

The response regulator 2 mediates ethylene signalling and hormone signal integration in *Arabidopsis*

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Hormones are important regulators of plant growth and development. In *Arabidopsis*, perception of the phytohormones ethylene and cytokinin is accomplished by a family of sensor histidine kinases including ethylene-resistant (ETR) 1 and cytokinin-response (CRE) 1. We identified the *Arabidopsis* response regulator 2 (ARR2) as a signalling component functioning downstream of ETR1 in ethylene signal transduction. Analyses of loss-of-function and ARR2-overexpressing lines as well as functional assays in protoplasts indicate an important role of ARR2 in mediating ethylene responses. Additional investigations indicate that an ETR1-initiated phosphorelay regulates the transcription factor activity of ARR2. This mechanism may create a novel signal transfer from endoplasmic reticulum-associated ETR1 to the nucleus for the regulation of ethylene-response genes. Furthermore, global expression profiling revealed a complex ARR2-involving two-component network that interferes with a multitude of different signalling pathways and thereby contributes to the highly integrated signal processing machinery in higher plants.

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Introduction

Hormones mediate the communication between cells and organs in plants and animals. In recent years, the molecular action of the phytohormones like ethylene and cytokinin has been intensively studied in plants (Kakimoto, 2003; Guo and Ecker, 2004). The gaseous molecule ethylene affects many aspects of the plant life cycle, including seed germination, abscission and fruit ripening (Guo and Ecker, 2004). Moreover, reactions of plants to biotic and abiotic stresses involve ethylene. Insights into the ethylene-response pathway have mainly arisen from molecular studies in the model plant *Arabidopsis thaliana* using genetic screens based on the 'triple response'. Several *etr*, *ers* and *ein* mutants have been identified in *Arabidopsis*, which exhibit a reduced or no triple response in the presence of ethylene or its biochemical precursor 1-aminocyclopropane-1-carboxylic acid (ACC). Cloning of the genes and characterization of mutant alleles have defined the framework of the signalling pathway leading from ethylene perception to changes in gene expression (Guo and Ecker, 2004). The first gene cloned was ETR1 (ethylene-resistant 1), which represents an endoplasmic reticulum (ER)-associated ethylene receptor with similarity to receptor histidine kinases of two-component signalling systems. Ethylene perception in *Arabidopsis* involves four additional ETR-related proteins. For all of them, binding of ethylene with similar affinity has been shown (Wang *et al.*, 2003). The next known downstream signalling component interacting with ETR1 and ethylene response sensor (ERS) 1 is CTR1. CTR1 encodes a Raf mitogen-activated protein (MAP) kinase kinase kinase and functions as a negative regulator of ethylene responses. Recent data indicate that CTR1 regulates a protein kinase cascade consisting of the MAP kinase kinase SIMKK and the MAP kinases MPK6 and MPK13 (Ouaked *et al.*, 2003). Therefore, sensor histidine kinase-like ethylene receptors link the perception of the hormone to the activity of a MAP kinase phosphorylation cascade. The integral membrane protein EIN2, which has similarity to Nramp metal ion transporters, is thought to function downstream of the CTR1-regulated protein kinase cascade (Ouaked *et al.*, 2003; Guo and Ecker, 2004). EIN2 regulates the activity of specific transcription factors by a yet unknown mechanism. Two families of ethylene-responsive transcription factors have been described: EIN3 and EIN3-like proteins and ethylene response element-binding proteins (EREBPs; Guo and Ecker, 2004). EIN3 and other members of the EIN3-like family bind to a conserved *cis*-regulatory element in the promoter of the EREBP-encoding gene *ethylene response factor (ERF) 1* (Solano *et al.*, 1998). As recently shown, ethylene regulates EIN3 activity by SCF^{EBF1/EBF2}-dependent proteolysis (Guo and Ecker, 2003; Potuschak *et al.*, 2003; Yanagisawa *et al.*, 2003). EIN3 activates transcription of the *ERF1* gene, and ERF1 in turn binds to a conserved *cis*-acting sequence within promoters of secondary target

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genes, which eventually mediate ethylene responses (Solano *et al*, 1998). However, although CTR1 and the other above-described proteins are central components in ethylene signal transduction, there are several indications for the existence of additional ethylene signalling pathways (reviewed in Hass *et al*, 2004).

The hormone cytokinin regulates physiological and developmental processes overlapping with those of ethylene. The cloning of the cytokinin receptor CRE1 (cytokinin-response 1) and its relatives the *Arabidopsis* histidine kinases 2 and 3 revealed that again a sensor histidine kinase family is involved in hormone perception (Kakimoto, 2003). Although the signal transduction cascade downstream of the cytokinin receptors awaits further elucidation, recent observations suggest that a two-component signalling circuit may mediate cytokinin signal transmission from the plasma membrane to the nucleus (Kakimoto, 2003; Hass *et al*, 2004). In addition to the receptors, this cytokinin-regulated circuit appears to comprise several response regulators including *Arabidopsis* response regulator (ARR) 1 and ARR2 (Hwang and Sheen, 2001; Sakai *et al*, 2001; Tajima *et al*, 2004).

A number of additional two-component elements have been discovered in plants within recent years. These include additional histidine kinases, histidine phosphotransfer (HPT) proteins and response regulators (Hwang *et al*, 2002). However, although the histidine kinase activity of ETR1 and of the cytokinin receptor CRE1 has been experimentally verified (Kakimoto, 2003; Hass *et al*, 2004), evidence for the precise mode of action of multistep phosphorelays in plant hormone responses remains elusive.

We have shown that ARR2 represents a transcriptional regulator of the GARP transcription factor family and binds to the 5'-GAT-3' sequence motif conserved in the promoters of nuclear genes coding for components of the *Arabidopsis* mitochondrial respiratory chain complex I (*nCI*; Lohrmann *et al*, 2001). Although preferentially found in pollen grains, ARR2 is expressed in all organs of the adult *Arabidopsis* plant as well as in seedlings (Lohrmann *et al*, 2001; Tajima *et al*, 2004). Whereas the C-terminal output domain of ARR2 mediates DNA binding and transactivation of target genes, the receiver module interacts with the *Arabidopsis* histidine phosphotransfer protein (AHP) 1 and AHP2 and contains the conserved aspartate residue (Asp80) predicted to be phosphorylated (Supplementary material; Sakai *et al*, 2000; Lohrmann *et al*, 2001). Hence, it was proposed that ARR2 constitutes a part of a two-component signalling pathway that in addition to the response regulator consists of at least one HPT protein and a hybrid sensor kinase (Lohrmann and Harter, 2002).

Here we describe the phenotypes of *Arabidopsis arr2* loss-of-function and ARR2-overexpressing lines. These data as well as results from functional analyses in protoplasts reveal that ARR2 contributes not only to cytokinin but also to ethylene signalling. Further analyses including biochemical approaches and genome-wide expression profiling suggest that an ETR1-dependent phosphorelay regulates the transactivation capacity of ARR2 thereby establishing a novel branch in ethylene signalling. Moreover, our data suggest that ARR2-including two-component signalling systems are responsible for fine-tuning and crosstalk of a multitude of signalling pathways in higher plants.

Results

Characterization of an *arr2* loss-of-function mutant from *Arabidopsis*

To elucidate the function of ARR2, we identified a *dissociation* (DS) transposon-tagged *Arabidopsis* line (SGT4387) in the IMA collection (Parinov *et al*, 1999). Characterization of this line revealed that the DS element is integrated in the ARR2 coding region at codon 19 in the first exon (Supplementary material). Southern blot and cosegregation analyses demonstrated the presence of a single transposon insertion in the genome of line SGT4387 and its linkage to the ARR2 locus. RT-PCR-based expression analysis revealed that the transposon insertion caused a complete absence of the ARR2 transcript defining this line as an *arr2* null mutant (Supplementary material).

Growth and development of the *arr2* mutant were monitored in comparison to the wild type. At early seedling stage, we did not observe major phenotypic differences. However, 20 days after sowing, the *arr2* mutant displayed retarded growth and development and early flowering (Figure 1A and data not shown). The *arr2* mutant phenotype was partially complemented by expression of the wild-type ARR2 cDNA under the control of the constitutive viral 35S promoter, indicating that the response regulator is responsible for the observed phenotype (Figure 1A). The incomplete complementation is very likely due to the 35S promoter, which may not perfectly establish the expression pattern of the native ARR2 promoter. The *arr2* mutant phenotype was only observed in homozygous *arr2* lines, indicating the recessive nature of the mutant allele. These analyses revealed that the *arr2* loss-of-function allele causes aberrations in growth and development of *Arabidopsis* plants, which could be due to an altered hormone homeostasis.

ARR2 also functions in ethylene signalling

We investigated in detail whether the *arr2* knockout mutant affects hormone signalling by analyzing the hypocotyl growth response in seedlings. As shown in Figure 1B, ACC- and cytokinin-treated *arr2* seedlings displayed a reduced response compared to wild type, whereas the application of other hormones did not affect growth. Dose-response analyses indicated that the observed altered hypocotyl elongation response of *arr2* seedlings is caused by a reduced sensitivity to ethylene and cytokinin (Figure 1C and D). The hyposensitive response of *arr2* seedlings to ethylene is comparable to that of the ethylene signalling mutant *ein3-1* and opposite to the weak hypersensitive reaction of the *etr1-7* loss-of-function receptor mutant (Figure 1E; Cancel and Larsen, 2002). To further elucidate the role of ARR2 in hormone signalling, we studied *arr2* loss-of-function seedlings that express the ARR2 cDNA under the control of the 35S promoter. As shown in Figure 1F and G, expression of ARR2 in the *arr2* mutant complemented the ethylene and cytokinin hyposensitive phenotype. These results demonstrate that the loss of the ARR2 gene product is responsible for the observed hormone hyposensitive phenotype and, furthermore, that ARR2 acts not only in cytokinin signal transduction (Hwang and Sheen, 2001) but also in ethylene signalling.

The *primary ethylene-responsive* (PER) element of the *ERF1* promoter harbors two GAT boxes that are essential for ethylene regulation of *ERF1* expression (Solano *et al*, 1998).

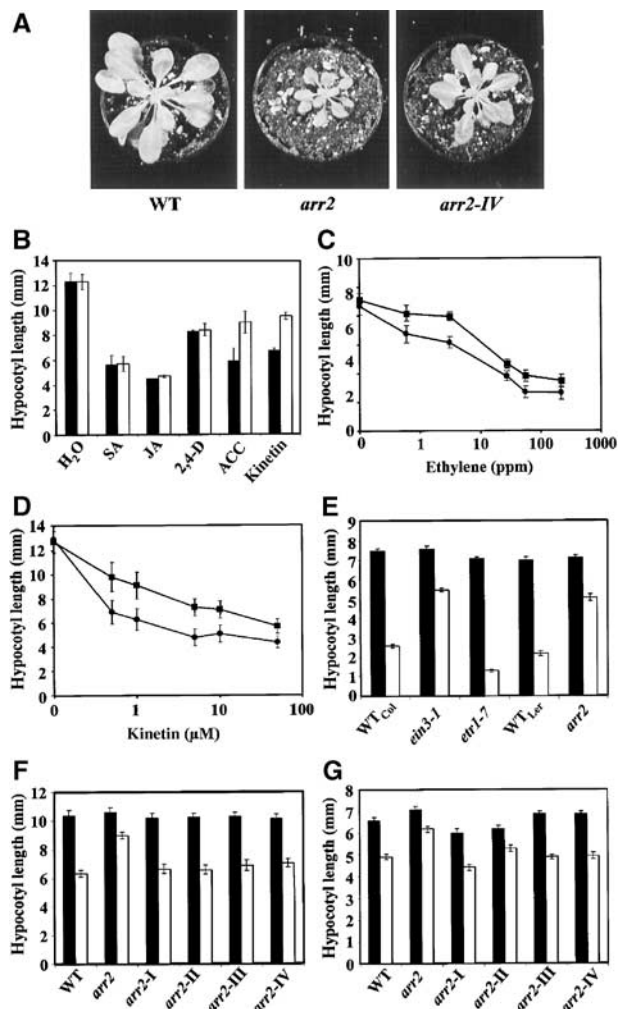


Figure 1 ARR2 is involved in ethylene and cytokinin signalling in *Arabidopsis*. (A) Wild-type and representative plants of homozygous *arr2* loss-of-function mutant (*arr2*) and ARR2 overexpressors (*arr2-IV*) 30 days after sowing. Bars, 2.5 cm. (B) Hypocotyl elongation of wild-type (black) and *arr2* (white) seedlings exposed to different plant hormones. Seedlings were grown on filter papers containing no hormone (control) or 1 mM salicylic acid (SA), 1 μ M jasmonic acid (JA), 1 μ M auxin (2,4-D), 10 μ M ACC or 0.5 μ M cytokinin (kinetin). (C) Dose dependence of ethylene-induced hypocotyl growth inhibition in wild-type (circles) and *arr2* (squares) seedlings. A 0 ppm concentration of ethylene means no gas supplied. (D) Dose dependence of cytokinin-induced hypocotyl growth inhibition in wild-type (circles) and *arr2* (squares) seedlings. Seedlings were grown in darkness on filter papers containing the indicated concentrations of kinetin. (E) Hypocotyl elongation of wild type (WT_{Col} , WT_{Ler}), the *ein3* mutant (*ein3-1*), the *etr1-7* mutant (*etr1-7*) and the *arr2* loss-of-function (*arr2*) in the absence (black) or presence of 10 μ M ACC (white). (F) Hypocotyl elongation of wild-type (WT), the *arr2* null mutant (*arr2*) and four independent ARR2-overexpressing lines in the *arr2* genetic background (*arr2-1* to *arr2-IV*) in the absence (black) or presence of 10 μ M ACC (white). (G) Hypocotyl elongation of wild-type (WT), the *arr2* null mutant (*arr2*) and four independent ARR2-overexpressing lines in the *arr2* loss-of-function background (*arr2-1* to *arr2-IV*) in the absence (black) or presence of 0.5 μ M kinetin (white).

As depicted in Figure 2A, ARR2 binds not only to the GAT box in the promoter of the *PSST* gene—a representative member of the *nCI* gene family serving as positive control—but also to the *PER* element of the *ERF1* promoter. To elucidate the participation of ARR2 in *ERF1* gene regulation, we used a leaf

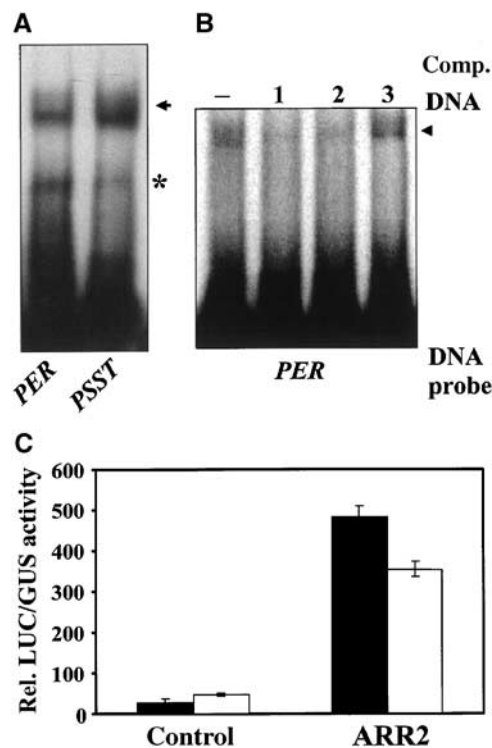


Figure 2 Transcriptional regulation of the ethylene-regulated *ERF1* gene by ARR2. (A) ARR2 binds to the conserved *PER* element of the *ERF1* promoter and to the *GAT* box of the *PSST* gene promoter *in vitro*. Gel-shift experiments were performed with the indicated radioactively labelled probes and recombinant ARR2. The arrow indicates the position of the ARR2:DNA complex. *, unspecific band. (B) Binding of ARR2 to the *PER* element is sequence specific. For competition experiments, a 50 \times molar excess of nonlabelled *PER* (lane 1), *PSST* (lane 2) element or DNA without a *GAT*-box motif (lane 3) was added to the binding reaction. (–), no competitor DNA. The arrow indicates the position of the ARR2:DNA complex. (C) Transcriptional regulation of *ERF1::LUC* by ARR2. *Arabidopsis* mesophyll protoplasts were cotransfected with *UBQ10::GUS* (internal standard), the *ERF1::LUC* reporter gene and an effector plasmid expressing HA-tagged ARR2. Nonfunctional GFP was used as a control. The transfected protoplasts were incubated without (black bars) or with 10 μ l/l ethylene (white bars).

cell assay on the basis of ethylene-regulated transcription in *Arabidopsis* mesophyll protoplasts (Hwang and Sheen, 2001). In transfected protoplasts, the activity of an *ERF1::LUC* reporter gene was very low and doubled by ethylene treatment (Figure 2B, control). Expression of ARR2 enhanced the activity of *ERF1::LUC* 20-fold. Treatment of protoplasts with ethylene in the presence of ARR2 reduced *ERF1::LUC* activity (Figure 2B). These data indicate that the response regulator ARR2 contributes to the regulation of *ERF1* gene expression in *Arabidopsis* protoplasts.

To further examine the role of ARR2, we carried out genome-wide expression profiling of the *arr2* null mutant compared to wild type. Total RNA from rosette tissue of four independently grown 30-day-old plants was combined, extracted and subjected to microarray analysis using the Affimetrix Genome Genechip representing about 26 370 *Arabidopsis* genes (Zhu, 2003, ArrayExpress accession numbers are A-MEXP-79 and E-MEXP-125). Around 370 of these genes showed a significant alteration of expression levels in the *arr2* null mutant compared to wild type (Table I and

Supplementary material). Among them, a major fraction is known to play a crucial role in responses of plants to phytohormones or is related to pathogen defense or reactions to stress (Table I and Supplementary material; Cheong *et al*, 2002; Lorenzo *et al*, 2003). The majority of these genes are downregulated in the *arr2* null mutant compared to wild type (Table I). However, we also observed a parallel up- and downregulation of genes within the same category (e.g. defense- and stress-related category; Table I). The microarray data were verified for a few selected genes by semiquantitative RT-PCR (Supplementary material). These results implicate a crucial role of ARR2 in the integration of plant signal transduction pathways.

ARR2 function depends on aspartate phosphorylation

Because ARR2 represents a response regulator, we considered that ARR2 activity may be regulated by phosphorylation. To investigate whether the conserved aspartate residue (Asp80) within the receiver domain of ARR2 may become phosphorylated in plants, a cell-free phosphorelay system on the basis of evacuated protoplasts (*AtEP*) from *Arabidopsis* mesophyll tissue was established (Figure 3A). These cells enabled the isolation of total extracts as well as of microsomal and cytosolic fractions as demonstrated by Western blot analyses using compartment-specific antisera (Figure 3B). For *in vitro* phosphorelay experiments, Strep-tagged ARR2 was incubated in the presence of [γ - 32 P]ATP either with microsomes, cytosol or both fractions. Then, ARR2 was recovered from the reaction mixture by affinity purification and analyzed for incorporation of radioactive phosphate. Phosphorylation of ARR2 was only detected when both the microsomal and cytosolic fractions were present in the reaction mixture (Figure 3C).

Mutation of the highly conserved phosphorylatable Asp to Glu within receiver domains of response regulators usually abolishes phosphorylation (Stock *et al*, 2000). To verify this for ARR2, the conserved Asp80 in the receiver domain was mutated to Glu generating ARR2^{D80E}. Whereas wild-type ARR2 was efficiently phosphorylated, a modification of ARR2^{D80E} was not observed (Figure 3D). These data show that the cell-free phosphorelay system from plant cells transfers a phosphoryl group from ATP to the receiver domain of ARR2. Furthermore, this system comprises at least two additional activities besides ARR2, which reside in two different subcellular compartments.

The membrane-associated phosphotransfer activity observed in the plant cell-free phosphorelay system very likely originates from sensor histidine kinases like ETR1. To investigate the linkage between ethylene perception and ARR2 function, we prepared protein fractions from *AtEP* of the ethylene receptor mutant *etr1-7* and of a transgenic *Arabidopsis* line expressing ETR1 under its own promoter in the *etr1-7* background ($P_{ETR1}::ETR1/etr1-7$; Gamble *et al*, 2002). *Etr1-7* is a loss-of-function allele of *ETR1* that arises from a stop codon at Trp74 (Hua and Meyerowitz, 1998). The protein fractions from evacuated protoplasts of *etr1-7*, $P_{ETR1}::ETR1/etr1-7$ and wild-type plants were supplemented with Strep-tagged ARR2 and [γ - 32 P]ATP, and phosphotransfer assays were performed as described above. Phosphorylation of ARR2 was strongly reduced in the presence of the *etr1-7* fraction as compared to wild type (Figure 3D). In contrast, if the extract from *AtEP* of $P_{ETR1}::ETR1/etr1-7$ plants was used, ARR2 phosphorylation was restored (Figure 3D). These

results indicate that ETR1 is an upstream regulator of ARR2 phosphorylation and is biochemically linked to the response regulator.

Asp80 phosphorylation is crucial for ARR2 function in vivo

To analyze the functional relevance of the Asp80-to-Glu mutation *in planta*, we generated wild-type and *arr2* null mutant plants expressing ARR2 or ARR2^{D80E} under the control of the constitutive viral 35S promoter (Figure 4A). Whereas plants carrying the ARR2 construct were almost indistinguishable from the corresponding wild type, expression of ARR2^{D80E} caused severe pleiotropic aberrations in growth and development (Figure 4B). These alterations included apparent disturbances in the primary vegetative meristem manifested in a disturbed rosette and an extremely aberrant leaf shape (Figure 4B and C). Moreover, around 50% of the primary ARR2^{D80E}-expressing transformants in wild-type background died before flowering. The ARR2^{D80E}-induced phenotype was even more severe in the *arr2* mutant background (Figure 4B and C), and surviving plants were infertile. Most remarkably, ARR2^{D80E}-overexpressing seedlings showed a triple response phenotype in the absence of ACC (Figure 4D). Further treatment with ACC could not enhance this reaction in ARR2^{D80E}-overexpressing plants, whereas wild-type seedlings displayed the expected ethylene-dependent phenotype (Figure 4D). In contrast to wild-type and the *ethylene overproduction 1 (eto1)* mutant (Chae *et al*, 2003) but comparable to *ctr1*, inhibition of ethylene biosynthesis by aminoethoxyvinyl glycine (AVG) could not significantly suppress the constitutive triple response, indicating that the phenotype seen in the ARR2^{D80E} overexpressor is not due to a strongly enhanced ethylene production (Figure 4D). These results implicate that ARR2^{D80E} (and ARR2) functions downstream of ethylene perception.

Subsequently, we performed an additional transcriptome analysis to elucidate the gene expression pattern giving rise to the phenotype induced by ARR2^{D80E}. Total RNA from four independently grown 30-day-old transgenic plants overexpressing to a similar extent either ARR2 or ARR2^{D80E} (Figure 4A) was hybridized to the whole genome exon GeneChip array. This analysis revealed a significant expression change in around 1.400 genes in the ARR2^{D80E} overexpressors compared to plants ectopically expressing wild-type ARR2. These included genes related to signal transduction of various phytohormones and light, responses to biotic and abiotic stress, protein degradation and folding, and development (Table II and Supplementary material). Remarkably, mRNA levels of several genes known to be induced by treatment of plants with auxin (e.g. Aux/IAA genes), cytokinin (e.g. type A ARR genes) and ethylene (e.g. EREBP genes) were up- or downregulated in ARR2^{D80E}-overexpressing plants (Table II and Supplementary material). The microarray data were verified for a few selected genes by semiquantitative RT-PCR (Supplementary material). These data indicate that the substitution of the conserved Asp80 to Glu creates a dominant-active form of ARR2 that causes plants to become partially independent of exogenously applied phytohormones. Taken together, our findings indicate that Asp80 phosphorylation of ARR2 apparently occurs in plants and plays an important role for the appropriate function of the response regulator.

Table 1 Differentially regulated genes in the *arr2* null mutant compared to wild type (selection)

AGI no.	Putative function	Fold change ^a
<i>ABA (abscisic acid)-related</i>		
AT4G24960	Abscisic acid-induced-like protein	-2.1
AT4G01600	ABA-responsive protein	2.4
AT5G15960	Cold- and ABA-inducible protein kin1	-2.2
<i>Abiotic stress-related</i>		
AT2G42540	Cold-regulated protein cor15a precursor	-3.0
AT3G12580	Heat shock protein 70	-5.8
AT3G47440	Aquaporin protein	-2.5
AT3G25760	ERD12	-8.5
AT3G46230	Heat shock protein 17	-2.3
AT4G30650	Low-temperature- and salt-responsive protein	-3.3
AT4G11650	Osmotin precursor	7.4
AT4G37220	Cold acclimation protein	2.7
AT4G21100	UV-damaged DNA-binding protein	-2.1
AT5G56010	Heat shock protein 90	-2.8
AT1G16410	Cytochrome P450	-3.2
AT2G22330	Cytochrome P450	-4.7
<i>Auxin-related</i>		
AT1G51760	IAA-Ala hydrolase (IAR3)	-3.2
AT1G78370	2,4-D-inducible glutathione S-transferase	-3.0
AT1G05670	Indole-3-acetate beta-glucosyltransferase	-3.0
AT4G15490	Indole-3-acetate beta-glucosyltransferase-like protein	-2.1
AT5G13370	Auxin-responsive-like protein	-3.9
<i>Ethylene-related</i>		
AT1G28370	Ethylene-responsive element-binding protein (EREBP)	-2.5
AT1G05010	ACC oxidase (ACO)	-2.8
AT2G44840	Ethylene-responsive element-binding protein (EREBP)	-3.4
AT2G31230	Ethylene-responsive element-binding protein (EREBP)	-2.7
AT3G24500	Ethylene-responsive transcriptional coactivator	-2.2
AT3G23240	Ethylene-responsive element-binding factor 1 (ERF1)	-2.0
AT3G23150	Ethylene receptor (ETR2)	-2.2
AT4G11280	ACC synthase (AtACS-6)	-2.8
<i>Defense-related</i>		
AT1G72900	Virus resistance protein	-5.8
AT1G80840	WRKY transcription factor	-5.1
AT1G61560	Mlo protein	-2.3
AT1G75380	Wound-responsive protein	2.2
AT1G73260	Trypsin inhibitor	6.8
AT2G46400	WRKY transcription factor	-5.8
AT2G29470	Glutathione S-transferase	3.0
AT2G14610	Pathogenesis-related PR-1-like protein	19.8
AT2G38470	WRKY transcription factor	-3.6
AT3G57240	beta-1,3-Glucanase	3.4
AT3G57260	beta-1,3-Glucanase 2 (PR-2)	4.7
AT3G04670	Elicitor response element-binding protein WRKY3 isolog	-2.5
AT3G04720	Hevein-like protein precursor (PR-4)	2.8
AT4G39410	WRKY transcription factor	-2.0
AT4G12880	Blue copper-binding protein	-2.7
AT5G13080	WRKY transcription factor	3.8
AT1G60270	beta-Glucosidase	-3.5
AT5G44420	Antifungal protein-like (PDF1.2)	3.5
<i>Jasmonate-related</i>		
AT1G17420	Lipoxygenase	-4.1
AT3G16470	Lectin, similar to jasmonate-inducible protein	-4.3
AT3G45140	Lipoxygenase AtLOX2	-2.8
<i>Phenylpropane biosynthesis-related</i>		
AT1G74100	Flavonol sulfotransferase	-3.7
AT1G74710	Isochorismate synthase (icsI)	-2.7
AT2G37040	Phenylalanine ammonia lyase (PAL1)	-3.6
AT2G38240	Anthocyanidin synthase	-6.5
AT3G21230	4-coumarate:CoA ligase 2	-2.8
AT3G53260	Phenylalanine ammonia-lyase	-5.5

^aAverage channel intensity ratio of *arr2* null mutant sample over wild-type sample.

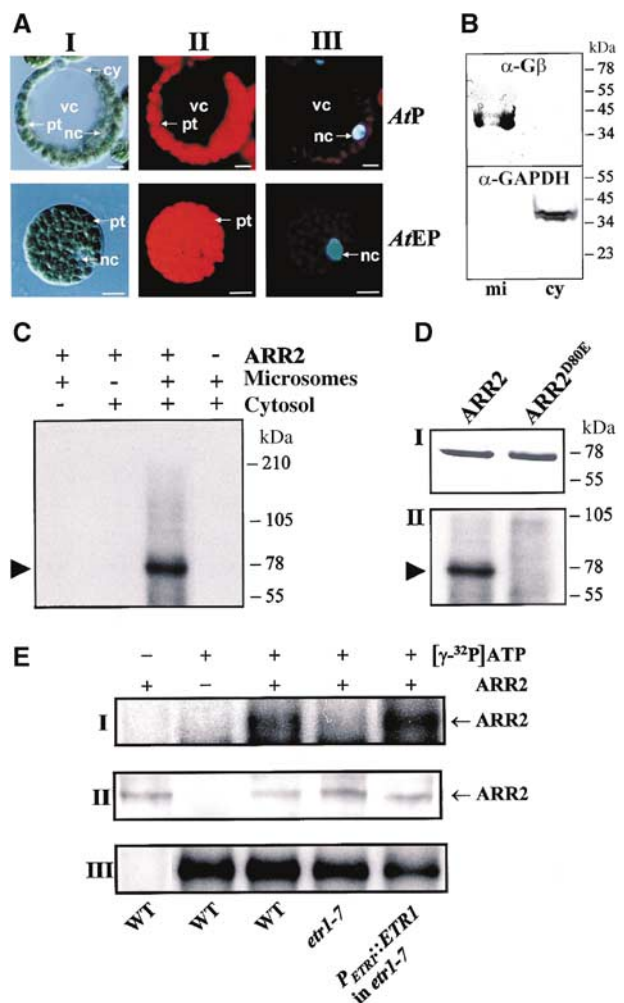


Figure 3 Phosphorylation of ARR2 by a plant cell-free phosphorylation system depends on Asp80 and the presence of ETR1. (A) Generation of evacuated protoplasts from mesophyll tissue of *Arabidopsis*. AtEP were generated from protoplasts (AtP) by high-speed centrifugation on Percoll gradients (I). Chlorophyll fluorescence of the chloroplasts (II) and DAPI staining of the nucleus (III) were analyzed by epifluorescence microscopy. cy, cytoplasm; nc, nucleus; vc, vacuole. Bars, 10 μm. (B) Western blot analysis of microsome (mi) and cytosolic (cy) fractions prepared from AtEP using compartment-specific antisera. α-Gβ, antiserum against membrane-associated β subunit of *Arabidopsis* G protein; α-GAPDH, antiserum against cytosolic glyceraldehyde-3-phosphate dehydrogenase. (C) Phosphorylation of ARR2 is mediated by microsomal and cytosolic activities. Strep-tagged ARR2 was incubated in a reaction mix containing [³²P]ATP and the indicated subcellular fractions. ARR2 was recovered from the mix by affinity purification and analyzed for incorporation of radioactively labelled phosphate by autoradiography. The arrow indicates the position of ARR2. (D) Phosphorylation of ARR2 targets Asp80. Equal amounts of Strep-tagged ARR2 and Strep-tagged ARR2^{D80E} (I) were analyzed for incorporation of radioactively labelled phosphate in the presence of microsomal and cytosolic fractions (II) as outlined above. The arrow indicates the position of ARR2. (E) ETR1 contributes to Asp80 phosphorylation of ARR2. Protein extracts from AtEP of wild type (WT), the *etr1-7* loss-of-function mutant (*etr1-7*) and the *etr1-7* mutant overexpressing ETR1 under the control of the *ETR1* promoter (*P_{ETR1}::ETR1* in *etr1-7*) were coincubated with Strep-tagged ARR2 (ARR2) and [³²P]ATP as indicated. After purification, phosphorylation of ARR2 was analyzed by autoradiography (panel I). An equal amount of ARR2 in the reaction mixture was tested by Western blot using an ARR2 antiserum (panel II). Nearly identical total phosphorylation activity is indicated in panel III, which shows a representative labelled protein in the extracts of evacuated protoplasts. Phosphorylation experiments were conducted at least twice with similar results.

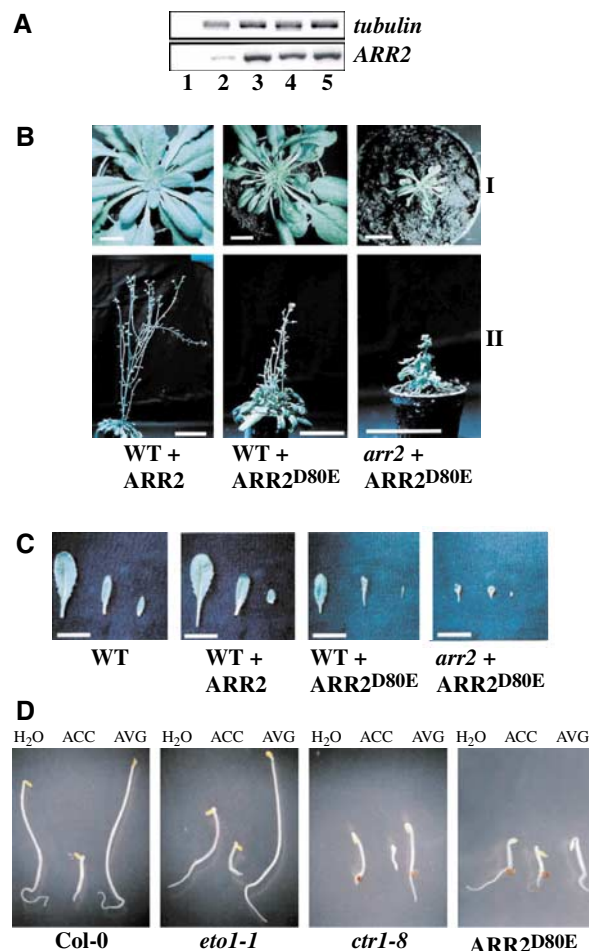


Figure 4 Phosphorylation of Asp80 is essential for appropriate function of ARR2 in plants. (A) ARR2, ARR2^{D80E} and tubulin transcript levels in seedlings from wild type (lane 2), ARR2 over-expressors in a wild-type background (lane 3) and ARR2^{D80E} over-expressors in a wild-type (lane 4) and *arr2* null mutant background (lane 5) as determined by RT-PCR. Lane 1, control PCR without reverse transcription. (B) Phenotypes of representative wild-type plants (WT) and the *arr2* null mutant (*arr2*) expressing either ARR2 (+ ARR2) or ARR2^{D80E} (+ ARR2^{D80E}) 50 days (I) and 70 days (II) after sowing. Bars, 1 cm in I and 5 cm in II. (C) Phenotypic comparison of representative leaves of different age from wild-type (WT) and *arr2* null mutant plants (*arr2*) expressing either ARR2 (+ ARR2) or ARR2^{D80E} (+ ARR2^{D80E}). Bars, 2 cm. (D) Phenotypes of 4-day-old dark-incubated wild-type (WT), *ctr1-8*, *eto1-1* and ARR2^{D80E}-overexpressing seedlings (WT+ARR2^{D80E}) grown in the absence (H₂O) or presence (+) of 50 μM ACC (ACC) or 10 μM AVG (AVG). Bars, 0.25 cm.

Phosphorylation modulates the transactivation capacity and stability of ARR2

The results presented in the previous sections and published data (Kakimoto, 2003) provide biochemical and functional evidence for a link of the histidine kinases ETR1 and CRE1 to ARR2 in mediating hormone signalling. Furthermore, Asp80 phosphorylation in the receiver domain of ARR2 is essential for its function. To determine how phosphorylation might regulate the activity of ARR2, we investigated whether the Asp80-to-Glu mutation affects its intracellular partitioning, DNA-binding transactivation capacity and stability. For the analysis of the intracellular distribution, we generated transgenic plants expressing ARR2-GFP and ARR2^{D80E}-GFP fusion

Table II Differentially regulated genes in ARR2^{D80E} compared to ARR2 overexpressors (selection)

AGI no.	Putative function	Fold change ^a
<i>Abscisic acid-related</i>		
AT4G26080	Protein phosphatase ABI1	2.2
AT5G13200	ABA-responsive protein	2.2
AT5G57050	Protein phosphatase ABI2	2.3
AT5G59220	ABA-induced protein phosphatase (PP2C)	6.4
<i>Abiotic stress-related</i>		
AT1G08830	Superoxidase dismutase	2.4
AT1G67970	Heat shock transcription factor (HSF)	2.7
AT2G17820	Histidine kinase (AtHK1)	2.2
AT2G46140	Desiccation-related protein	3.1
AT3G53800	Hsp70-binding protein HspBP1	-2.7
AT4G09350	Heat shock protein (HSP)	-3.0
AT4G15910	Drought-induced protein	2.1
AT4G16660	Heat shock protein (HSP)	2.9
AT4G18880	Heat shock transcription factor (HSF)	3.2
AT4G24190	Heat shock protein 90 (HSP90)	2.8
AT4G25100	Superoxide dismutase	-2.7
AT4G35970	Ascorbate peroxidase	-2.0
AT4G36680	Salt-inducible protein	2.3
AT4G36990	Heat shock transcription factor 4 (HSF4)	6.2
AT5G59610	Heat shock protein 40 (HSP40)	-3.1
AT5G05410	DREB2A	3.8
AT5G09590	Heat shock protein 70 (Hsc70-5)	2.4
AT5G49480	NaCl-inducible Ca ²⁺ -binding protein	2.4
AT5G52300	Low-temperature-induced 65 kDa protein	2.3
AT5G56010	Heat shock protein 90 (HSP90)	2.1
AT5G60950	Phytochelatin synthetase	3.5
<i>Auxin-related</i>		
AT1G04250	Auxin-induced protein	2.7
AT1G19840	Auxin-induced protein	3.6
AT1G23080	Auxin transport protein	-2.6
AT1G29440	Auxin-induced protein	-3.0
AT1G75580	Auxin-induced protein	3.9
AT1G77850	Auxin response factor	2.3
AT1G80390	Auxin-responsive protein IAA2	-3.6
AT2G21210	Auxin-regulated protein	-2.6
AT4G27260	GH3-like protein	2.8
AT4G34750	Auxin-regulated gene	3.3
AT4G34810	Small auxin up RNA (SAUR-AC1)	5.7
AT4G37390	Auxin-responsive GH3-like protein	5.0
AT4G38840	Auxin-induced protein	-2.9
AT4G38860	Auxin-induced protein	-2.4
AT5G13320	Auxin-responsive protein	6.2
AT5G18010	Auxin-induced protein	-3.2
AT5G18050	Auxin-induced protein	-4.5
AT5G18080	Auxin-induced protein	-3.2
<i>Cytokinin-related</i>		
AT1G19050	Two-component response regulator (ARR7)	2.0
AT1G74890	Two-component response regulator (ARR15)	3.8
AT2G07440	Two-component response regulator (ARR24)	2.6
AT2G40670	Two-component response regulator (ARR16)	10.7
AT2G41310	Two-component response regulator (ARR8)	2.4
AT3G48100	Two-component response regulator (ARR5)	9.2
AT3G56380	Two-component response regulator (ARR4)	6.0
AT5G56970	Cytokinin oxidase	14.1
<i>Defense-related</i>		
AT1G05760	Disease resistance protein RTM1	-2.2
AT1G05850	Class I chitinase	-3.3
AT1G53290	Avr9 elicitor response protein-like	-2.2
AT1G65390	Disease resistance protein RPS4	-4.5
AT1G72230	Blue copper protein	-2.2
AT1G72920	Virus resistance protein	2.0
AT1G72930	Flax rust resistance protein	3.3
AT1G73260	Trypsin inhibitor	13.2
AT1G75280	NADPH oxidoreductase	-2.3
AT2G21900	WRKY-type DNA-binding protein	3.1
AT2G26010	Antifungal protein	-2.6
AT2G33050	Leucine-rich repeat disease resistance protein	-2.2
AT2G40000	Nematode resistance protein	2.0

Table II (continued)

AGI no.	Putative function	Fold change ^a
AT2G40740	WRKY-type DNA-binding protein	3.2
AT2G43570	Endochitinase	13.4
AT2G46400	WRKY-type DNA-binding protein	5.4
AT3G01970	WRKY-type DNA-binding protein	9.0
AT3G48720	Hypersensitivity-related hsr201 protein	-2.9
AT3G55470	Elicitor-responsive protein (FIERG2)	2.6
AT3G56400	WRKY-type DNA-binding protein 4 (WRKY4)	4.0
AT4G13920	Disease resistance Cf-2-like protein	2.2
AT4G19530	TMV resistance protein N-like	-2.4
AT5G13080	WRKY-type DNA-binding protein	6.1
AT5G14930	Disease resistance protein EDS1	3.0
AT5G26170	WRKY-type DNA-binding protein 1 (WRKY1)	3.3
AT5G44420	Antifungal protein (PDF1.2)	-2.6
AT5G45110	Regulatory protein NPR1-like	2.1
AT5G46350	WRKY-type DNA-binding protein	2.7
AT5G62740	Hypersensitive-induced response protein HIR3	3.2
<i>Degradation-related</i>		
AT1G20140	SKP1 ASK1 (At4)	3.1
AT1G23410	Ubiquitin extension protein	2.9
AT1G68050	F-box protein (FKF1)	2.3
AT1G77000	F-box protein (AtFBL5)	3.3
AT2G20160	SKP1 ASK1 (At17)	6.7
AT3G17000	E2 ubiquitin-conjugating enzyme	2.1
AT3G54650	F-box protein (AtFBL17)	-2.3
AT4G38930	Ubiquitin fusion-degradation protein	2.5
AT5G24810	Ubiquitin biosynthesis EIN AARF	2.7
AT5G27920	F-box protein	2.3
AT5G55170	Ubiquitin	2.7
AT5G57480	AAA-type ATPase	4.7
AT5G57900	SKP1 interacting partner 1 (SKIP1)	2.0
<i>Development-related</i>		
AT1G01010	NAC domain protein	17.1
AT1G02220	NAM (no apical meristem) protein	9.7
AT1G02250	NAM protein	11.4
AT1G52690	Late embryogenesis-abundant protein	8.9
AT1G52890	NAM protein	7.2
AT1G77450	GRAB1-like protein	2.1
AT2G22850	Embryo-abundant protein	-2.6
AT4G00180	YABBY3 axial regulator	-2.1
AT5G06760	Late embryogenesis-abundant protein	4.7
AT5G07190	Embryo-specific protein 3 (ATS3)	-2.1
AT5G20240	PISTILLATA-like	3.9
AT5G39610	NAM protein	2.6
<i>Ethylene-related</i>		
AT3G12500	Basic chitinase	2.7
AT3G16050	Ethylene-inducible protein	2.5
AT3G23150	Ethylene receptor (ETR2)	2.4
AT3G23240	Ethylene response factor 1 (ERF1)	3.9
AT3G50260	EREBP-3-like	2.9
AT4G23340	ACC oxidase	-2.6
AT4G26200	ACC synthase	2.4
AT5G20400	Ethylene-forming enzyme dioxygenase	2.9
AT5G43410	EREBP-3-like	-3.1
<i>Folding-related</i>		
AT1G06330	Copper chaperone	-13.9
AT1G21750	Protein disulfide isomerase	3.8
AT1G23100	Chaperonin	2.8
AT2G47470	Disulfide isomerase	2.2
AT3G18190	Chaperonin	2.0
AT3G56070	Peptidyl-prolyl isomerase	3.0
AT5G45680	FKBP-type peptidyl-prolyl isomerase	-2.7
AT5G55220	Trigger factor	-2.4
<i>Gibberellin-related</i>		
AT1G22690	GAST1-like protein	-4.5
AT1G74670	GAST1-like protein	-2.4
<i>Jasmonate-related</i>		
AT1G55020	Lipoxygenase	8.8

Table II (continued)

AGI no.	Putative function	Fold change ^a
AT3G16400	Jasmonate-inducible protein	6.0
AT3G16420	Jasmonate-inducible protein (lectin)	5.5
AT3G16430	Jasmonate-inducible protein	5.1
AT3G16450	Jasmonate-inducible protein (lectin)	31.0
AT3G16460	Jasmonate-inducible protein (lectin)	11.4
AT5G48180)	Jasmonate-inducible protein	3.0
<i>Light-related</i>		
AT1G06680	OEC protein (23 kDa)	-2.1
AT1G14280	Phytochrome kinase substrate 1	-3.4
AT1G19150	Chlorophyll <i>a b</i> -binding protein (LHCP)	-4.0
AT1G51890	Light-repressible receptor protein kinase	2.6
AT3G15570	Nonphototropic hypocotyl protein	-2.1
AT4G14110	COP9	3.0
AT5G11260	HY5	2.3
AT5G54190	Protochlorophyllide oxidoreductase	-2.9
AT5G58140	Nonphototropic hypocotyl 1-like	-2.4

^aAverage channel intensity ratio of ARR2^{D80E} overexpressors over ARR2 overexpressors.

constructs. These plants displayed the same phenotype as described for ARR2- and ARR2^{D80E}-expressing plants, respectively, indicating that the GFP fusion proteins are present and functional *in vivo* (Figure 5A). Epifluorescence images of hypocotyl cells from these plants revealed that ARR2-GFP as well as ARR2^{D80E}-GFP is exclusively localized to the nucleus (Figure 5B).

South-Western analyses were performed to investigate a possible phosphorylation dependence of the ARR2 DNA-binding activity. Strep-tagged ARR2 and ARR2^{D80E} were incubated with ATP in the cell-free phosphorelay system derived from *AtEP*, affinity purified from the reaction mixture and dotted onto PVDF membranes. Nonphosphorylated but otherwise equally treated ARR2 and, as a control, ATP-treated BSA were dotted onto the membranes as well. The membranes were incubated with either a radioactively labelled *PSST* or *PER* promoter fragment and afterwards subjected to autoradiography. Independent of whether phosphorylated or nonphosphorylated ARR2 were present on the membrane, no differences in DNA binding were detected (Figure 5C). We also did not observe an altered affinity of dominant-active, nonphosphorylatable ARR2^{D80E} to the *PSST* and *PER* promoter fragments, indicating that regulation of DNA-binding capacity by phosphorylation plays a minor role in ARR2 regulation.

Whether Asp80 phosphorylation interferes with the transactivation capacity of ARR2 was addressed in protoplasts using the ethylene-regulated *ERF1::LUC* as reporter gene. As already presented in Figure 2C, expression of wild-type ARR2 in *Arabidopsis* mesophyll protoplasts resulted in basal expression of *ERF1::LUC*. Application of ethylene inhibited the activity of *ERF1::LUC* (Figures 2C and 5D). When ARR2^{D80E} was present, we observed a strong repression of *ERF1::LUC* gene in the absence of ethylene (Figure 5D). Treatment of protoplasts with ethylene further reduced *ERF1::LUC* expression (Figure 5D).

To elucidate whether protein stability may play a role in the regulation of *ERF1::LUC* activity, we determined ARR2 and ARR2^{D80E} levels in the protoplasts. As shown in Figure 4E, the amounts of ARR2 and ARR2^{D80E} decreased within the time period of 4 h with ARR2^{D80E} appearing to be slightly less stable than ARR2 (Figure 5E). Ethylene treatment

of protoplasts expressing ARR2 did not alter the destruction rate, whereas ARR2^{D80E} seems to be less stable under these conditions.

As shown recently, ARR2 induces *ARR6::LUC* reporter gene expression in protoplasts in a cytokinin-dependent way (Supplementary material; Hwang and Sheen, 2001). Remarkably, protoplasts expressing ARR2^{D80E} displayed *ARR6::LUC* activity, which was as high as in cytokinin-treated, ARR2-expressing cells. Exposure of ARR2^{D80E}-expressing protoplasts to cytokinin further enhanced *ARR6::LUC* activity (Supplementary material).

Taken into account that the DNA-binding activity and the intracellular distribution of ARR2 remained unaltered, these results indicate that phosphorylation of Asp80 regulates the transcriptional capacity and/or the stability of the response regulator.

Discussion

ARR2 functions as a transcriptional regulator in cytokinin signalling (Hwang and Sheen, 2001; Sakai *et al*, 2001) and binds to the GAT-box motif within the promoters of *nCI* genes (Lohrmann *et al*, 2001). Here we show that ARR2 is also able to bind to the *PER* promoter element of the *ERF1* gene and mediates upregulation of *ERF1::LUC* gene activity in *Arabidopsis* protoplasts. Application of exogenous ethylene results in repression of *ERF1::LUC* in protoplasts. Because of *ERF1* being an ethylene-triggered gene (Solano *et al*, 1998; Lorenzo *et al*, 2003), these data indicate that in *Arabidopsis* mesophyll protoplasts ARR2 negatively interferes with ethylene signal transduction. To further elucidate the contribution of ARR2 in ethylene signalling, we analyzed an *Arabidopsis arr2* loss-of-function mutant in which the expression of the response regulator is abolished. In seedlings, the *arr2* loss-of-function mutant renders plants less sensitive to cytokinin and ethylene. Furthermore, the overexpression of dominant-active ARR2^{D80E} induces a triple response-like phenotype in the absence of ethylene and in the presence of the ethylene biosynthesis inhibitor AVG. Again, these results urge that ARR2 contributes to ethylene signalling in *Arabidopsis*. However, whereas ARR2 is a negative regulator of ethylene signalling in protoplasts, the hyposensitive hypocotyl growth

phenotype of the recessive *arr2* loss-of-function mutant implicates that ARR2 positively contributes to this response in seedlings. This effect becomes also visible when the activity of ARR2^{D80E} is compared in protoplasts and transgenic plants. In protoplasts ARR2^{D80E} represses *ERF1::LUC* activity, while in ARR2^{D80E} overexpressors *ERF1* transcript accumulation is elevated. Furthermore, ARR2 and ARR2^{D80E} positively regulate the activity of *ARR6::LUC* in both protoplasts and transgenic plants. These data suggest that the way and direction (negative versus positive) of ARR2 action seem to depend on tissue-, cell- and even development-specific coacting factors that contribute to the activity and specificity of ARR2 function. In *Arabidopsis* protoplasts, which already produce ethylene and are responsive to the hormone (Yanagisawa *et al*, 2003), an additional exogenous supply in the presence of ARR2 may induce a negative feedback regulation. At the molecular level, this could result in inhibition of ARR2 transcription factor activity by an interacting repressor. Likewise, ARR2^{D80E} appears to be less stable in

protoplasts compared to wild-type ARR2. This instability may contribute to the reduction of the *ERF1::LUC* activity and adds further complexity to the regulation of ARR2.

We established a plant cell-free phosphorelay system that implements a phosphotransfer from ATP to the conserved Asp80 in the receiver domain of ARR2. The microsomal activity for ARR2 phosphorylation derives from membrane-associated histidine kinases. The necessity for a soluble component may reflect the participation of HPt proteins in ARR2 modification. The cell-free phosphorelay system enabled us to investigate the functional interplay of ARR2 and ETR1. Compared to wild type, subcellular fractions from the *etr1-7* mutant are significantly less efficient in phosphorylating ARR2. In extracts from *etr1-7* plants expressing wild-type ETR1, phosphorylation of ARR2 was restored. These data indicate that the *in vitro* transfer of the phosphoryl group from ATP to the Asp80 of ARR2 depends to a certain extent on ETR1. These results not only suggest that ARR2 is involved in ethylene signalling, but also that the underlying mechanism is a two-component phosphorelay.

Our experiments suggest that the mutation of Asp80 to a Glu residue creates a constitutively active form of ARR2 (ARR2^{D80E}). This finding is in agreement with observations in prokaryotic systems, where Asp-to-Glu mutations in the receiver domain of response regulators often generate constitutive active forms (Stock *et al*, 2000). Moreover, ARR2^{D80E} expression also causes major phenotypic aberrations in transgenic plants. These results implicate that Asp phosphorylation of response regulators like ARR2 and, hence, phosphorelays play a crucial role in hormone signalling in plants.

Until now it has been assumed that ethylene signal transduction downstream of the receptors exclusively depends on CTR1 (Huang *et al*, 2003; Guo and Ecker, 2004). However, *ctr1* null mutants still display residual ethylene responsiveness and an incomplete activation of ethylene-triggered responses (Guo and Ecker, 2003; Potuschak *et al*, 2003), suggesting the existence of an additional branch for ethylene

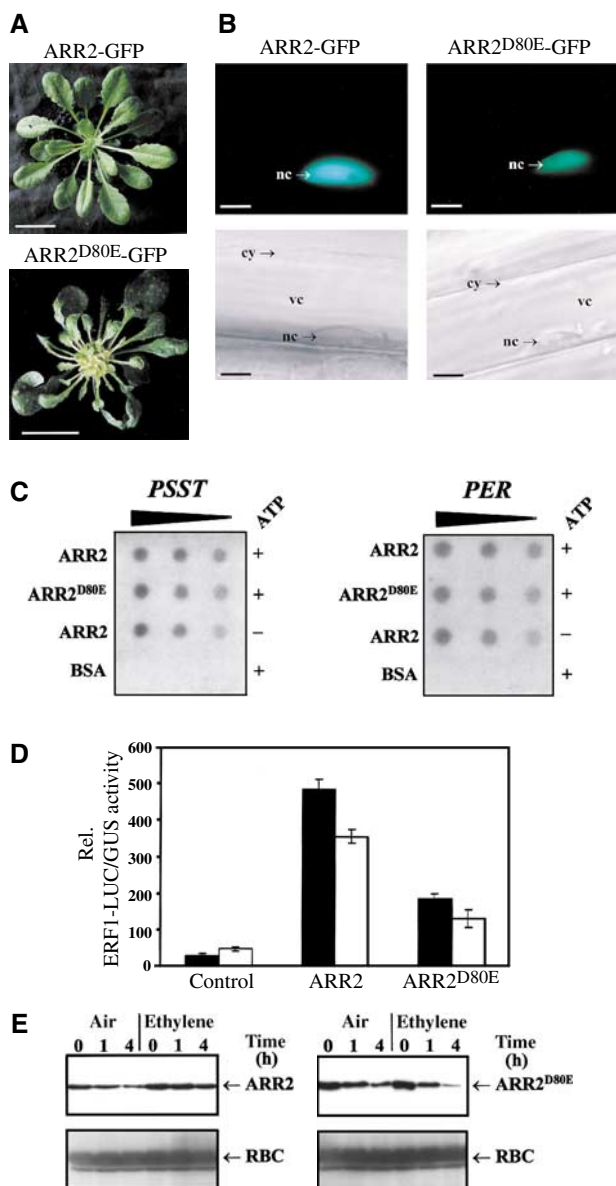


Figure 5 Asp80-to-Glu mutation regulates the transactivation capacity of ARR2. (A) Representative 40-day-old transgenic *Arabidopsis* plants (wild-type background) expressing either ARR2-GFP (left) or ARR2^{D80E}-GFP (right). Bars, 2.5 cm. (B) ARR2-GFP and ARR2^{D80E}-GFP are both localized to the nucleus. Presented are epifluorescence and bright-field images of hypocotyl cells from the transgenic plants described in (A). cy, cytoplasm; nc, nucleus; vc, vacuole. Bars, 10 μ m. (C) *In vitro* phosphorylated and nonphosphorylated ARR2 and ARR2^{D80E} display identical DNA-binding activities. Recombinant Strep-tagged ARR2 and ARR2^{D80E} were treated in the cell-free phosphorelay system in the presence (+ATP) or absence of ATP (-ATP). After recovery by affinity purification, decreasing amounts of ARR2, ARR2^{D80E} and, as a control, BSA were dotted onto PVDF membranes. The membranes were incubated with radioactive PSST and PER promoter fragments. Binding to DNA was analyzed by autoradiography. (D) Transactivation capacity of ARR2 is phosphorylation dependent. Protoplasts were transfected with *UBQ10::GUS* (internal standard) and the *ERF1::LUC* reporter construct and effector plasmids expressing either ARR2 or ARR2^{D80E} (both HA tagged). Nonfunctional GFP was used as a control. The transfected protoplasts were incubated without hormone (black bars) or with ethylene (10 μ l/l, upper panel, white bars). (E) Kinetics of ARR2 and ARR2^{D80E} protein levels in *Arabidopsis* mesophyll protoplasts in the absence (air) or presence of ethylene (ethylene). The first sample (time 0 h) was harvested 30 min after protoplast transformation. ARR2 and ARR2^{D80E} were detected using an HA-tag-specific antibody. RUBISCO (RBC) served as a control and was detected with a specific antiserum.

signalling. Based on the evidence presented here, it is conceivable that a two-component signalling system represents such a branch. In this branch, ethylene binding may induce histidine kinase activity and autophosphorylation of ETR1. Phospho-ETR1 then could initiate a phosphorelay cascade that may include a shuttling HPt protein. The phosphotransfer could finally result in the transcriptional regulation of ARR2 inside the nucleus (Lohrmann and Harter, 2002). Thus, ETR1 may have a dual functional role in the initiation of ethylene signal transduction: whereas the CTR1-dependent pathway is negatively regulated (Guo and Ecker, 2004), the ARR2-dependent pathway is subject to positive regulation. The proposed phosphorelay is difficult to reconcile with observations that histidine kinase activity of ETR1 and ERS1 may not be required to mediate ethylene signalling (Guo and Ecker, 2004). However, it is of interest that the ethylene receptors with proven (ETR1) or proposed (ERS1) histidine kinase activity play a predominant role in the regulation of ethylene responses (Guo and Ecker, 2004). This relative importance of ETR1 and ERS1 could potentially be because of the presence of histidine kinase activity. Furthermore, it is impossible to observe an ARR2-dependent two-component mechanism in all of those ETR1 and ERS1 mutant plants, in which the receptors are present in a form that abolishes transphosphorylation capacity. Intramolecular transphosphorylation of the transmitter domains is a prerequisite for histidine kinases to initiate phosphorelay mechanisms (Stock *et al*, 2000; Ames *et al*, 2002). Thus, further biochemical and genetic experiments have to be carried out to clarify the relationship of the CTR1- and ARR2-dependent ethylene signal transduction pathways.

Genechip experiments were performed to investigate the expression network influenced by ARR2. We first compared the expression pattern of the *arr2* null mutant with the corresponding wild type. Besides changes in the expression of genes related to ethylene signalling, we also observed alterations in the activity of genes associated with auxin, abscisic acid, jasmonic acid and gibberellin signal transduction. Although these genes may not be the primary targets of ARR2, their altered expression may explain the complex mutant phenotype of the *arr2* loss-of-function plants that is different from those of plants solely impaired in ethylene (and cytokinin) perception. As a consequence of impaired hormone balance, genes related to defense and abiotic stress signalling and adaptation show altered expression as well (Zhong and Burns, 2003). Remarkably, significant changes in the expression of typical cytokinin-responsive genes like those encoding A-type response regulators (Rashotte *et al*, 2003) were not observed in the *arr2* loss-of-function mutant. Obviously, in plants that have not been treated with exogenous cytokinin, absence of ARR2 does not cause significant alterations in the expression of early cytokinin-induced genes. Thus, endogenous variations in cytokinin levels may either induce slight, nondetectable changes in the accumulation of these cytokinin-induced genes or cytokinin signalling occurs by post-transcriptional mechanisms. Upregulation of *type-A* ARR genes in response to exogenous cytokinin may therefore reflect an adaptation process rather than a primary signalling event.

We also performed a comparative expression profiling with RNA from plants that express either wild-type ARR2 or ARR2^{D80E}. The strongly altered expression of a variety of

hormone-regulated genes indicates that the dominant-active ARR2^{D80E} protein affects the entire two-component signalling network of *Arabidopsis*. As a consequence, the hormone-sustained homeostasis of gene expression pattern seems to be completely disturbed in ARR2^{D80E}-expressing plants. This fundamental change in hormone homeostasis induces expression alterations of a multitude of genes related to many different signalling pathways. This also explains why the ARR2^{D80E}-induced phenotype is distinct from the phenotype of the *arr2* loss-of-function mutant. In conclusion, our expression studies suggest that the two-component network may directly or indirectly modulate many signal transduction pathways initiated by phytohormones and other endogenous and exogenous stimuli. An independent example for a modulating action of two-component systems is the stabilization of the active form of the phytochrome B photoreceptor by the type-A response regulator ARR4 (Sweere *et al*, 2001; To *et al*, 2004). In summary, these data suggest that two-component signalling systems may not always represent the primary mechanisms for signal transduction pathways but rather may establish a complex network that is predominantly responsible for maintaining fine-tuning and crosstalk of signalling pathways.

Materials and methods

Characterization of the *arr2* insertion mutant

DNA and RNA manipulations and RT-PCR were performed as described previously (Lohrmann *et al*, 2001). The transposon insertion site within the *ARR2* gene was verified by PCR using the DS-specific primers DS3' and DS5' (Parinov *et al*, 1999) and two *ARR2*-specific primers followed by sequencing of the PCR product.

Arabidopsis plants were grown for the indicated time periods on soil under short-day conditions (8 h light/16 h dark). Hormone treatments were performed as follows: seedlings were grown for 96 h on moisturized filter paper and treated with the indicated concentrations of hormones. For ethylene analyses, seedlings were germinated for 24 h on moisturized filter paper in open glass vessels. The vessels were closed and ethylene was injected. Seedlings were further grown for 72 h and ethylene concentration in the airspace was measured at the end of the 72 h growth period by gas chromatography. Ethylene gas was applied to protoplasts at a concentration of 10 μ l/l for 9 h beginning 30 min after transfection. Air was applied as control. Physiological data are presented as the mean and standard deviation of at least 50 seedlings. Dose-response analyses were repeated at least three times and the means and the standard error of the mean are shown.

Plasmid construction and expression of recombinant proteins

For construction of binary constructs, wild-type ARR2 cDNA was cloned into the pPCV812 derivative pPCVB downstream of the (2 \times 35S) promoter and upstream of the *mGFP4* gene. For expression of ARR2 without C-terminal GFP tag, a stop codon was introduced in front of the *mGFP4* gene. The *ERF1::LUC* reporter construct was generated by fusing a 2.1 kbp 5'-flanking region of the *ERF1* gene (At3g23240) to the firefly luciferase cDNA. The *ARR6::LUC* construct has been described previously (Hwang and Sheen, 2001). Site-directed mutagenesis of Asp80 to Glu was performed using the QuickChangeTM XL Mutagenesis System (Stratagene). PCR-generated cDNAs were verified by sequencing. Expression of Strep-tagged ARR2 and ARR2^{D80E} in *Escherichia coli* and affinity purification on StrepTactin (IBA) were performed according to Lohrmann *et al* (2001).

Expression profiling

Total RNA from rosettes of four independently grown 30-day-old *Arabidopsis* plants was extracted using Qiagen RNeasy columns. Total RNA (5 μ g) was used to synthesize cDNAs. Labelled cRNA, synthesized from the cDNA, was hybridized to the *Arabidopsis* whole genome exon GeneChip array according to the procedure

described previously (Zhu *et al*, 2001). The custom *Arabidopsis* whole genome array used contains 42 Affymetrix control probe sets, three transgene control probe sets, four QC probe sets and 26 412 probe sets representing 26 367 *Arabidopsis* genes. On average, each gene contains 15 perfect match probes. These 25-mer oligonucleotide probes were selected from the 3' end of 133 397 exons (Zhu, 2003). The hybridization signal of each probe set was quantified using the value of weighted average of all probes in a set, subtracting bottom 5% of average intensity of the entire array. The overall intensity of all probe sets of each array was further scaled to a target intensity of 100, so hybridization intensity of all arrays was equivalent. Genes corresponding to probe sets with a minimum detected expression level of 50 and a minimum change of 2.0-fold compared to wild type were selected and defined as genes with significant alteration of expression. The significant expression level threshold of 50 was determined based on the 100% above noise level from all negative controls on the microarray according to the determined scaling target 100. The reproducibility of the microarray was characterized by comparing data generated in parallel from 10 pairs of total RNA samples. The detection sensibility and specificity of the GeneChip microarray were characterized by spiking in a serial dilution of equimolar concentration of negative control transcripts from BioB, BioC and CreX to 10 µg *Arabidopsis* cRNAs and hybridizing to the 26K *Arabidopsis* whole genome array. The data are available at ArrayExpress with the accession numbers A-MEXP-79 for the array design and E-MEXP-125 for the experiment.

Generation of evacuated protoplasts, cell fractionation and immunoblotting and transformation techniques

Protoplasts preparation, evacuation of protoplasts, protein extraction and fractionation were carried out as described (Harter *et al*, 1994). The fractions were characterized by immunoblotting with antibodies against cytoplasmic GAPDH and the β subunit of plant G proteins (Obrdlik *et al*, 2000). Transformation of *Arabidopsis* plants was conducted according to Lohrmann *et al* (2001). Transfection of *Arabidopsis* mesophyll protoplasts has been described previously (Hwang and Sheen, 2001). Microscopic work was carried out as reported by Lohrmann *et al* (2001).

In vitro phosphotransfer assays

A 2 µg portion of recombinant Strep-tagged ARR2 was incubated with 5 µg of total extract, 1.5 µg of microsomal fraction or 4 µg of

cytosolic fraction, and 20 µCi [γ -³²P]ATP for 60 min at 4°C in a total volume of 75 µl standard phosphorylation buffer (50 mM Tris-HCl, 0.5 mM EDTA, 2 mM DTT, 50 mM KCl, 5 mM MgCl₂, 5 mM MnCl₂, 10% glycerol). The protein was recovered from the reaction mixture by affinity purification on StrepTactin beads (IBA). After washing three times with 0.5 ml of washing buffer (100 mM Tris-HCl pH 7.4, 150 mM NaCl), the ARR2 proteins were released from the beads by addition of 50 µl of washing buffer containing 2.5 mM desthiobiotine. The ARR2 proteins were subjected to SDS-PAGE, autoradiography and Western blot as described by Harter *et al* (1994).

EMSA and South-Western analysis

³²P-labelling of the *PSST* (Lohrmann *et al*, 2001) and *PER* DNA probe (nucleotides -1213 to -1178 of the *ERF1* promoter), EMSA and competition experiments were carried out as described previously (Lohrmann *et al*, 2001). For South-Western analyses, a dilution series (1:1, 1:2, 1:4) starting from 60 µl of affinity-purified, phosphorylated or control-treated ARR2 proteins was generated in 60 µl of washing buffer. The protein solutions were dotted on PVDF membranes. As a control, BSA (0.5, 1.0, 2.0 µg per dot) was dotted as well. The membrane was prehybridized for 2 h at 4°C in hybridization buffer (gel-shift binding buffer (Lohrmann *et al*, 2001) supplemented with 0.25% (w/v) BSA, 5 µg/ml salmon sperm DNA). Hybridization in 2 ml of hybridization buffer containing 0.4 ml of radioactively labelled *PER* or *PSST* promoter element was performed overnight at room temperature. Afterwards, the membrane was washed several times in hybridization buffer, dried and exposed to X-ray film (Kodak).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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