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Crossroads of stress responses, development and flowering regulation—the multiple roles of Cyclic Nucleotide Gated Ion Channel 2

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Abbreviation: CNGC, cyclic nucleotide-gated ion channel; SA, salicylic acid.

The Arabidopsis autoimmune mutant, *defense-no death 1* (*dnd1*) is a null mutant of *CYCLIC NUCLEOTIDE-GATED ION CHANNEL2* (*AtCNGC2*). *dnd1* exhibits constitutive pathogen resistance responses including higher levels of endogenous salicylic acid (SA), which is an important signaling molecule for pathogen defense responses. Recently we have reported that *dnd1* exhibits a significantly delayed flowering phenotype, indicating the involvement of *AtCNGC2* in flowering transition. However, since SA has been known to influence flowering timing as a positive regulator, the delayed flowering phenotype in *dnd1* was unexpected. In this study, we have asked whether SA is involved in the *dnd1*-mediated delayed flowering phenotype. In addition, in order to gain insight into the involvement of SA and CNGCs in flowering transition, we analyzed the flowering transition of *cpr22*, another CNGC mutant with a similar autoimmune phenotype as *dnd1* (including high SA accumulation), and null mutants of several other CNGCs. Our data suggest that *dnd1* does not require SA or SA signaling for its delayed flowering phenotype, while SA was responsible for the early flowering phenotype of *cpr22*. None of the other CNGC mutants besides *AtCNGC4*[1] displayed an alteration in flowering transition. This indicates that *AtCNGC2* and *AtCNGC4* have a unique role controlling flowering timing and this function is independent from its role in pathogen defense.

Cyclic nucleotide-gated ion channels (CNGCs) are non-selective cation channels that were first identified in animals, where they play key roles in light and olfactory signaling. In mammals, there are six genes that encode CNGCs and the typical mammalian CNGC consists of 4 CNGC subunits. The predicted structures of plant CNGCs are similar to their animal counterparts; however, in plants an expansion of the CNGC family occurred. The *Arabidopsis thaliana* genome has 20 members in the CNGC family, which are classified into four groups (group I-IV), where group IV is further divided into subgroup IVA and IVB.[2] This expansion may indicate diverse biological roles of CNGCs in plants. They have been implicated in a diverse range of biological phenomena such as defense responses, pollen tube growth, ion homeostasis and thermo-tolerance.[2,3] In addition, recent electrophysiological studies showed that plant CNGCs are likely Ca^{2+} permeable channels that are involved in a variety of physiological phenomena.[3-5]

Group IVB comprises only the 2 most divergent plant CNGCs, *AtCNGC2* and *AtCNGC4*. Both are reported to be involved in pathogen defense responses, as loss-of-function mutants of *AtCNGC2* or *AtCNGC4* show remarkably similar autoimmune phenotypes. The null mutant of *AtCNGC2*, "*defense, no death1*" (*dnd1*), has been extensively characterized and is known as a rare autoimmune mutant with impaired hypersensitive responses (HR).[6] The HR is a characteristic defense response, which is a type of programmed cell death around the sites of pathogen entry. Despite the impairment of HR upon pathogen infection, the *dnd1* mutant displays constitutive defense responses, such as elevated expression of *Pathogenesis-Related* (*PR*) genes, high levels of salicylic acid (SA) - an important signaling molecule for resistance against biotrophic pathogens, and conditional HR-like spontaneous lesions without pathogen infection. Consequently, *dnd1* plants show enhanced broad spectrum resistance against several taxonomically unrelated pathogens. In addition, it exhibits characteristic morphological phenotypes, such as small stature and senescence-like chlorosis at the tips of the leaves, indicating roles of *AtCNGC2* in both defense and development.[7]

Recently, we discovered a novel phenotype in *dnd1,* which is a delayed flowering transition observed under both long and short day conditions, although enhanced in the latter condition[1] (**Fig. 1A**). Flowering transition is tightly regulated by endogenous and external cues. In addition, it is known that various

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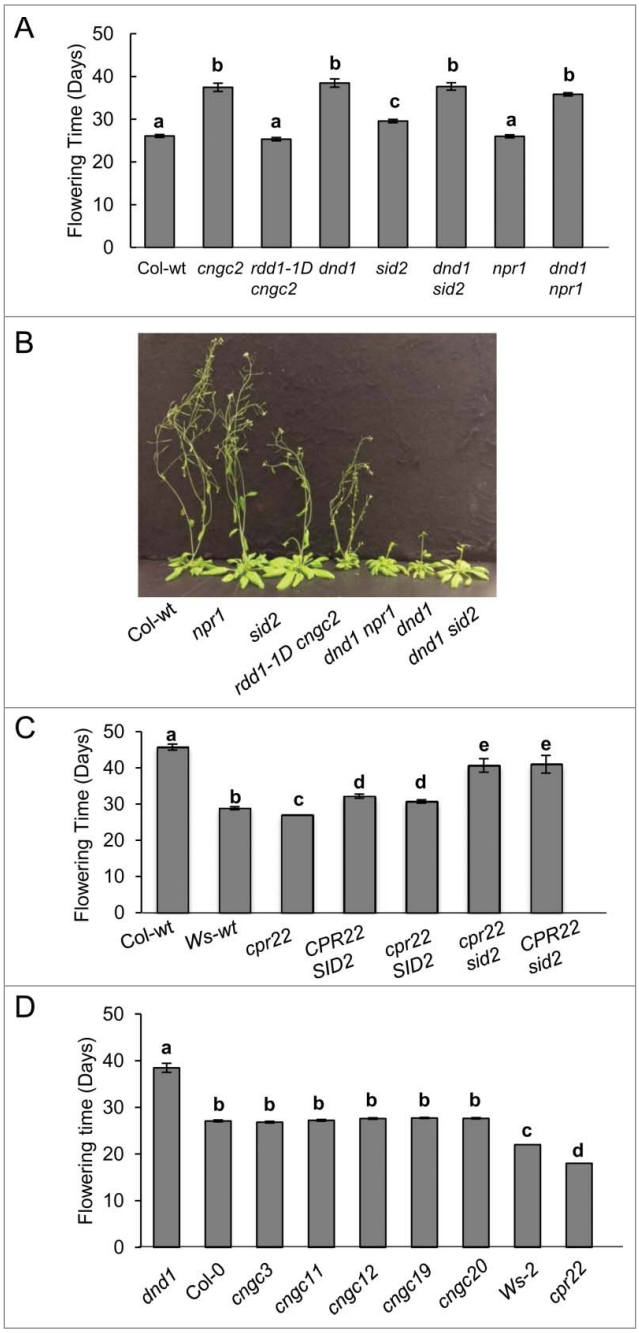

Figure 1. Flowering phenotypes in various CNGC-related mutants. (**A**) The delayed flowering phenotype in *dnd1* is SA/NPR1 independent. *rdd1–1D cngc2,* the suppressor of *dnd1,* served as a control. n = 12–25. (**B**) Flowering phenotype of about 5 week old Col-wt and mutant plants. (**C**) The early flowering phenotype of *cpr22* is SA-dependent. *cpr22* has Ws and *sid2* has Col ecotype background. n = 10–35. (**D**) Flowering phenotype of various CNGC T-DNA Insertion lines, n = 21–31. Flowering time was measured as described in Chin et al. (2013). Error bars = SE, Bars marked with the same letter indicate no significant difference (Student's t-test, $P < 0.05$). Plants were grown in Sunshine Mix #1 with a photoperiod of 16 h light and 8 h dark. All experiments have been repeated at least 3 times with similar results.

stresses, such as ultraviolet-C radiation, pathogen infection and extreme temperatures can promote flowering.[8] Interestingly, it has been reported that SA positively regulates flowering timing in Arabidopsis.[9] SA-deficient mutants, such as *nahG*, *sid2* and *eds5/sid1,* exhibit late flowering phenotypes, while SA hyper-accumulating mutants, such as *acd6* or *siz1* show early flowering transition, supporting this notion.[9,10,11]

Contrary to the positive role of SA, HOPW1-INTERACTING3 (WIN3), which positively regulates broad-spectrum disease resistance through SA signaling, supresses flowering transition.[10] Thus, the relationship of SA, defense activation and flowering timing is complex. This raises several questions regarding the delayed flowering phenotype in *dnd1* in spite of the high levels of SA accumulation: 1) does SA play a role in the delayed flowering transition phenotype in *dnd1*?, 2) is the flowering transition phenotype in *dnd1* a by-product of hyper-activation of defense signaling*?,* and 3) are other CNGCs also involved in the regulation of flowering? To address the first and second questions, we monitored the timing of flowering transition in double mutants of *dnd1* with SA-deficient and defense signaling mutants. *SID2* (*ICS1*) is a major SA biosynthesis gene for defense responses; thus, *dnd1 sid2* exhibits reduced levels of SA compared to the *dnd1* single mutant.[12] *NPR1* is a major component of SA signaling and *npr1* mutants show a deficiency in SA-induced defense responses.[13] It has been reported that *dnd1 npr1* exhibits similar susceptibility to wild type plants against pathogens; thus, the enhanced pathogen resistance of *dnd1* is *NPR1*-dependent.[12] As shown in **Fig. 1A and B**, both double mutants, *dnd1 sid2* and *dnd1 npr1,* exhibited no significant difference in flowering transition from the *dnd1* single mutant, indicating that the delayed flowering transition phenotype in *dnd1* is independent from SA accumulation or *NPR1*-mediated defense activation.

To test whether other CNGC mutants that are related to SA and defense responses also show similar delayed flowering transition phenotypes, we monitored *constitutive expresser of PR genes22* (*cpr22*). *cpr22* displays autoimmune phenotypes with increased SA accumulation and constitutive *PR* gene expression, similar to *dnd1*.[14] It is a gain-of-function mutant and its phenotype is due to the expression of the chimeric *AtCNGC11/12* gene.[14] As show in **Fig. 1C**, *cpr22* does not show delayed flowering transition. Rather we observed a consistent early flowering phenotype in *cpr22* compared to its wild type, (Wassilewskija (Ws)). This indicates that elevated SA levels in *cpr22* promote flowering transition, as expected by the positive role of SA in flowering transition.[9] To further address this question, we monitored flowering transition in the double mutant of *cpr22* and *sid2*. Since *cpr22* has a Ws and *sid2* has a Columbia ecotype background, we used mixed background lines from a *cpr22* x *sid2* cross for this analysis. As expected, *cpr22 SID2* showed earlier flowering transition than *CPR22 SID2* wild type by a few days (**Fig. 1C**). Also, *CPR22 sid2* showed delayed flowering, as expected. Interestingly, the double mutant *cpr22 sid2* showed almost the same flowering transition as *CPR22 sid2,* indicating that the earlier flowering phenotype in *cpr22* is due to its SA accumulation. This agrees well with the reported positive role of SA in flowering transition, unlike what we observe in *dnd1*.

Although they share similar autoimmune phenotypes, *cpr*22 (*AtCNGC11/12*) is a gain-of-function and *dnd1 (atcngc2)* is a loss-of-function mutant of CNGCs. In addition, the loss-of-function mutants for *AtCNGC11 and 12* (*atcngc11* and *atcngc12*) show a partial breakdown of pathogen resistance.[14]. These data indicate a striking difference in the molecular mechanisms that govern defense signaling mediated by *AtCNGC11* and *12* from that of *AtCNGC2*.[14,15] Thus, the flowering phenotype difference between *cpr22* and *dnd1* is not surprising. However, it is possible that some CNGC members share a common role in flowering transition and the loss-of-function of any CNGCs (loss of their channel function) might cause a similar late flowering phenotype that is not related to SA. To address this point, we have monitored various CNGC loss-of-function mutants including *cngc11* and *cngc12*. However, as shown in **Figure 1D**, knockout mutants for *AtCNGC3, 11, 12, 19* and *20* did not exhibit any significant delay in flowering transition, suggesting that it is not a common feature in *CNGC* knockout mutants. Recently, we showed that null mutants of *AtCNGC4,* which display very similar autoimmune phenotypes as *dnd1,* also have delayed flowering phenotypes, and that *AtCNGC2* and *4* likely form a channel complex together.[1] In other words, these data suggested that the 2 class IVB CNGCs have a unique role in flowering transition.

The extensive analyses of the *dnd1* mutant makes *At*CNGC2 the best-characterized CNGC member and it has been suggested that *AtCNGC2* transduces the Ca^{2+} signal after pathogen infection upon recognition of Pathogen Associated Molecular Patterns (PAMPs).[16] In this work, we demonstrate that the novel delayed flowering phenotype in *dnd1* is not a by-product of its autoimmune phenotype or SA accumulation. It is likely another authentic biological role of *AtCNGC2* (and *AtCNGC4*) and is likely unique among CNGCs. Further analysis of the molecular mechanism of the delayed flowering transition in *dnd1* and *cngc4* will shed light on this novel biological function of CNGCs in flowering.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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