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Addendum

Acquiring competence for shoot development in Arabidopsis

ARR2 directly targets A-type ARR genes that are differentially activated by CIM preincubation

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Shoots can be regenerated from roots in Arabidopsis by treating root explants with cytokinin, however, shoot regeneration requires preincubation on callus induction medium (CIM) prior to induction on cytokinin-rich shoot induction medium (SIM). A cytokinin-inducible marker gene, *RESPONSE REGULATOR 15* (*ARR15*), was identified through a "CIM dropout experiment" with similar requirements for CIM preincubation. The requirements for *ARR15* contrasted to *ARR5*, another cytokinin-inducible ARR gene that does not require CIM preincubation. We show here that despite their differences, both *ARR5* and *ARR15* are direct targets of the transcriptional B-type response regulator, *ARR2*. This was demonstrated by identifying genes upregulated following β estradiol induced nuclear relocation of an *ARR2*-estradiol receptor fusion protein. The differences in CIM preincubation requirements for *ARR5* and *ARR15* expression indicate an additional layer of control for these A-type *ARR* genes during SIM incubation. For *ARR15*, the CIM requirement is a transcriptional effect, because the expression of *ARR15* promoter:GUS reporter gene constructs is also affected by CIM preincubation. A testable model is that transcription of *ARR15*, but not *ARR5*, is blocked by a repressor and that the effects of the repressor are relieved by CIM preincubation.

Shoots can be regenerated from roots in Arabidopsis by a process of indirect organogenesis in which root explants are preincubated on an auxin-rich callus induction medium (CIM) and then are transferred to a cytokinin-rich shoot induction medium (SIM) for shoot formation.[1] During CIM preincubation, root explants "acquire competence" to form shoots during subsequent incubation on SIM.[2] We have examined the impact of competence acquisition on the unfolding of the program of gene expression that underpins the shoot development process. To do so we conducted a "CIM dropout experiment" whereby we compared the program of gene expression during SIM incubation with and without CIM preincubation.[3]

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Many genes were affected in the CIM dropout experiment, but *ARR15* (At1g74890), encoding an A-type response regulator, was of special interest because *ARR15*, like several other A-type response regulator genes, can be induced by cytokinin.[4] In our system, both *ARR15* and *ARR5* (At3g48100), another A-type ARR, are upregulated during incubation on SIM, but *ARR15* was dependent on CIM preincubation. Using an *ARR15* promoter:GUS reporter construct, we demonstrated that the dependency of *ARR15* on CIM preincubation was transcriptional.[3] We found subsequently, that another A-type ARR, *ARR16* (At2g40670) was actually downregulated in response to CIM preincubation (Che, unpublished observations).

Several other A-type ARRs are known to be upregulated by cytokinin[5-13] and activated by B-type ARRs, the transcriptional regulators in the cytokinin signaling pathway.[10] For example, the A-type *ARR6* has been shown to be directly activated by B-type ARRs, *ARR1* (AT3G16857) and/or *ARR2* (AT4G16110).[6,7] In particular, Hass et al[14] demonstrated that overexpression of a constitutively active form of *ARR2* upregulated the expression of *ARR5*, *-15* and *-16*. We were particularly interested in knowing whether *ARR5, -15* and *-16*, which respond so differently to CIM preincubation, are directly activated by the same B-type ARR.

To do so, we fused *ARR2ΔDDK* (a constitutively active form of *ARR2*[7]) to the β-estradiol receptor and expressed the construct in transgenic plants with the 35S promoter. Steroid receptor fusion proteins have been used in Arabidopsis by a number of investigators to identify immediate transcription factor targets.[7,15-19] In response to β-estradiol treatment, the fusion protein, which accumulates in the cytoplasm, should be translocated to the nucleus. To identify genes directly activated by the *ARR2ΔDDK-ER* fusion, seedlings were treated (or not treated) with β-estradiol and cycloheximide (CHX) to block subsequent protein synthesis, then analyzed by Affymetrix DNA chip analysis. As a control for β-estradiol effects, CHX-treated non-transgenic seedlings were further treated or not treated with β-estradiol. Genes were rank ordered by the fold change between β-estradiol treated and untreated seedlings when controlling the false discovery rate at the level of 0.10 (Table 1).

By these criteria, the top genes most highly activated by *ARR2ΔDDK-ER* are some of the A-type response regulators, including *ARR4*, *-6*, *-7*, *-9*, *-15* and *-16*. These genes are affected very little by β-estradiol treatment in the wild type control, but are significantly upregulated by β-estradiol treatment in transgenic seedlings bearing *ARR2ΔDDK-ER* constructs (Table 1). The data show that *ARR15*

Table 1 **Immediate transcriptional targets of ARR**

Locus	non-transgenic untreated mean	non-transgenic β-est treated mean	FC non-transgenic	Adjusted FC non-transgenic	ARR2-ER transgenic untreated mean	ARR2-ER transgenic β-est treated mean	FC ARR2-ER transgenic	pvalue	qvalue	FC-ARR2 transgenic adjusted FC-non transgenic	Gene description
At1g74890	197.5	185.4	0.94	1.00	102.5	1802.4	17.58	1.47E-04	6.91E-02	17.58	ARR15
At1g34330	5.7	2	0.35	1.00	1.4	23.7	16.63	3.49E-03	1.30E-01	16.63	putative peroxidase
At1g10460	19.8	18.1	0.91	1.00	4.5	49.5	10.89	3.49E-03	1.30E-01	10.89	germin-like protein
At2g14270	11.2	1.9	0.17	1.00	1.8	16.7	9.10	2.30E-03	1.17E-01	9.10	putative protein phosphatase 2C
At5g62920	300.1	310.1	1.03	1.03	228.5	1906.3	8.34	4.04E-04	7.96E-02	8.07	ARR6
At3g28890	23.4	5.4	0.23	1.00	2.8	19.6	6.90	5.80E-03	1.50E-01	6.90	leucine-rich repeat family protein
At4g19770	28	33.9	1.21	1.21	2.4	19.2	7.99	1.11E-03	1.05E-01	6.59	glycosyl hydrolase family 18 protein
At5g45830	56.6	33.9	0.60	1.00	12	78.1	6.54	3.03E-03	1.23E-01	6.54	expressed protein
At1g04840	42.1	28	0.66	1.00	5.5	36	6.49	7.49E-06	3.82E-02	6.49	pentatricopeptide (PPR) repeat protein
At5g45650	215.1	194.1	0.90	1.00	402.7	2536	6.30	2.76E-07	2.33E-03	6.30	subtilisin-like protease
At2g40670	378.2	338.6	0.90	1.00	516.4	3072.7	5.95	3.38E-05	5.89E-02	5.95	ARR16
At5g56970	146.3	192.7	1.32	1.32	159.2	1217.2	7.64	5.83E-03	1.50E-01	5.80	cytokinin oxidase family protein
At1g10470	1715.8	1511.8	0.88	1.00	1150	6186.8	5.38	3.13E-04	7.33E-02	5.38	ARR4
At1g16530	98.7	137.8	1.40	1.40	138.8	1033.8	7.45	9.55E-04	1.02E-01	5.33	lateral organ boundaries protein 3
At3g48100	1219.9	1554.9	1.27	1.27	1043.5	6345.8	6.08	5.40E-03	1.47E-01	4.77	ARR5
At3g57040	681.3	581.6	0.85	1.00	490.4	2284	4.66	1.70E-04	6.91E-02	4.66	ARR9
At2g01890	412.5	243.1	0.59	1.00	432.2	1938.8	4.49	8.31E-08	1.40E-03	4.49	putative purple acid phosphatase
At4g26010	63.1	53.8	0.85	1.00	94.2	402.3	4.27	4.11E-03	1.40E-01	4.27	putative peroxidase ATP13a
At3g42380	27.1	6.5	0.24	1.00	8.2	34.3	4.15	5.43E-03	1.47E-01	4.15	hypothetical protein
At1g68360	173.9	176.6	1.02	1.02	120.2	505.7	4.21	4.28E-03	1.40E-01	4.14	putative zinc finger protein
At2g39920	111	126.3	1.14	1.14	130.8	604.9	4.63	8.84E-04	9.92E-02	4.06	acid phosphatase class B protein
At5g15190	271.7	311.9	1.15	1.15	210.6	912.3	4.33	1.21E-04	6.91E-02	3.77	putative protein
At4g13560	22	7.2	0.33	1.00	4.6	17.3	3.73	5.21E-04	8.60E-02	3.73	LEA domain-containing protein
At1g13740	388.7	271.8	0.70	1.00	259.1	957.6	3.70	9.90E-04	1.02E-01	3.70	expressed protein
At5g46230	384	314.5	0.82	1.00	346.2	1266.6	3.66	4.33E-03	1.41E-01	3.66	expressed protein
At3g57010	112.6	66.4	0.59	1.00	63.8	223.4	3.50	5.54E-04	8.60E-02	3.50	strictosidine synthase family protein
At5g05860	337.7	303.8	0.90	1.00	320.8	1115.9	3.48	2.19E-03	1.15E-01	3.48	UDP-glucosyl transferase family protein
At2g07630	1.4	1.1	0.77	1.00	1	3.4	3.23	2.87E-03	1.22E-01	3.23	expressed protein
At2g01830	185.6	199.7	1.08	1.08	215.7	712.7	3.30	8.12E-04	9.63E-02	3.07	his kinase cytokinin receptor (AHK4)
At3g15720	68	76	1.12	1.12	191	654.2	3.43	1.76E-03	1.07E-01	3.06	glycoside hydrolase family 28
At1g13420	18.8	4.3	0.23	1.00	32.1	97.5	3.04	8.26E-04	9.63E-02	3.04	steroid sulfotransferase
At5g23270	38	49.5	1.30	1.30	33.3	128.2	3.85	3.98E-03	1.38E-01	2.96	sugar transporter
At1g28100	190.3	186.4	0.98	1.00	186	543.3	2.92	1.64E-04	6.91E-02	2.92	expressed protein
At1g69040	1368.1	1466.6	1.07	1.07	1113.5	3466.2	3.11	8.75E-04	9.92E-02	2.90	ACT domain containing protein (ACR4)
At5g41890	17.4	4.9	0.28	1.00	6.2	17.3	2.79	2.65E-03	1.21E-01	2.79	GDSL-motif lipase/hydrolase protein
At2g43550	537.8	362	0.67	1.00	262.2	698.6	2.66	1.59E-03	1.07E-01	2.66	defensin-like family protein
At4g23750	941.7	1054.5	1.12	1.12	1337.4	3845.3	2.88	4.55E-04	8.45E-02	2.57	ERF/AP2 transcription factor family
At1g19050	5149.1	5267.9	1.02	1.02	4038	10567.9	2.62	1.54E-04	6.91E-02	2.56	ARR7

To identify genes directly activated by the ARR2ΔDDK-ER fusion, seedlings were treated (or not treated) with β-estradiol and cycloheximide (CHX) to block subsequent protein synthesis, then analyzed by Affymetrix DNA chip analysis. As a control for β-estradiol effects, CHX-treated non-transgenic seedlings were further treated or not treated with β-estradiol. Each of the treatments was duplicated, employing a total of eight DNA chips. Estimated means of the MAS 5.0 signal intensities are shown based on back-transformation of log-scale data. A two-way ANOVA was performed on log-scale data to identify genes exhibiting significant interaction between genotype (wild type vs. transgenic) and treatment (β-estradiol vs. no β-estradiol). Q-values were computed according to Storey and Tibshirani (2003). Data are sorted by the ratio of the fold change (FC) between the treated and untreated transgenic plants divided by the adjusted FC between the treated and untreated non-transgenic plants. The FC for the non-transgenic plants was adjusted to 1 for any values <1 to prevent inflation of the ratio of the FCs when the mean for the β-estradiol treated controls was less than the untreated controls.

is more than 17-fold upregulated by β-estradiol treatment, *ARR16* nearly 6 fold and *ARR5* nearly 5 fold. These observations support the proposition that several A-type *ARR* genes, noting *ARR5*, *ARR15* and *-16*, in particular, are direct transcriptional targets of the B-type *ARR2*. Comparable results have been obtained by Taniguchi et al[20] with a constitutively active construct involving another B-type ARR, *ARR1ΔDDK-GR* (in which GR=glucocorticoid receptor). Thus, both *ARR1* and *ARR2* have very broad control over genes which otherwise have been shown to be cytokinin regulated.

The finding that CIM preincubation was required for *ARR15* upregulation during subsequent incubation on SIM was unexpected and interesting. From what was discussed above, it was anticipated that *ARR15* would be induced on cytokinin-rich SIM, whether or not explants had been preincubated on CIM. Other A-type ARRs, such as *ARR5* and *ARR6*, which are also normally upregulated in SIM, did not require CIM preincubation. The response of *ARR15* and *-16* to CIM preincubation was particularly intriguing because the expression of both appear to depend on the function of *AHK4* as a receptor. Kiba et al.[21] showed that *ARR15* and *ARR16* expression is markedly reduced in *cre1-1*, a loss-of-function mutation in *AHK4*.

One major difference between *ARR15* and *-16* expression (noted by Kiba et al., ref. 21) is that a *ARR15* promoter:GUS construct is expressed in the vasculature of roots treated with cytokinin (t-zeatin), while *ARR16* promoter:GUS is expressed in the endodermis. On the other hand, we have found in other experiments that *ARR5* and *-15* appear to be expressed in the same root tissue (Che unpublished), yet they too differ in their dependence on CIM preincubation. A possible explanation for this is that *ARR5* and *-15* may be activated by different branches of the cytokinin signaling pathway—*ARR5* expression in seedlings is not dependent on *AHK4*, while *ARR15* expression is. As pointed out above, Kiba et al[21] found that *ARR15* expression was downregulated in *cre1-1* (*AHK4* loss-of-function mutant), but ARR5 expression was relatively unaffected. In addition, we found that *ARR15* was highly upregulated by β-estradiol treatment of *ARR2ΔDDK-ER* seedlings, but *ARR5* was not.

The fact that *ARR5*, *-15* and *-16* are direct targets of *ARR1* and *ARR2,* yet their regulation in response to CIM preincubation differs, indicates an additional layer of control for these A-type ARR genes during SIM incubation. For *ARR5* and *ARR15*, that regulation is exercised at a transcriptional level, because the expression of promoter:GUS reporter gene constructs is also affected by CIM preincubation. A testable model for the control is that transcription of *ARR15* is blocked by a repressor and that the effects of the repressor are relieved by CIM preincubation. If that kind of control can be generalized

to other genes that are expressed during SIM incubation, then the function of CIM preincubation might be to overcome a major gene expression checkpoint in shoot regeneration.

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