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EHB1 and AGD12, two calcium-dependent proteins affect gravitropism antagonistically in *Arabidopsis thaliana*

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ABSTRACT

The ADP-RIBOSYLATION FACTOR GTPase-ACTIVATING PROTEIN (AGD) 12, a member of the ARF-GAP protein family, affects gravitropism in *Arabidopsis thaliana*. A loss-of-function mutant lacking AGD12 displayed diminished gravitropism in roots and hypocotyls indicating that both organs are affected by this regulator. AGD12 is structurally related to ENHANCED BENDING (EHB) 1, previously described as a negative effector of gravitropism. In contrast to *agd12* mutants, *ehb1* loss-of function seedlings displayed enhanced gravitropic bending. While EHB1 and AGD12 both possess a C-terminal C2/CaLB-domain, EHB1 lacks the N-terminal ARF-GAP domain present in AGD12. Subcellular localization analysis using Brefeldin A indicated that both proteins are elements of the *trans* Golgi network. Physiological analyses provided evidence that gravitropic signaling might operate via an antagonistic interaction of ARF-GAP (AGD12) and EHB1 in their Ca^{2+}-activated states.

1. Introduction

Tropic bending of plant organs depends on and correlates with polarized auxin transport. Its intercellular redistribution is mediated by influx carriers of the AUX1/LAX family (Bennett et al., 1996; Swarup et al., 2008) and efflux carriers of the PIN FORMED (PIN) family (Adamowski and Friml, 2015). There is growing evidence that PIN3 redistribution involves ARF proteins (ADP-ribosylation factor; not to be mistaken in this context with auxin response factors) that mediate vesicle coating within the *trans* Golgi network. ARF belongs to a group of small GTPases, which play a prominent role in plants by mediating vesicle trafficking (Fujimoto and Tsutsumi, 2014; Fujimoto and Ueda, 2012; Li et al., 2011; Vernoud et al., 2003). ARF activity is affected by ARF-GEF proteins, like GNOM (Geldner et al., 2001; Grebe et al., 2000; Kleine-Vehn et al., 2008a; Steinmann et al., 1999). These proteins are required for GTP loading. Mutants lacking GNOM show decreased root gravitropism (Geldner et al., 2004). However, ARF-mediated membrane traffic requires not only GTP exchange, but also needs GTP to GDP hydrolysis, mediated by a corresponding ARF-GAP element (Yorimitsu et al., 2014). Thus, it is likely that not only associated GTPase regulators of the ARF-GEF family, i.e. GNOM, but also ARF-GAPs might play an important role in gravitropic bending.

Given the prominent role small G-proteins play in auxin-mediated tropisms, it would be relevant to know, whether or not EHB1, an early signaling effector in light triggered bending (Knauer et al., 2011), could also operate *via* small G-protein interaction. It was previously shown that EHB1 acts in Arabidopsis as a negative regulator in gravitropism. (Knauer et al., 2011). EHB1 was recently reclassified as a member of the subfamily of C2-domain proteins related to the ABA response (CAR), although data that this protein is involved in ABA related processes are still lacking (Rodriguez et al., 2014). Members of this particular CAR subfamily contain

Abbreviations: AGD, ADP-ribosylation factor GTPase-activating protein; ARF, ADP-ribosylation factor; ARF-GAP, ARF GTPase-activating protein; ARF-GEF, ARF guanine-nucleotide exchange factor; BFA, brefeldin; CaLB, calcium/lipid-binding; ER, endoplasmic reticulum; g, gravity vector; EHB1, enhanced bending1; PIN, pin formed; rpm, rounds per minute.

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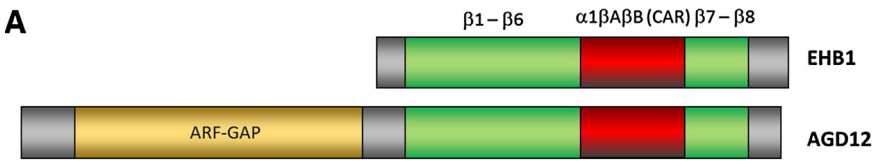

B

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                  1                                                    50
* At3g17980(CAR)  .......... .......... .......... .......... ..........
At1g70800(EHB1)   .......... .......... .......... .......... ..........
At4G22160(AGD12)  MSYSGAGLGK PGSGKRRIRD LLTQSDNRVC ADCGAPDPKW ASANIGVFIC

                  51                                                   100
At3g17980(CAR)    .......... .......... .......... .......... ..........
At1g70800(EHB1)   .......... .......... .......... .......... ..........
At4G22160(AGD12)  LKCCGVHRSL GSHISKVLSV TLDEWSDEEV DSMIEIGGNA SANSIYEAFI

                  101                                                  150
At3g17980(CAR)    .......... .......... .......... .......... ..........
At1g70800(EHB1)   .......... .......... .......... .......... ..........
At4G22160(AGD12)  PEGSSKPGPD ASHDQRMRFI RSKYEHQEFL KPSLRITSVR GSSTKTPAFL

                  151                                β1                200
At3g17980(CAR)    .......... ...MTTACPA RTSSLMDDLL GLLRIRIKRG VNLAVRDISS
At1g70800(EHB1)   .......... ......MEKT EEEVEMKELV GLVRILVKRG IDLARRDALS
At4G22160(AGD12)  SSSLSKKIVD SFRTNSSSQQ PQLEGMVEFI GLLKVTIKKG TNMAIRDMMS

                  201 β2      β3                  β4            β5   250
At3g17980(CAR)    SDPYVVVKMG KQKLKTRVIN KDVNPEWNED LTLSVTDSNL TVLLTVYDHD
At1g70800(EHB1)   SDPFVVITMG PQKLKSFTVK NNCNPEWNEE LTLAIEDPNE PVKLMVYDKD
At4G22160(AGD12)  SDPYVVLTLG QQKAQSTVVK SNLNPVWNEE LMLSVPHNYG SVKLQVFDYD

                        β6          α1                βA                300
At3g17980(CAR)    MFSKDDKMGD AEFEIKPYIE ALRMQLDGLP SGTIVT.TVK PSRRNCLAEE
At1g70800(EHB1)   TFTADDKMGD AQIDMKPFLD VHKLGLKELP HGKELK.RIV PTRDNCLSED
At4G22160(AGD12)  TFSADDIMGE AEIDIQPLIT SAMAFGDPEM FGDMQIGKWL KSHDNALIED

                  βB          β7                β8          341
At3g17980(CAR)    SRVTWVDGKL VQDLVLRLRH VECGEVEAQL QWIDLPGSKG L
At1g70800(EHB1)   SIIVSDNGKI VQDMILLLKN VECGKVEIQL EWLKNPGGSG L
At4G22160(AGD12)  SIINIADGKV KQEVQIKLQN VESGELELEM EWLPLEQ... .
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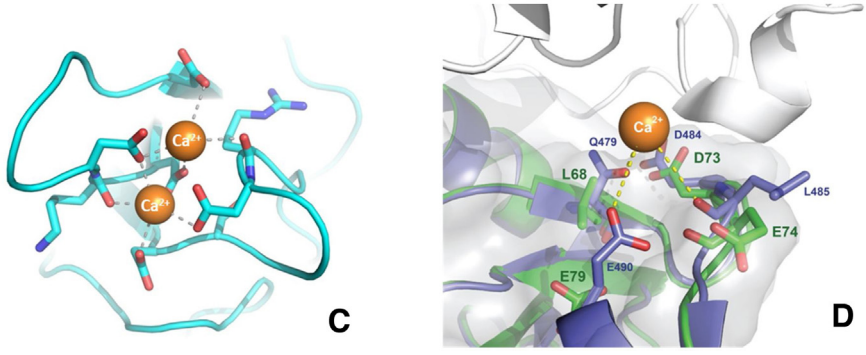

Fig. 1. (A) Domain structure of EHB1 (177 aa) and AGD12 (337 aa). Both proteins contain a calcium-dependent lipid binding domain (C2) with two loops for Ca^{2+}-binding (green). Both domains include a CAR element (in red) previously described for ABA-responsiveness (Rodriguez et al., 2014). EHB1 lacks entirely the N-terminal ARF-GAP domain (in yellow). (B) Sequence alignment of EHB1 and AGD12 compared to CAR4 (AT3G17980), a canonical CAR domain containing protein used by Rodriguez et al. for sequence comparison. Alignment was generated by using the Vector NTI software. Amino acids involved in Ca^{2+}-binding (Shao et al., 1996; Rizo and Sudhof, 1998) are highlighted in orange. (C) Structural models for Ca^{2+} bound state of the C2 domain and (D) of the ARF-GAP domain of AGD12 in comparison to ASAP3 (dark blue; Ismail et al., 2010). Homology models of EHB1 and AGD12 revealed each two putative calcium-binding sites for membrane binding and for AGD12 an additional pocket involved in Ca^{2+}-mediated ARF/ARF-GAP interaction. The AGD12 and EHB1 models (cyan in C, green in D) were generated by MODELLER9.4 using the structure of human ASAP3 (PDB code: 3LVQ/3LVR, seq. identity 45%) and Arabidopsis CAR4 (4V29, seq. identity 51%), respectively, as templates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)