *w71w8 + 28RNAi-8* would no longer act antagonistically against the inhibition by NTL8, BFT and DELLAs, and thus *FT* and *LFY*



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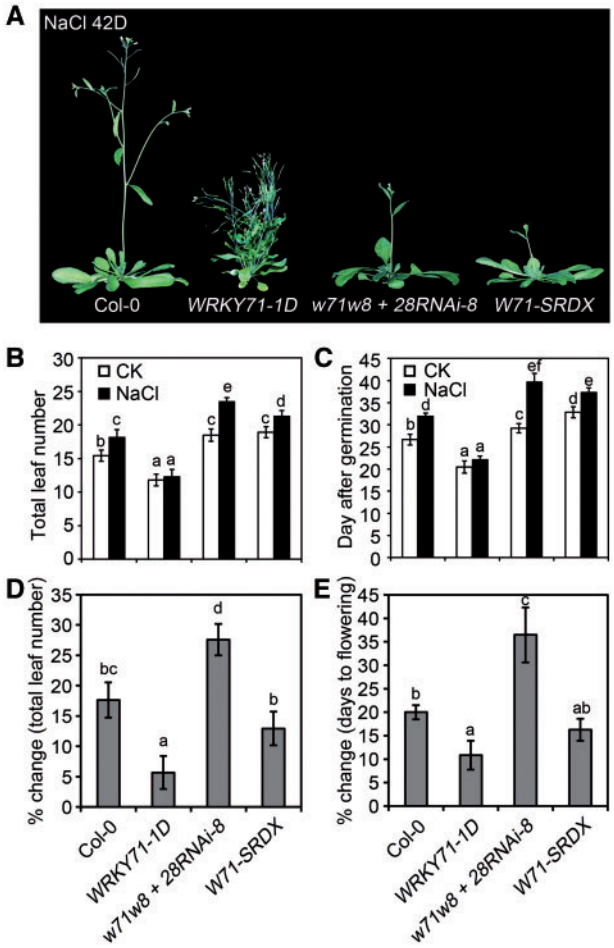


**Fig. 4** The effect of high salinity on the flowering phenotype of Col-0, *WRKY71-1D*, *w71w8 + 28RNAi* and *W71-SRDX* plants. (A–C) The flowering phenotype (A) and flowering time (B and C) of the Col-0, *WRKY71-1D*, *w71w8 + 28RNAi* and *W71-SRDX* plants exposed to 200 mM NaCl. CK, non-stressed plants; NaCl, NaCl-stressed plants. (D and E) Relative increase in number of total leaves (D) and days to flowering (E) of the plants exposed to 200 mM NaCl. Error bars in (B–E) represent the SEM ($n = 3$). Different lower case letters in (B–E) above the columns represent significant differences (one-way ANOVA, $P < 0.01$).

transcript abundance in the salt-stressed *w71w8 + 28RNAi-8* was more greatly reduced than in the WT (**Fig. 5**). Therefore, the elimination or decrease of *WRKY71*, *-8* and *-28* would aggravate the salinity-induced delay to flowering, while just like in the presence of *WRKY71*, *-8* and *-28* in the WT, the delay of flowering was not serious (**Figs. 4A–E**, **6**). Ectopic expression of *WRKY71* was able to promote *FT* and *LFY*; although the transcripts of *FT* and *LFY* expression were inhibited by high salinity in *WRKY71-1D*, they remained at a higher than the WT level, even the non-stressed WT level (**Fig. 5**), consistent with the early flowering of *WRKY71-1D* under salt stress conditions (**Figs. 2A, B**, **6**). However, intolerance to salt stress of *WRKY71-1D* suggested that WRKY71 helped Arabidopsis escape salt stress but did not make them tolerant to it.

Overall, the present research revealed a distinct salt-responsive early flowering pathway, in which WRKY71, -8 and -28 acted antagonistically against salt-delayed flowering in Arabidopsis, provided a sound theoretical basis for understanding the escape strategy of higher plants in salinity environments and offered opportunities to adopt a rational genetic engineering approach towards enhancing the ability of crop species to escape salt stress.

## Materials and Methods

### Plant materials and growth conditions

The mutant *aba3-1* was obtained from the Nottingham Arabidopsis Stock Centre. The other Arabidopsis lines used in the present study were previously described. *WRKY71-1D* is an activation tagging line in which WRKY71 is overexpressed; *w71w8 + 28RNAi-8* is the line in which *WRKY71* and *WRKY8* are knocked out and *WRKY28* is silenced; *W71-SRDX* is a line generated by chimeric repressor gene-silencing technology; *wrky71-1*, *wrky8*, *wrky28*, *w71 w8*, *w71 w28*, *w8 w28* and *w71 w8 w28* have been introduced in our previous study (Yu et al. 2016). Seeds were synchronized on wet filter paper at 4°C for 3 d, and then sown in soil. They were kept in a greenhouse at 20–22°C with 45% relative humidity under long-day (LD) conditions (16 h light/8 h dark, 110 μmol photons $m^{-2}$ $s^{-1}$ of white light). The other seeds were first surface-sterilized, and then placed on a half-strength Murashige and Skoog (1/2 MS) agar plate. To generate the transgenic plants with the *GUS* reporter gene, an approximately 3 kb fragment of the *WRKY71* promoter was cloned with primers (pW71-F: AAAAGCAGGCTCGGAAAGTGACAACCGTGCACC and pW71-R: AGAAAGCTGGGTTTGAAGAAGAGGAGAAAGAGAA) and then inserted into pKGWFS7; the recombinant plasmid were introduced into Col-0 through the floral dip method (Clough and Bent 1998). Transgenic lines were obtained by screening on MS agar plates with 40 μg $ml^{-1}$ kanamycin.

### Abiotic stress and hormone treatments

Abiotic stress and hormone treatments were applied to plants grown on agar plates at 10 DAG . The salinity treatment involved transferring the plants into a MS solution containing 0 or 200 mM NaCl, and maintaining them under constant agitation (60–80 r.p.m.) under a 24 h photoperiod. Drought stress was imposed by setting the plants on a dry or wet (control) filter paper and maintaining them under LDs. Cold stress was imposed by holding the plants at −4 or 22°C (control) under LDs. The hormone treatments were applied by transferring the plants to a MS liquid culture supplemented with 10 μM of either ABA, IAA, BL, gibberellic acid, NPA or SA, and maintaining them with constant agitation (60–80 r.p.m.) under a 24 h photoperiod; the control materials were transferred to a MS liquid culture. The whole plants were harvested at the indicated time for RNA isolation.

For the measurement of flowering time, tissue sectioning and salt stress phenotype, 7 DAG plants grown in soil (25×50×8 $cm^3$ plastic plates with 32 small holes, 6×6×7 $cm^3$ for each hole) were watered twice with 1,000 ml of 200 mM NaCl solution at 7 and 14 DAG. To assay the effect of salinity on gene expression, 7 DAG soil-grown plants (25×50×8 $cm^3$ plastic plates with 32 small holes, 6×6×7 $cm^3$ for each hole) were watered once with 1,000 ml of 200 mM NaCl, then the aerial parts of plants were harvested at 13 DAG for RNA isolation. For determination of the growth rate of roots, the seeds were surface-sterilized and placed on a MS plate, held at 4°C for 3 d and then transferred to LDs at 20–22°C. Two days later, the control seedlings were held for up to 10 d, while the other seedlings were transferred to a MS plate containing 100 mM NaCl and held for up to 10 d. Finally, at least 10 seedlings of each line were measured for the data statistics.

### GUS staining

Plants grown on MS agar plates were exposed at 7 DAG to 200 mM NaCl solution (water for control) for 1 and 2 h. Next, they were fixed in 90% acetone for 20 min on ice, and then subjected to GUS staining following the protocol described previously (Seo et al. 2009). After staining, samples were washed with 70% (v/v) ethanol, and then were observed under a light microscope (BX51, Olympus).

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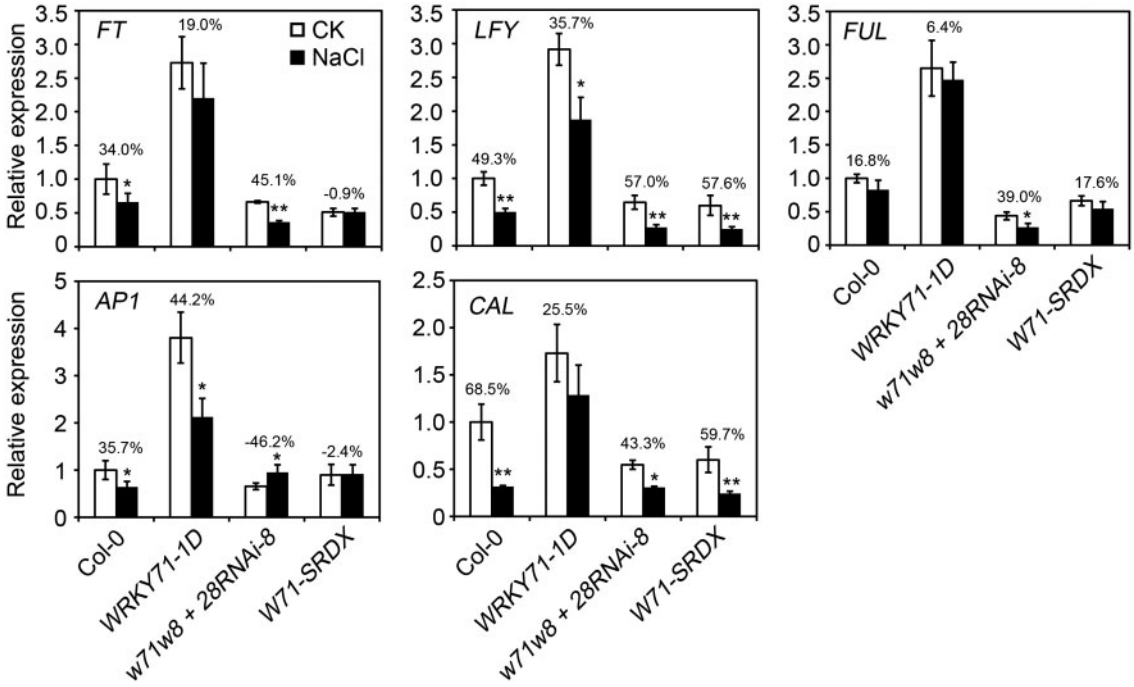


**Fig. 5** Comparison of transcription of flowering-related genes between non-stressed and NaCl-stressed plants. CK, non-stressed plants; NaCl, NaCl-stressed plants. The percentages above the columns indicate the change in gene transcription levels. Error bars represent the SEM ($n = 3$). Statistical significance was evaluated by Student's *t*-test (*$P < 0.05$, **$P < 0.01$).

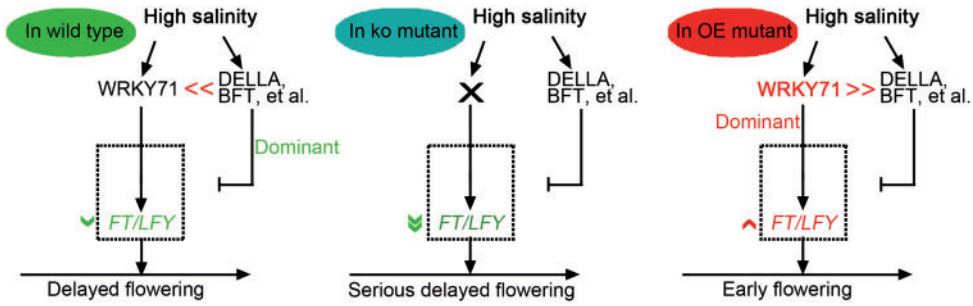


**Fig. 6** Proposed model of WRKY71 function in salinity-induced early flowering. In the WT plants, high salinity would promote DELLAs and BFT, etc., and thereby inhibit *FT* and *LFY* expression, while in the other pathway, WRKY71 was also induced by high salinity, and it would then promote *FT* and *LFY* expression to act against their inhibition by DELLAs and BFT, etc. However, the inhibition pathway was dominant; thus, the transcript abundance of *FT* and *LFY* was repressed. Finally, the WT displayed a late flowering phenotype under high salinity conditions. In gene knockout mutants, the promotion of *FT* and *LFY* by WRKY71 vanished. Therefore, *FT* and *LFY* expression was very repressed compared with the WT, and the flowering of the mutant was seriously delayed. In contrast, in the *WRKY71-1D* plants, the elevation of *FT* and *LFY* became dominant, so their transcription remained at a high level under high salinity conditions. The result is that *WRKY71-1D* showed an early flowering phenotype under high salinity conditions.

## Flowering time measurement and germination rate

When the first floral bud appeared, total (rosette add cauline) leaf numbers were counted to estimate flowering time. Simultaneously, the consumption time (days) was taken as the other scale for flowering time measurement. At least 30 plants was counted and averaged for the data statistics. For germination rate, air-dried seeds were placed on a MS agar plate containing 0 or 150 mM NaCl, held at 4°C for 3 d and then germinated at 20–22°C under LDs. The morphological marker of germination was the emergence of visible radicles. At least 100 seeds of each line were counted for the measurement.

## Tissue sectioning

The NaCl-stressed and control plants was harvested at the indicated time point, the leaves and roots were removed, and the left shoot apex was fixed in FAA solution (formaldehyde : acetic acid : 70% ethanol, 5 : 5 : 90, by vol.), and dehydrated by passing through an ethanol and a dimethylbenzene series. The dimethylbenzene was gradually replaced by paraplast plus (Sigma) chips at 60°C, and finally the plant materials were embedded for sectioning. The sectioning was performed according to the description in our previous study (Yu et al. 2016). More than 100 plants for each line were observed for transition rate statistics.

## Transcription analysis

Relative transcription levels were examined by quantitative reverse transcription–PCR (RT–qPCR). To ensure that the estimation was reproducible and accurate, total RNA isolation, DNA elimination, cDNA synthesis and PCR were performed according to a previous study (Udvardi and Scheible 2008). RT–qPCR was performed with the LightCycler 480 SYBR Green I Master (Roche) by using a Bio-Rad Real-Time PCR detection system (Bio-Rad). Gene-specific primers are listed in **Supplementary Table S1**; the *TUB2* gene was used as the internal control. All



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RT–qPCRs were repeated three times, using a template prepared from three independent plants grown under identical conditions.

## Supplementary Data

Supplementary data are available at PCP online.

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## Disclosures

The authors have no conflicts of interest to declare.

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