Journal of Experimental Botany, Vol. 62, No. 14, pp. 4995–5002, 2011
doi:10.1093/jxb/err199 Advance Access publication 24 June, 2011
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Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

CRFs form protein–protein interactions with each other and with members of the cytokinin signalling pathway in *Arabidopsis* via the CRF domain

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Received 5 May 2011; Revised 5 May 2011; Accepted 23 May 2011

Abstract

Cytokinin is a plant hormone essential for growth and development. The elucidation of its signalling pathway as a variant of the bacterial two-component signalling system (TCS) has led to a better understanding of how this hormone is involved in general plant processes. A set of cytokinin-regulated transcription factors known as cytokinin response factors (CRFs) have been described as a potential branch emanating from the TCS, yet little is known about how CRFs actually interact with each other and with members of the TCS pathway. Here the interactions of CRF proteins (CRF1–CRF8) using the yeast two-hybrid system and bimolecular fluorescence complementation *in planta* assays are described. It was found that CRFs are readily able to form both homo- and heterodimers with each other. The first analysis of CRF versus TCS pathway protein interactions is also provided, which indicates that CRFs (CRF1–CRF8) are able specifically to interact directly with most of the *Arabidopsis* histidine-phosphotransfer proteins (AHP1–AHP5) further solidifying their link to the cytokinin signalling pathway. In addition, the region of CRF proteins involved in these interactions was mapped and it was determined that the clade-specific CRF domain alone is sufficient for these interactions. This is the first described function for the CRF domain in plants.

Key words: *Arabidopsis*, CRF, cytokinin, cytokinin signalling pathway, protein–protein interaction.

Introduction

Cytokinin is an essential plant hormone for plant growth and developmental processes (Mok and Mok, 2001; Kieber, 2002; Heyl and Schmülling, 2003). Understanding the process of cytokinin signalling in plants is a crucial part of knowing how this hormone functions during development (Werner and Schmülling, 2009). Numerous studies in *Arabidopsis* and other plant species have provided a detailed picture of how several of the proteins involved in cytokinin signalling, namely the receptor histidine kinases (AHKs), histidine-phosphotransfer protein (AHPs), and type-A and type-B response regulators (ARRs), interact, supporting a multistep two-component signalling model, which seems to be well conserved in land plants (Ferreira and Kieber, 2005; Dortay *et al.*, 2006, 2008; To and Kieber, 2008; Pils and Heyl, 2009; Hellmann *et al.*, 2010). Using comparative microarray analyses and cytokinin-regulated nuclear localization in different mutant backgrounds of the two-component cytokinin signalling (TCS) pathway, another class of proteins, the cytokinin response factors (CRFs), have also been shown to interact functionally with this pathway (Rashotte *et al.*, 2006). Specifically, CRFs were placed downstream in the signalling pathway of AHPs and probably function in parallel with the type-B ARRs in their action on cytokinin-regulated targets (Rashotte *et al.*, 2006).

CRFs are members of the AP2/ERF family of transcription factors, existing as the B-5 or VI phylogenetic clade of the AP2/ERF proteins containing a single AP2–DNA-binding domain, distinct from both DREB and AP2

proteins (Sakuma *et al.*, 2002; Nakano *et al.*, 2006; Rashotte and Goertzen, 2010). CRFs (CRF1–CRF6) have been previously examined and shown to be regulated by cytokinin and affect leaf and cotyledon development, although there has been no examination of how CRFs might be involved in interactions at a protein level (Rashotte *et al.*, 2006). One level of regulation of AP2/ERF transcription factor family members such as CRFs may occur through the homo- and heterodimerization of different proteins, possibly though differential binding of specific protein dimer combinations to *cis*-element regulator sites.

In this study, CRF protein–protein interactions were examined in order to better understand the function of CRFs. This was done in experiments to determine the ability of CRFs (CRF1–CRF6) to homo- and heterodimerize using both the yeast two-hybrid system (Y2H) and bimolecular fluorescence complementation (BiFC) analyses. Then using a combination of protein regions, the CRF domain of CRF2, and natural variation among CRF proteins (CRF7 and CRF8), the CRF protein–protein interaction domain was narrowed down to the region of the CRF protein containing the CRF domain. In addition it was intended to determine if CRF proteins could directly interact with any members of the TCS pathway. The results of interaction experiments between CRF1–CRF8 and various parts of the TCS pathway are shown, along with results of what region of the CRF protein is responsible for pathway interactions which were found.

Materials and methods

Plasmid construction and recombinant proteins

All plasmids were generated using the Invitrogen GATEWAY™ system according to the manufacturer's instructions. Entry plasmids were generated for CRF1–CRF8 and the CRF domain using gene-specific PCR primers designed with attB1 and attB2 recombination sequences as adaptor sites such that these PCR products could be cloned into the entry vector pDONR207 or pDONR221 via a BP reaction (Invitrogen, Carlsbad, CA, USA). All full-length CRF genes were generated in this manner such that their stop codon was removed. All TCS gene cDNAs used were from previously generated entry clone plasmids (Dortay *et al.*, 2006). The entry clones of the full-length AHKs were obtained from different sources (Heyl *et al.*, 2007; Stolz *et al.*, 2011).

Yeast two-hybrid assays

The yeast two-hybrid analysis was done using a LexA DNA-binding domain-based bait vector (*pBTM116c-D9*; a kind gift of E. Wanker, MDC Berlin, Germany) and a Gal4 activation domain-encoding prey vector (*pACT2*; Clontech, Montain View, CA, USA), both adapted to the GATEWAY™ system (Dortay *et al.*, 2006). The respective cDNAs were shuttled into these vectors via *in vitro* recombination. Based on the LiAc method, yeast transformations were conducted as described earlier (Bürkle *et al.*, 2005). Weak autoactivation of some hybrid proteins was suppressed by supplementing the interaction medium with 5 mM 3-amino-1,2,4-triazole (3AT), whereas strong autoactivating hybrid proteins were tested in the presence of up to 40 mM 3AT. All yeast clones were scored for interaction after a 7 d incubation period at 30 °C.

Bimolecular fluorescence complementation

BiFC was performed using entry clones for CRF genes (CRF1–CRF8) and CRFD (the first 180 bp of CRF2, containing the CRF domain) that were transferred to the destination clone vectors pSAT4-DEST-n(1–174)EYFP-C1 and pSAT5-DEST-c(175-end)EYFP-C1(B) using the LR reaction (Invitrogen) (Tzfira *et al.*, 2005). Entry clones containing TCS genes (AHK2, 3, 4; AHP1, 2, 3, 4, 5; and ARR5, 7, 10, 12) were transferred to the destination clone vector pSAT4-DEST-n(1–174)EYFP-C1 using the LR reaction (Invitrogen) for potential BiFC interaction with CRF genes in pSAT5-DEST-c(175-end)EYFP-C1(B) vectors. Empty vectors (EVs) were also used for both N- and C-terminal constructs (EVn and EVc) as controls, transformed into protoplasts in conjunction with every potential functional interacting construct that was generated, and examined as detailed below and for selected constructs in *Agrobacterium*-transformed tobacco leaf examinations.

BiFC in leaf mesophyll protoplasts transformed via electroporation

Arabidopsis thaliana plants (Columbia ecotype) used for protoplast *in planta* BiFC experiments were grown under a long day cycle of 16 h light 22 °C, 8 h dark 18 °C. Leaf mesophyll protoplasts were prepared from leaves of 14- to 21-day-old plants prior to bolting as modified from Rashotte *et al.* (2006). Briefly, leaves were removed from plants, gently stacked, and sliced into 1 mm strips. Leaf strips were placed in enzyme solution [1% cellulase R10, 0.25% macerozyme R10, 0.4 M mannitol, 20 mM KCl, 20 mM MES pH 5.7 that was heated to 55 °C for 10 min, then cooled to room temperature before adding 10 mM $CaCl_2$ and 1% bovine serum albumin (BSA), and finally passed through a 0.45 mm filter] under a vacuum for 30–60 min. Afterwards, leaf strips were shaken very gently at 40 rpm for 90 min, before being shaken more rapidly at 80–90 rpm for 10 min to release protoplasts from the leaf. Protoplasts were then filtered from larger plant material through a 40 μM cell sifter and centrifuged at 150 *g* so that they were gently pelleted. Enzyme solution was separated from the protoplast pellet, which was then very gently resuspended in 2 ml of cold protoplast enzyme washing/transformation buffer (0.5 M mannitol, 4 mM MES pH 5.7, and 20 nM KCl). Protoplasts were centrifuged again, washing buffer was removed, and then protoplasts were resuspended in a final volume of washing/transformation buffer prior to transformation and placed on ice until transformation, usually within 1 h. A 100 μl aliquot of transformation buffer containing ~10^5 protoplasts along with ~40–50 μg of plasmid DNA for each plasmid used were given two rapid pulses of 300 V for electroporation in a 0.1 mm electroporation cuvette using an Eppendorf Electroporator 2510. Protoplasts were then immediately placed on ice and left in the dark at 22 °C for 18 h before examination using epifluorescence microscopy.

BiFC interactions in tobacco leaves transformed via Agrobacterium

Tobacco (*Nicotiana tabacum*) plants were grown under a long day cycle of 16 h light 26 °C, 8 h dark 22 ° C. Vectors used for transformation (CRF or TCS destination vectors or EVs, as described above) were transformed into *Agrobacterium tumefaciens* (LBA4404) using a method similar to that of Rashotte *et al.* (2006) leading to a floral dip. However, once properly antibiotic selected individual colonies had been identified, further grown up in liquid culture, and then spun down, they were then resuspended in infiltration medium (10 mM $MgCl_2$, 10 mM MES, 100 μM acetosyringone) and left at room temperature for 3 h similar to the method of Liu *et al.* (2002). The *A. tumefaciens* were then infiltrated into the abaxial side of 14- to 21-day-old plant leaves using a needle-less 2 ml syringe. Individual *A. tumefaciens*-transformed vectors were grown separately and then injected into unique sites within the same leaf. Plants were then examined for

transient transformation and specific BiFC protein–protein interactions 48–72 h after injection using epifluorescence microscopy.

Microscopy

BiFC in both *Arabidopsis* protoplasts and tobacco leaves was examined using a Nikon Eclipse 80*i* epifluorescence microscope with a UV source. A standard UV filter was used in addition to 1 ng ml^{-1} Hoechst 33342 dye initially to observe and identify nuclei in intact cells as a measure of the cell viability. A yellow fluorescent protein (YFP) filter that blocks both chlorophyll fluorescence and Hoechst 33342 fluorescence was used to examine localization of any split-YFP fusions that occur due to BiFC between proteins. Cytokinin [2 μM benzyladenine (BA)] was routinely added to protoplasts prior to examination, but was not required and not done in tobacco leaves. However, in assessment of any AHK interactions, predicted to be localized at the plasma membrane, examinations were made in both the presence and absence of cytokinin in order to avoid potential cellular compartmentalization of proteins that might physically limit possible interactions. All photographs were taken with a Qimaging Fast 1394 digital camera and are presented as composite images using Adobe Photoshop CS3 without altering the original integrity of the picture.

Results

CRF–CRF protein interactions

The ability of CRF proteins both to homodimerize and to heterodimerize was examined using a combination of Y2H and *in planta* BiFC methods to assess interactions. In each method, copies of CRF1–CRF8 proteins were placed in specific vectors (see the Materials and methods) such that potential interactions could be assayed. This was conducted through growth on selection media of transformed yeast or through epifluorescence examination of BiFC of either double transformation of *Arabidopsis* leaf mesophyll protoplasts or double *Agrobacterium*-infiltrated tobacco leaves. Results from these methods suggest that CRFs 1–8 are all able to form both homo- and heterodimers and no interactions are seen using any CRF and an empty vector (EV) control (Fig. 1). While all of these interactions could be seen using BiFC in protoplasts and many by Y2H, not every interaction could be detected by Y2H (Fig. 1A). Specific examples of CRF homo- and heterodimerization via BiFC

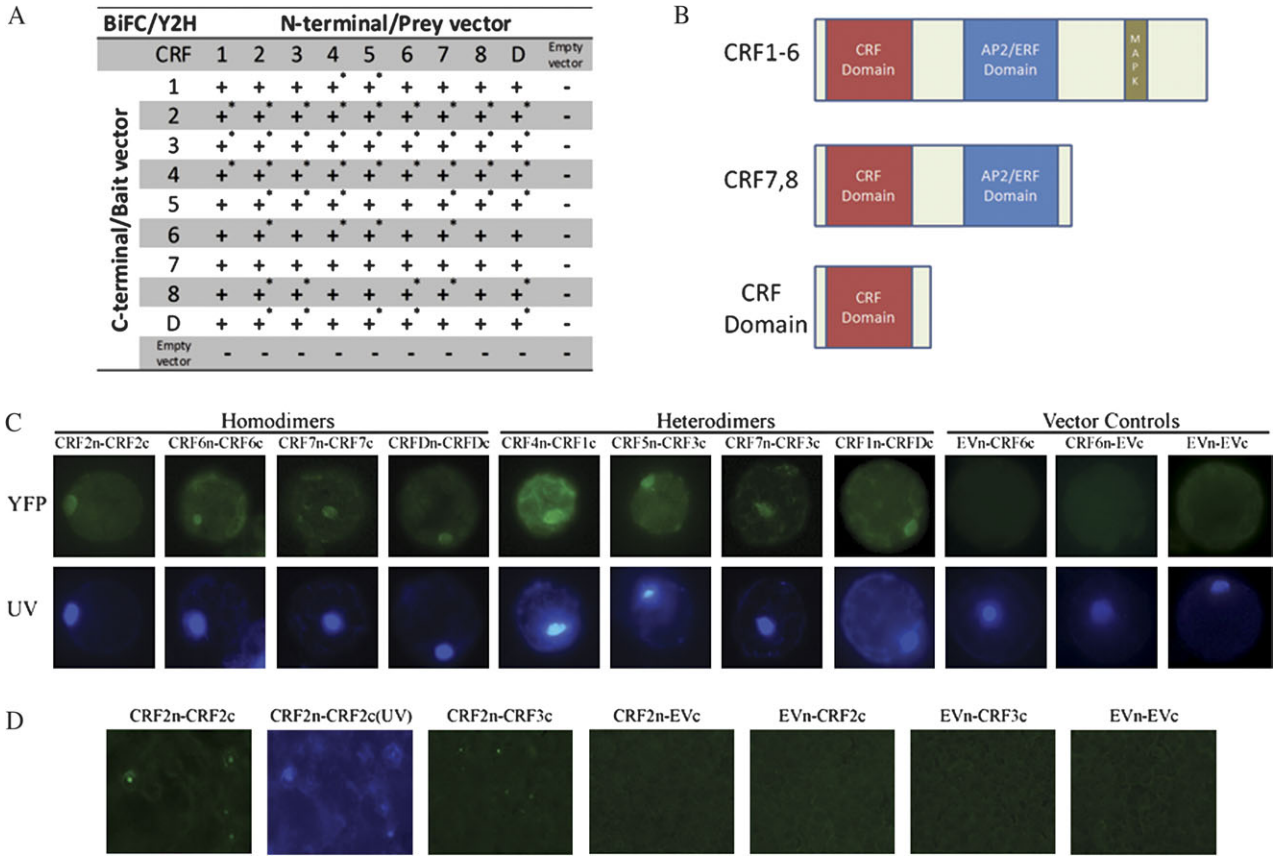

Fig. 1. CRF protein homo- and heterodimerization. (A) CRF proteins CRF1–CRF8 and just the CRF domain of CRF2 (otherwise noted as CRFD) were analysed for potential homo- and heterodimerization using both BiFC and Y2H. Positive interactions are noted as a (+), (+*) for both BiFC and Y2H together, and (–) for non-interactions. (B) A cartoon of the conserved regions of CRF1–CRF6 proteins, the highly related CRF7 and CRF8 proteins (lacking the C-terminal third of the protein, but containing both the CRF domain and the AP2/ERF domain), and CRFD (just the CRF domain of CRF2). (C, D) Representative examples of positive CRF protein homo- and heterodimerization by BiFC as indicated in A are shown both under UV light in the presence of Hoechst 33342 dye denoting the nucleus and using a YFP wavelength filter to visualize BiFC interaction. Additionally, representative examples of empty vector (EV) controls for both N- and C-terminal BiFC vectors (EVn and EVc) examined in A are shown versus various CRFs and each other. (C) BiFC interactions in protoplasts transformed via electroporation. (D) BiFC interactions in tobacco leaves transformed via *Agrobacterium*.

can be seen most clearly in the nucleus of the protoplasts examined, indicating that these interactions are probably occurring at or near the nucleus (Fig. 1C, D). It is possible that CRF–CRF interactions may also be occurring in the cytoplasm, although unlike easily detected interactions in the nucleus it can be difficult to discern cytoplasmic-specific changes in fluorescence between double transformed protoplasts and background levels. The lack of some interactions detected in Y2H compared with BiFC supports the idea that additional *in planta* factors, such as the presence of a functional TCS or potentially plant-specific post-translational modifications of CRFs, may be required for interactions detected in protoplasts that are lacking in the yeast system.

Mapping of CRF protein interactions to the CRF domain

In order to examine further if a specific region or domain of the CRF proteins might be involved in protein–protein interactions, use was made of both natural variation among CRF proteins and a truncated version of a CRF protein. A recent analysis of CRF protein sequences from all available organisms has revealed that CRFs are specific to land plant species (Rashotte and Goertzen, 2010). The analysis of >125 different CRF proteins from a wide range of species revealed the presence of two distinct domains that are always present in CRF proteins: the conserved AP2/ERF transcription factor DNA-binding domain and a phylogenetic clade-specific domain of no known function designated as the CRF domain. Additionally, many of the CRF proteins examined also have a putative MAPK phosphorylation site motif. Sequence alignments always place the CRF domain in the N-terminal third of the protein, the AP2/ERF DNA-binding domain near the middle, and the putative MAPK phosphorylation site motif in the C-terminal third of CRF proteins (Fig. 1B). The CRF1–CRF6 proteins that were examined each contain a CRF and a AP2/ERF domain as well as the putative MAPK motif. Two recently described CRF proteins, CRF7 (At1g22985.1) and CRF8 (At1g71130.1), are related to CRF1–CRF6, yet exist naturally without an extended C-terminal end present in many CRFs and thus lack the putative MAPK motif (Fig. 1B; Sakuma *et al.*, 2002; Nakano *et al.*, 2006; Rashotte and Goertzen, 2010). The ability of CRF7 and CRF8 to interact as homo- and heterodimers with other CRF proteins was examined using BiFC and Y2H. Both CRF7 and CRF8 were able to form homodimers by these methods (Fig. 1). Furthermore, CRF7 and CRF8 were able to form heterodimers with all other CRF proteins as seen by BiFC, and several also by Y2H (Fig. 1). Y2H-positive protein–protein interaction results were again only found for some of these interactions, also suggesting that additional *in planta* factors or plant-specific post-translational modifications are likely to be required for normal interactions to occur. However, as these CRF interactions occurred without the C-terminal region found in CRF1–CRF6, this indicates that the putative MAPK phosphorylation motif or any other feature in the C-terminal regions of these proteins are not necessary for CRF–CRF protein interaction.

To narrow further the potential region of CRF proteins involved in interaction, it was decided to investigate the CRF domain, as it has no known function, yet it is specifically found only in this clade grouping of AP2/ERF proteins (Rashotte and Goertzen, 2010). A truncated version of CRF2 was generated containing only the CRF domain (CRFD) to determine if this domain alone is sufficient for CRF protein interaction (Fig. 1B). The CRFD was examined for its ability to homo- and heterodimerize with other CRFs similar to the full-length CRFs above. CRFD was able to form as a homodimer and also can heterodimerize with each of the other CRF proteins (1–8) as seen by BiFC and supported in several cases by Y2H in a similar manner to other CRFs and even the full-length CRF2 (Fig. 1). This indicates that the AP2/ERF domain is not necessary for dimerization to occur and that the CRF domain alone is sufficient for CRF–CRF protein interactions.

CRF protein interactions with members of the cytokinin signalling pathway

Genetic analysis of TCS mutants has shown CRFs to be a side branch emanating from the multistep TCS pathway downstream of the AHPs and parallel to type-B ARRs in the regulation of cytokinin target genes (Rashotte *et al.*, 2006). In order to better resolve the role of CRF proteins in the TCS pathway, the ability of the previously studied CRF1–CRF6 proteins to interact with proteins from each part of the TCS pathway: the receptors AHK2, 3, and 4; AHP1, 2, 3, 4, and 5; type-A ARR5 and 7; and type-B ARR10 and 12 was examined (Fig. 2). No interactions were observed between CRF1–CRF6 and AHK2, 3, and 4 *in planta*, indicating that CRFs probably do not interact with the cytokinin receptors. In contrast, almost every CRF interacted with each of the AHPs (Fig. 2). Only CRF2 and CRF3 showed no interaction with AHP2. This result strongly suggests that CRFs and AHPs directly interact in a number of different combinations. As these results parallel previous results of ARRs interacting with AHPs, they indicate that CRFs are directly connected to the cytokinin signalling pathway. CRFs showed almost no interactions with type-A ARRs, although both an ARR7–CRF1 and ARR7–CRF2 interaction could be detected. Similarly there were almost no interactions of CRFs with the tested type-B ARRs. However, interactions between these two transcription factor groups could be detected for ARR12–CRF1, ARR12–CRF2, and ARR10–CRF6. Similar experiments were conducted in the Y2H experimental system. Also with this method interactions were detected between CRFs and the only tested AHP, AHP5. Some weak interactions between CRFs and ARR1 were also identified. However, in both cases the interactions were weak and just above background (Supplementary Table S1 available at *JXB* online). These results corroborate what has been found before, namely that not all of the CRF interactions detected in the BiFC system can be detected in the heterologous yeast system as well.

A

BiFC	AHK2	AHK3	AHK4	AHP1	AHP2	AHP3	AHP4	AHP5	ARR5	ARR7	ARR10	ARR12
CRF1	-	-	-	+	+	+	+	+	-	+	-	+
CRF2	-	-	-	+	-	+	+	+	-	+	-	+
CRF3	-	-	-	+	-	+	+	+	-	-	-	-
CRF4	-	-	-	+	+	+	+	+	-	-	-	-
CRF5	-	-	-	+	+	+	+	+	-	-	-	-
CRF6	-	-	-	+	+	+	+	+	-	-	+	-
CRF7	-	-	-	+	+	+	+	+	-	-	-	-
CRF8	-	-	-	+	+	+	+	+	-	-	-	-
CRFD	-	-	-	+	+	+	+	+	-	+	-	-
Empty Vector	-	-	-	-	-	-	-	-	-	-	-	-

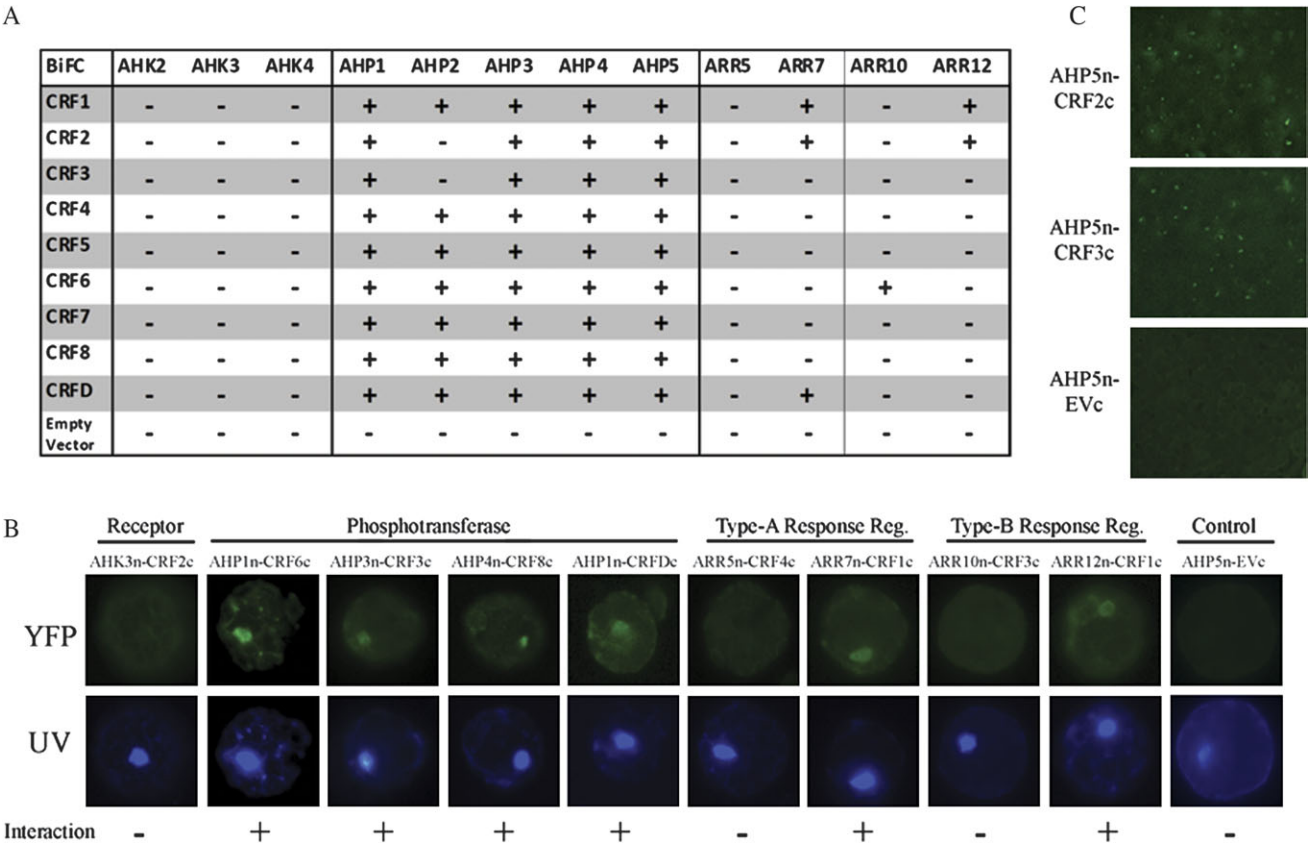

Fig. 2. CRF protein interactions with members of the cytokinin signalling pathway. (A) CRF proteins, CRF1–CRF8, and CRFD (C-terminal) were analysed for potential interactions with TCS pathway proteins (N-terminal) using BiFC. Positive interactions are noted as (+) and non-interactions as (–). (B, C) Representative examples of positive and negative CRF protein interactions with TCS proteins as well as an empty vector control (EVc: C-terminal) by BiFC as indicated in A are shown both under UV light in the presence of Hoechst 33342 dye denoting the nucleus and using a YFP wavelength filter to visualize BiFC interaction. (B) BiFC interactions in protoplasts transformed via electroporation. (C) BiFC interactions in tobacco leaves transformed via *Agrobacterium*.

Examinations of the specific region or motif of CRF proteins that is involved in CRF–CRF protein–protein interaction was also conducted in a similar manner for interactions with TCS proteins. Both CRF7 and CRF8 were used as natural variants lacking the C-terminal region found in CRF1–CRF6, which contains a putative MAPK motif, to determine if this or any other C-terminal feature was necessary for interactions between CRFs and parts of the TCS pathway. CRF7 and CRF8 showed similar interaction patterns between parts of the TCS pathway as compared with CRF1–CRF6 (Fig. 2). CRF7 and CRF8 were found to interact with most of the AHPs (1–4), and not with any of the receptor AHKs or either tested type-A or -B ARRs. This again indicates that the putative MAPK phosphorylation motif is not necessary for any interactions that have been examined for CRF proteins. Further analysis was conducted with CRFD to determine if the CRF domain alone was sufficient for interaction with parts of the TCS pathway using BiFC analysis. CRFD was found to interact in a manner similar to the full-length CRF proteins examined. CRFD did not interact with any of the receptors or type-B ARRs, but did interact with AHP1–AHP5 and the type-A ARR, ARR7, similarly to the full-length form of the CRF2 protein (Fig. 2). These results, coupled with the CRFD versus other CRF protein interactions, indicate that it is the CRF domain itself that is involved in interactions that were observed between other CRF proteins and parts of the TCS pathway.

Discussion

CRFs are member of the AP2/ERF protein family that have been previously shown to be linked to the multistep TCS pathway, which is known to regulate cytokinin responses in plants (Ferreira and Kieber 2005; Rashotte *et al.*, 2006; To and Kieber, 2008). CRFs were found to be genetically downstream of the AHPs of the TCS pathway and appeared to act either in concert or in parallel with the type-B ARRs to regulate a series of cytokinin-regulated target genes (Rashotte *et al.*, 2006).

This study examined whether CRFs had the ability to form dimers, either in homo- or in hetero- configuration, as a potential means to regulate their activity. It was found that CRFs are readily able to form homodimers, and in all cases were able to do so *in planta* (Fig. 1). Additionally, CRFs readily form heterodimers, forming all possible CRF–CRF heterodimer forms that were examined (Fig. 1).

This suggests that specific homo- and heterodimer forms are unlikely to be involved in any regulatory control of downstream targets. However, previous work indicated that *crf5,crf6* double mutants are embryo lethal, yet single mutants are fully fertile, suggesting that these genes function in a redundant manner for this process (Rashotte *et al.*, 2006). It may be that CRF5 and CRF6 homodimers, which were seen to form in this study, can act functionally in a redundant manner to control their required embryo process if the other gene is knocked out.

The ability of CRF proteins to interact with parts of the TCS pathway was also examined to help better determine the role of CRFs in this pathway and in cytokinin signalling. Previous work has established that AHPs are the major interacting proteins linking all the TCS components in this pathway (Dortay *et al.*, 2006, 2008). AHPs interact with each of the receptor AHKs, and with most of both the type-A and type-B ARRs, consistent with a multistep phosphorelay model that is proposed for cytokinin signalling in *Arabidopsis* (Hwang and Sheen, 2001; Kakimoto, 2003). Based on previous work, CRFs have been placed into the signalling model as downstream of AHPs and potentially acting as transcription factors in concert with type-B ARRs (Rashotte *et al.*, 2006). Here it is identified that CRF proteins are able to interact directly with AHPs *in planta*, confirming that these proteins are directly linked to the TCS pathway. Nearly every CRF protein (CRF1–CRF8) was able to interact with nearly every AHP protein tested (AHP1–AHP5) (Fig. 2). This supports previous findings of AHPs being central players in the cytokinin signalling pathway (Ferreira and Kieber; 2005; Dortay *et al.*, 2006, 2008; To and Kieber, 2008; Werner and Schmülling, 2009). No interactions of CRFs with the receptor AHKs (AHK2–AHK4) were observed, similar to what was seen for other TCS components, namely that almost no type-A or -B ARRs directly interact with AHKs (Dortay *et al.*, 2006, 2008). Interactions of CRFs with the response regulators were observed only in rare instances *in planta*, such as ARR10–CRF6, and appear to be atypical of CRF–TCS pathway interactions. However, the interaction of both CRF1 and CRF2 with ARR7 and ARR12 may suggest a unique ability of the most closely related CRFs to be involved in specific interactions. The importance of these interactions will have to be examined further to determine what if any role they might play in cytokinin responses.

The ability of CRF7 and CRF8 to form heterodimers with CRF1–CRF6 provides the first hints that these two proteins might function in a similar biological process to the other, better characterized CRFs. It also indicates that the C-terminal third of CRFs 1–6 is not responsible for CRF–CRF interactions as CRF7 and CRF8 are shorter proteins lacking that C-terminal region. As the previously described putative MAPK phosphorylation motif found in many CRF proteins is contained within this C-terminal region of CRF1–CRF6, it also indicates that any phosphorylation of that motif or the motif itself is not required for CRF–CRF interactions. Moreover, the ability of just the N-terminal region of CRF2, containing only the CRF domain of this protein, to form both a homodimer and heterodimers with the other full-length CRFs (CRF1–CRF8) indicates that it is the CRF domain and not the AP2/ERF DNA-binding domain that is required for CRF–CRF interactions. This is the first function that can be experimentally attributed to this domain; in fact no known function has been attributed to this domain through any sequence motif analysis programme (Nakano *et al.*, 2006; Rashotte and Goertzen, 2010). Furthermore, the ability of the shorter, naturally C-terminal truncated CRF7 and CRF8 proteins to interact equally well as CRF1–CRF6 with the AHPs suggests that the putative MAPK phosphorylation motif found in CRF1–CRF6 is not required for CRF interaction in the TCS pathway. Additionally, the ability of the CRF domain alone to interact with parts of the TCS pathway in a similar manner to other CRFs and its full-length CRF2 version reveals that the CRF domain is the likely region of these proteins to be involved in CRF–TCS interactions. It is clear that the CRF domain alone is sufficient for CRF–TCS interactions and that the AP2/ERF domain is not required. Interestingly, there is another example of ERF proteins having a domain other than the AP2/ERF domain that allows these proteins to undergo protein–protein interactions: DRN/ERS1 and DRNL/ERS2 (Chandler *et al.*, 2007). These proteins were each shown to have a PAS-LIKE domain independent of the AP2/ERF domain that is sufficient for heterodimerization with several members of the class III HD-ZIP transcription factor family, including PHV. It is suggested that protein complexes formed by DRN, DRNL, and PHV through these protein interactions may act to control developmental patterning in the embryo (Chandler *et al.*, 2007). It is an attractive hypothesis to think that CRFs and parts of the TCS pathway might act as a complex in a similar manner to control cytokinin-regulated processes during development.

Surprisingly, more CRF interactions could be detected in the *in planta* BiFC system than with the heterologous Y2H method. While unusual, this observation seems to indicate that a plant-specific modification of the CRFs or another, as yet unidentified, plant-specific protein is facilitating the interactions of this protein family.

In summary, it was shown that CRF1–CRF8 proteins are able to form both homo- and heterodimers with themselves *in planta* (Fig. 3). Additionally, it was shown that each of the CRF proteins (CRF1–CRF8) is able to interact directly with almost all of the AHPs, (AHP1–AHP5), thus supporting the link to CRFs as a potential branch of the cytokinin signalling pathway. CRF protein interactions with TCS members occurs mostly between the AHPs, as CRF proteins appear not to interact directly with any receptor AHKs and may interact in limited cases with specific response regulators (Fig. 3). This pattern of interaction is similar to that observed for both AHKs and ARRs in that interactions are primarily with the AHPs (Dortay *et al.*, 2006, 2008). The regions of CRF proteins that are responsible for their interactions with other CRFs and parts of the TCS pathway were examined and it was determined that the clade-specific CRF domain alone is sufficient for

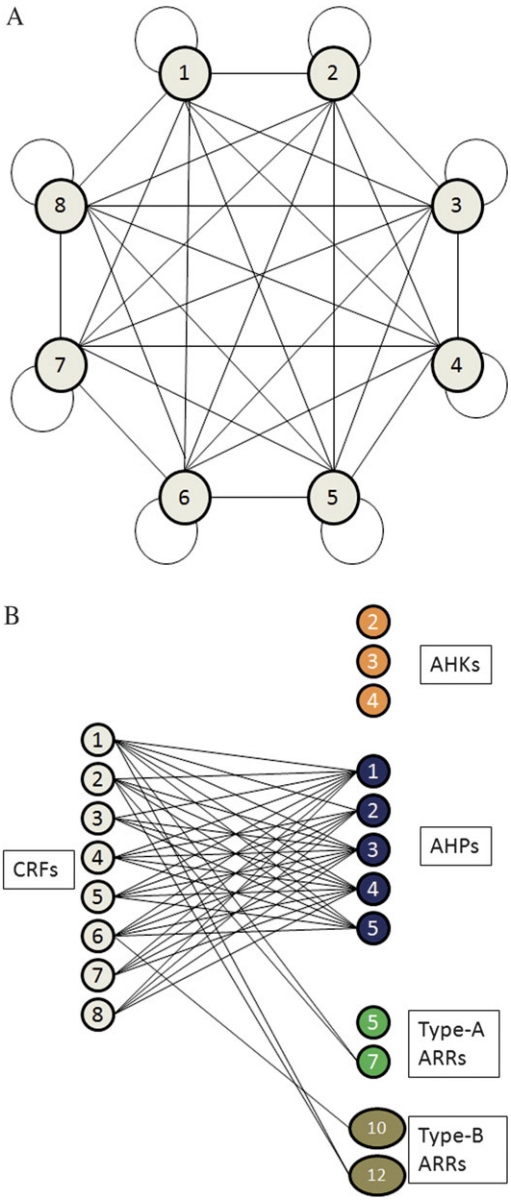

Fig. 3. Models of CRF protein interaction. (A) A model of CRF1–CRF8 proteins interacting with other CRF1–CRF8 proteins as indicated by the present results. CRFs are indicated by their number in a circle and are linked by a line to other CRFs with which they were found to interact. (B) A model of CRF1–CRF8 proteins interacting with members of the TCS pathway. Classes of proteins are labelled in boxes and the gene number designation of that class is noted in a corresponding circle. Interactions between proteins as indicated by the present results are shown as a solid line if positive, and the lack of a line indicates that no interaction was detected.

these interactions. This is the first described function for the CRF domain in plants.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. Interactions between CRFs and members of the two-component signalling system of *Arabidopsis thaliana* as detected in the yeast two-hybrid system.

Acknowledgements

We gratefully acknowledge technical support for the yeast interaction assays from Cordula Braatz. We also thank all members of the Rashotte and Heyl labs for critical reading of the manuscript. Support for this work was provided by Auburn University Cell and Molecular Biology Program to AMR and in part by a DAAD-RISE fellowship grant to JWC and an Elsa-Neumann-stipend to EH.

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