The Potassium-Dependent Transcriptome of Arabidopsis Reveals a Prominent Role of Jasmonic Acid in Nutrient Signaling[1][w]

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Full genome microarrays were used to assess transcriptional responses of Arabidopsis seedlings to changing external supply of the essential macronutrient potassium (K^+). Rank product statistics and iterative group analysis were employed to identify differentially regulated genes and statistically significant coregulated sets of functionally related genes. The most prominent response was found for genes linked to the phytohormone jasmonic acid (JA). Transcript levels for the JA biosynthetic enzymes lipoxygenase, allene oxide synthase, and allene oxide cyclase were strongly increased during K^+ starvation and quickly decreased after K^+ resupply. A large number of well-known JA responsive genes showed the same expression profile, including genes involved in storage of amino acids (VSP), glucosinolate production (CYP79), polyamine biosynthesis (ADC2), and defense (PDF1.2). Our findings highlight a novel role of JA in nutrient signaling and stress management through a variety of physiological processes such as nutrient storage, recycling, and reallocation. Other highly significant K^+-responsive genes discovered in our study encoded cell wall proteins (e.g. extensins and arabinogalactans) and ion transporters (e.g. the high-affinity K^+ transporter HAK5 and the nitrate transporter NRT2.1) as well as proteins with a putative role in Ca^{2+} signaling (e.g. calmodulins). On the basis of our results, we propose candidate genes involved in K^+ perception and signaling as well as a network of molecular processes underlying plant adaptation to K^+ deficiency.

Potassium (K^+) is the most abundant inorganic cation in plants, comprising up to 10% of a plant's dry weight (Leigh and Jones, 1984). K^+ is an important macronutrient for plants, which carries out vital functions in metabolism, growth, and stress adaptation. These functions can be classified into those that rely on high and relatively stable concentrations of K^+ in certain cellular compartments and those that rely on K^+ movement between different compartments, cells, or tissues. The first class of functions includes enzyme activation, stabilization of protein synthesis, and neutralization of negative charges on proteins (Marschner, 1995). The second class of functions of K^+ is linked to its high mobility. This is particularly evident where K^+ movement is the driving force for osmotic changes as, for example, in stomatal movement, light-driven and seismonastic movements of organs, or phloem transport. In other cases K^+ movement provides a charge-balancing counter-flux essential for sustaining the movement of other ions. Thus, energy production through H^+-ATPases relies on overall H^+/K^+ exchange (Tester and Blatt, 1989; Wu et al., 1991). The most general phenomenon that requires directed movement of K^+ is growth. Accumulation of K^+ (together with an anion) in plant vacuoles creates the necessary osmotic potential for rapid cell extension.

K^+ deficiency is of great agricultural importance (Laegreid et al., 1999). This fact was recognized early in plant physiological research and has led to a good description of K^+ starvation symptoms at the physiological level (Marschner, 1995). It is well established that K^+ starvation leads to (1) growth arrest due to the lack of the major osmoticum, (2) impaired nitrogen and sugar balance due to inhibition of protein synthesis, photosynthesis, and long-distance transport, and (3) increased susceptibility to pathogens probably due to increased levels of low M_r nitrogen and sugar compounds. In a natural environment low-K^+ conditions are often transient and therefore plants have developed mechanisms to adapt to short-term shortage of K^+ supply. One important aspect of plant adaptation to K^+ stress is cellular and tissue homeostasis of K^+, which involves transport of K^+ across various membranes in various tissues (Amtmann et al., 2004). K^+ transport mechanisms have been studied extensively at the molecular level (Véry and Sentenac, 2003). Much less is known about the molecular nature of adaptive responses at the level of metabolism and development. Furthermore, it is completely unknown how plants sense external K^+ concentration, how this information is communicated within the plant, and how physiological, biochemical, and molecular responses are integrated into a concerted adaptive response.

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Microarray technology allows us to approach K^+ deficiency again from a more integrative point of view considering all aspects of K^+ management in plants, including processes related to growth, development, metabolism, and stress resistance. It therefore provides us for the first time with a molecular picture of how plants manage one of its most important nutrients under conditions of shortage in supply. Since the obtained picture is limited to transcripts, it will be far from complete. Nevertheless, the identified genes should provide a framework of molecular processes involved in K^+ nutrition, which in future can be studied in more detail and complemented at the level of proteins and metabolites.

In this study we have used full genome microarrays to monitor the transcriptome of Arabidopsis seedlings exposed to long-term K^+ starvation and short-term K^+ resupply after starvation. Our experimental design ensures that we identify transcriptional responses that are specifically linked to the external K^+ supply. Furthermore, we have employed new analysis methods to avoid determination of transcriptional changes based on arbitrary cut-off of fold-changes. Rank products (RP) is a novel nonparametric statistics test that assesses the consistency and magnitude of expression changes (Breitling et al., 2004b). We also employ a novel algorithm to identify groups of functionally related genes with significant transcriptional changes, which facilitates the biological interpretation of the obtained dataset (Breitling et al., 2004a). Our results indicate that changes in external K^+ supply affect the transcription of genes within four major functional categories, namely genes related to the phytohormone jasmonic acid (JA), genes encoding cell wall proteins, genes involved in transport processes, and genes with possible function in cellular signaling.

Our study delineates a specific set of genes that respond rapidly and selectively to external K^+. It is hoped that this will facilitate further studies of K^+ signaling as screening for K^+ perception mutants was previously hampered by the fact that K^+-related physiological and morphological phenotypes appear late during the stress and are relatively unspecific. The identified set of specifically K^+-regulated genes, which respond quickly to changes in external K^+ supply, should be a useful tool for the further dissection of signaling pathways involved in perception or integration of K^+ changes at the cellular and tissue level.

RESULTS

Experimental Design and Analysis

Design and Phenotypic Characterization of the Experimental System

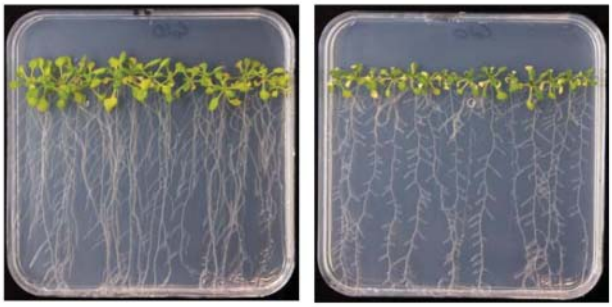
Biological conditions and experimental design were tailored to characterize molecular responses to changes in the external supply of the macronutrient K^+. To ensure that the biological material was sensitive to external K^+, we used 2-week-old plants, which have low internal K^+ storage capacity and exhibit high relative growth rate, thus requiring high rates of K^+ uptake from the external medium. Long-term starvation responses were assessed by comparing plants grown on K^+-free medium (K^+ starved) with plants grown on K^+-replete medium (control, 2 mM K^+). Short-term K^+ resupply responses were assessed by providing 10 mM KCl to K^+-starved plants for 2 and 6 h (Fig. 1). This treatment was compared to two control experiments, i.e. resupply of K^+-free solution (0) or resupply of 10 mM NaCl (Na). Transcriptional changes due to differences in ionic strength, osmotic potential, and chloride or sodium concentrations between the solutions were eliminated by identifying those genes that changed transcript levels upon K^+ resupply with respect to both control treatments (see below).

Plants grown on K^+-free medium developed visible K^+ starvation symptoms on day 10 (± 2) after germination, which included chlorosis of older leaves and a typical growth arrest of lateral roots (Fig. 1A). Consequences of K^+ starvation and resupply in terms of root and shoot ion contents were measured using inductively coupled plasma optical emission spectroscopy (ICP-OES). No significant changes in Mg, P, S, B, Fe, or Zn were observed during any of the treatments. K^+, Na^+, and Ca^{2+} tissue contents are shown in Figure 2. K^+-starved seedlings contained about 4 times less K^+ than K^+-sufficient seedlings, in both roots and shoots. A concomitant doubling in tissue Ca^{2+} contents during K^+ starvation was probably due to the increased Ca^{2+} concentration in the K^+-free medium (see "Materials and Methods"). K^+ resupply led to a slight but significant increase of root K^+ content within the first 6 h. No significant increase of shoot K^+ content was detected within this period of time, but after 24 h both tissues had regained a considerable amount of K^+, i.e. 40% to 60% of K^+ levels of plants grown in complete medium. Addition of the two control media (0 or Na) to K^+-starved plants did not affect their ion contents, in particular no increase of tissue Na^+ content was observed within 24 h after addition of 10 mM NaCl. We can conclude that our experimental design ensures that (1) responses to K^+ starvation are assessed at a stage were K^+ deficiency occurs but is not yet lethal, and that (2) responses to K^+ resupply are monitored at a stage where external K^+ is sensed by the plants but has not yet resulted in apparent morphological changes or a considerable increase of shoot K^+ content.

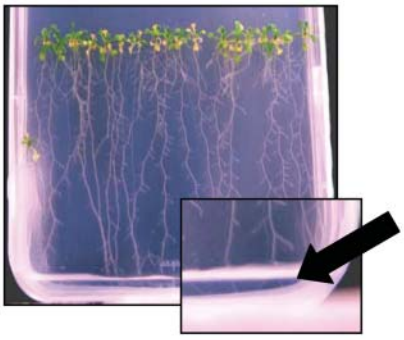
Identification of Differentially Expressed Genes

Three biological replicates were examined for all experimental conditions (Fig. 1C). The statistical analysis was based on three replicates of the long-term starvation experiment and six replicates for K^+ resupplied roots and shoots, merging the two replica sets obtained with 0 or Na^+ control media at each time point. (It should be emphasized that the two sets of

A



B



C

	K Re-supply						K Starvation	
	Roots 2h		Shoot 2h		Shoot 6h		Root	Shoot
Control	NaCl 10 mM	-K medium	NaCl 10 mM	-K medium	NaCl 10 mM	-K medium	Complete medium	Complete medium
Treatment	KCl 10 mM	KCl 10 mM	KCl 10 mM	KCl 10 mM	KCl 10 mM	KCl 10 mM	-K medium	-K medium

Figure 1. Biological system and experimental design. A, Phenotype of Arabidopsis seedlings grown vertically on petri dishes for 2 weeks after germination. Left, Complete medium (2 mM K^+). Right, K^+-free medium (−K). For composition of media see "Materials and Methods." B, Resupply experiments; 2-week-old K-starved seedlings were provided with 5 mL of sterile −K medium supplemented with 10 mM KCl. Control plants received fresh −K medium with or without additional 10 mM NaCl. C, Overview of microarray experiments. Each column represents one array. Control samples are shown in green, treated samples in red. All eight comparisons were carried out for three independent biological replicates (24 arrays in total).

experiments differing in their control treatment were not treated as replicates in a narrow sense. Rather our analysis method [see below] identified genes that showed significant transcriptional responses that were consistent over the six arrays. Thus, the response of these genes was indeed due to a change in the external K^+ concentration rather than to changes in Cl^- or Na^+ concentration, ionic strength, or osmotic potential.) For each treatment genes were sorted to produce a ranked list based on their fold-change (FC) compared to the control treatment (i.e. the gene with the highest FC is assigned rank 1 etc.). To identify genes with statistically significant expression changes, we used the RP test statistics (see "Materials and Methods;" Breitling et al., 2004b). We chose this recently developed, fully nonparametric test rather than a classical *t* test method (such as the one implemented in the Significance Analysis of Microarrays software; Tusher et al., 2001) because it does not require an estimate of measurement variation and is therefore more stable with regard to experimental noise and small data sets. Furthermore, it is applicable to low numbers of replicates, where traditional nonparametric methods (e.g. Wilcoxon rank test) fail due to the small number of possible permutations. Determination of expect-values and false discovery rate (E-values and FDR; see "Materials and Methods") provided a well-defined significance estimate for the RP values obtained from replicated experiments. For each comparison the analysis resulted in two lists of genes sorted by their respective RP values, one for up- and one for down-regulated genes.

Table I gives an overview of the number of genes whose expression was affected by K^+- treatment according to significance levels expressed in terms of FDR (minimum FC are shown in brackets). The first point to note is that the number of genes showing significant transcriptional changes varied considerably depending on the applied treatment and the examined tissue. In roots, expression of a very large number of genes was significantly changed already after 2 h of K^+ resupply (387 genes below 1% FDR, combining up and down-regulation). In shoots the same level of response was reached later (24 genes below 1% FDR at 2 h and 799 genes below 1% FDR at 6 h, combining up- and down-regulation). By contrast, 6 h incubation in K^+ resupplied medium did not further increase the number of regulated root transcripts (as compared to 2 h), and we therefore restricted the analysis of early responses to K^+ resupply in the roots to the 2 h time point. Expression level changes were stronger during starvation than after resupply (e.g. fold change of 2.14 or more at an FDR below 0.01%). The second point to make in relation to Table I is that

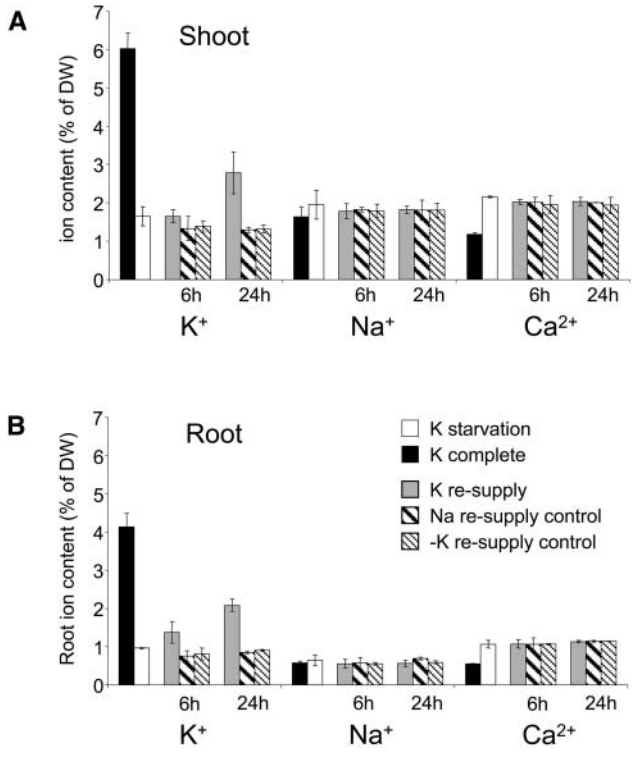


Figure 2. Seedling ion content after K^+ starvation and resupply. Potassium, sodium, and calcium levels were measured with ICP-OES in shoots (A) and roots (B) of 2-week-old seedlings grown either on complete medium (black boxes) or on $-K^+$ medium (white boxes). Ion content was also analyzed at 6 and 24 h after resupplying 10 mM KCl (gray boxes), 10 mM NaCl (bold dashed boxes), or a fresh K^+-free-solution (light dashed boxes) to K^+-starved plants. Fifty seedlings were pooled for each sample. Averages and SES of three independent experiments are shown.

our experimental design and analysis method allowed us to detect small transcriptional changes with high significance. For example, changes of only 1.3-fold were frequently detected at an FDR of 1% or lower.

Expression patterns of all genes listed in Table I with FDRs of up to 1% are available as supplemental material (www.plantphysiol.org). Furthermore, a fully annotated list of transcriptional changes with FDRs of up to 0.001%, sorted by putative physiological function, can be obtained from the web supplement. In both cases functional annotation was based on the Arabidopsis Information Resource (TAIR), Munich Information Center for Protein Sequences (MIPS), and The Institute for Genomic Research (TIGR). We also offer access to a searchable database of our results at http://www.brc.dcs.gla.ac.uk/~rb106x/arabidopsis_results.htm.

Identification of Functional Categories of Differential Gene Expression

To detect physiologically relevant patterns in the gene lists resulting from RP analysis we employed iterative group analysis (iGA; Breitling et al., 2004a). In a traditional approach, such patterns are defined manually by looking for groups of genes that share a certain function or participate in a common process and are over-represented among a predefined list of differentially expressed genes. iGA encodes this procedure in an objective and statistically rigorous algorithm. Genes are automatically assigned to functional groups based on a wide variety of available annotations (gene names, gene families, Gene Ontology classes, etc.) and each of these groups is then tested for differential expression. In doing so, the iterative nature of the algorithm allows significance estimates even for imperfect annotations or when only some members of a functional group are changed. In addition, iGA does not rely on an arbitrarily restricted list of significantly changed genes, but rather determines the optimal cut-off for each particular group. The result is a list of groups, sorted by statistical significance (minimal *P*-value), and the corresponding member genes that show relevant expression changes (Tables II–V). Only groups up to an expected FDR of 10% are shown in Tables II to V.

Together, the RP and iGA algorithms allowed us to obtain a dataset, which not only represents a highly structured overview of transcriptional responses to external K^+, but also provides an estimate of the statistical significance of the observed patterns.

Biological Classification of K^+-Responsive Genes

In this section we attempt to draw a comprehensive picture of the transcriptional adaptation occurring in response to changes in external K^+ supply. For this purpose we used two different approaches. Firstly, iGA results were examined to obtain a general view of genes that share a common physiological role and were, as a group, transcriptionally affected by our treatments. From this analysis emerged several functional super-categories of genes that appear to play an important role in the plants adaptation to K^+ stress. We then proceeded by looking at individual genes that showed highly significant transcriptional changes in response to K^+ (i.e. FDR of 0.001% or lower) and identified those that are functionally linked to the iGA-based super-categories. This allowed us to include genes within the super-categories that are highly significant in their responsiveness to K^+, but might have been overlooked by the iGA due to imperfect annotation.

K^+-Responsive Functional Groups

The group lists resulting from iGA of the K^+ resupply experiments revealed a number of recurring and statistically highly significant patterns (Tables II–V). Many of the functional groups identified in the K^+ resupply experiments were also found when iGA was applied to the starvation experiments (with group

Table I. *Significance analysis of genes differentially expressed in response K^+ starvation and resupply*

Cumulative numbers of differentially expressed genes according to FDRs ranging from <0.001% to <10%. FDRs were calculated by dividing E-values by the position of the gene in the RP list obtained from three (for K^+ starvation) or six (for resupply) replicate experiments. E-values were assigned by comparing the RP of each gene to the RP calculated from 10,000 random permutations of the original dataset (see "Materials and Methods" for details). Numbers in brackets indicate the minimal FC at each level of significance.

FDR %	Down	Up	Down	Up
	Starvation (Shoots, $n = 3$)		Starvation (Roots, $n = 3$)	
<0.001	1 (2.91)	4 (7.57)	1 (3.08)	1 (4.1)
<0.01	1 (2.91)	19 (2.75)	4 (2.14)	11 (2.63)
<0.1	8 (1.62)	45 (1.89)	22 (1.72)	35 (1.95)
<1	17 (1.45)	99 (1.52)	150 (1.49)	149 (1.53)
<10	43 (1.33)	224 (1.3)	473 (1.30)	417 (1.30)
	Resupply (2 h Roots K(Na) and K(0), $n = 6$)			
<0.001	23 (1.59)	13 (1.51)		
<0.01	40 (1.45)	25 (1.45)		
<0.1	72 (1.36)	55 (1.40)		
<1	177 (1.28)	210 (1.21)		
<10	658 (1.14)	801 (1.10)		
	Resupply (2 h Shoots K(Na) and K(0), $n = 6$)		Resupply (6 h Shoot K(Na) and K(0), $n = 6$)	
<0.001	5 (1.51)	0	104 (1.55)	50 (1.57)
<0.01	7 (1.45)	0	152 (1.45)	89 (1.44)
<0.1	14 (1.33)	0	249 (1.37)	175 (1.35)
<1	23 (1.29)	1 (1.38)	436 (1.27)	363 (1.27)
<10	57 (1.18)	5 (1.21)	935 (1.18)	934 (1.15)
	Resupply (2 and 6 h Shoot K(Na) and K(0), $n = 12$)			
<0.001	126 (1.31)	64 (1.33)		
<0.01	200 (1.25)	122 (1.24)		
<0.1	297 (1.18)	222 (1.21)		
<1	499 (1.16)	507 (1.13)		
<10	1020 (1.06)	1217 (1.08)		

members being regulated in the opposite direction; data not shown). The most prominent groups of genes down-regulated upon K^+ resupply shared functional annotations related to either JA biosynthesis and known jasmonic acid-dependent processes (mostly in shoots; Table II) or synthesis of secondary metabolites (in both roots and shoots; Tables II and III). Furthermore, several gene groups encoding proteins functioning in sulfur metabolism (in particular linked to the homo-Cys cycle) were significantly down-regulated by K^+ resupply and were detected as a group in both root and shoot datasets. K^+ resupply also triggered down-regulation of genes known to be involved in stress adaptation (e.g. glutathione transferases, dehydroascorbate reductases, cold responsive genes, and polyamine synthesis). Another interesting result of the iGA was the detection of a number of genes encoding Ca^{2+}-binding proteins (mainly calmodulins), which as a group showed a significant down-regulation in roots after resupply of K^+. Significant groups among transcripts up-regulated upon K^+ resupply overlapped between shoots and roots (Tables IV and V). In particular both tissues showed up-regulation of transporters (aquaporins) and cell wall related genes (arabinogalactans). By contrast, two groups of up-regulated genes linked to primary metabolism were shoot specific (Table IV).

The iGA results highlighted four main functional super-categories: (1) genes related to the JA signaling pathway, (2) genes encoding cell wall proteins, (3) genes with a putative function in transport, and (4) genes encoding Ca^{2+}-binding proteins. Figures 3 and 4 show expression profiles of all genes in these categories that were differentially expressed upon K^+ resupply with high significance (FDR of 0.001% or lower). Note that while adding genes to the functional categories, the figures omit some of the group members identified by iGA. This has two reasons. Firstly, the chosen significance cut-off was extremely rigorous due to space limitations. Secondly, significant group responses can include changes in individual transcripts that have much lower significance when analyzed outside the group context. For comprehensive visualization, genes were sorted according to their expression profile.

JA- and Defense-Related Genes

The largest functional category detected by iGA concerned genes related to the plant hormone JA. Our

Table II. *Groups of functionally related genes down-regulated in shoots after K^+ resupply*

K^+-responsive groups of functionally related genes were identified by iGA based on RP lists of shoot genes sorted for down-regulation over 12 experiments (2 and 6 h K^+ resupply, three replicates with two controls each). Significantly affected gene groups up to an FDR of 10% are shown. Some of the groups may overlap completely because genes are classified similarly by various annotations, and in these cases they are combined for the presentation in Tables II to V. For rank and evidence details, see footnote of Table IV.

Groups		AGI	Code	Annotation	Rank	Category
Myrosinase Binding Proteins		At1g54020		Myrosinase-associated protein	13	JA-Related
Minimal *P*-value	Evidence	At1g52030	MBP2	MYROSINASE-BINDING PROTEIN 2	24	JA-Related
1.53E−08	K	At1g52040	MBP1	MYROSINASE-BINDING PROTEIN 1	36	JA-Related
		At5g38540		Myrosinase binding protein-like	37	JA-Related
		At2g39330		Putative myrosinase-binding protein	71	JA-Related
Lectins (Myrosine Binding Protein-Like)		At3g16470	JR1	Putative lectin (JR1)	47	JA-Related
Minimal *P*-value	Evidence	At3g16400		Putative lectin	66	JA-Related
7.15E−08	G, K	At3g16410		Putative lectin	76	JA-Related
		At3g16390		Putative lectin	82	JA-Related
Vegetative Storage Proteins						
Minimal *P*-value	Evidence	At5g24780	VSP1	VEGETATIVE STORAGE PROTEIN 1	2	JA-Related
1.40E−07	K (2x), M	At5g24770	VSP2	VEGETATIVE STORAGE PROTEIN 2	3	JA-Related
Jasmonic Acid Biosynthesis		At3g45140	LOX2	LIPOXYGENASE 2	11	JA-Related
Minimal *P*-value	Evidence	At5g42650	AOS	ALLENE OXIDE SYNTHASE	14	JA-Related
1.99E−06	G	At3g25760	AOC	ALLENE OXIDE CYCLASE	87	JA-Related
Tryptophan Catabolism/CYP79B						
Minimal *P*-value	Evidence	At4g39950	CYP79B2	Cytochrome P450 (CYP79B2)	57	Stress
6.44E−06	G, F	At2g22330	CYP79B3	Cytochrome P450 (CYP79B3)	61	Stress
Trypsin Inhibitors		At2g43530		Putative trypsin inhibitor	38	Stress
Minimal *P*-value	Evidence	At2g43510		Putative trypsin inhibitor	50	Stress
8.59E−06	K, G	At2g43520		Putative trypsin inhibitor	93	Stress
Degradation of Tyrosine						
Minimal *P*-value	Evidence	At2g24850	TAT3	Tyrosine aminotransferase (TAT3)	5	JA-Related
8.96E−06	M (2x), K	At4g23600	CORI3	CORONATINE INDUCED 1 (CORI3)	7	JA-Related
Arginase/Biosynthesis of Polyamines						
Minimal *P*-value	Evidence	At4g08870		Putative arginase	17	JA-Related
1.62E−05	K, M (2x)	At4g08900		Arginase	72	JA-Related
Phenylpropanoid Biosynthesis						
Minimal *P*-value	Evidence	At3g53260	PAL2	Phenylalanine ammonia-lyase PAL2	127	Stress
4.27E−05	G (2x)	At2g37040	PAL1	Phenylalanine ammonia-lyase PAL1	151	Stress
Cold Acclimation						
Minimal *P*-value	Evidence	At1g20440	COR47	COR47	40	Stress
7.47E−05	G	At5g15960	KIN1	KIN1	65	Stress
Dehydroascorbate Reductase						
Minimal *P*-value	Evidence	At1g19570		Dehydroascorbate reductase	23	Stress
9.11E−05	K, M	At5g36270		Dehydroascorbate reductase	96	Stress
Sulfate Reduction, APS Pathway						
Minimal *P*-value	Evidence	At3g22890	APS1	ATP sulfurylase	85	Sulfur
1.16E−04	G	At4g04610	APR1	5-Adenylylsulfate reductase	163	Sulfur
Gluconolactonase Activity						
Minimal *P*-value	Evidence	At5g24420		6-Phosphogluconolactonase-like protein	1	Growth
1.35E−04	G					
Harpin-Induced Proteins						
Minimal *P*-value	Evidence	At5g06320		Harpin-induced protein-like	42	Stress
1.40E−04	K	At2g35460		Harpin-induced protein-like	116	Stress
Other Extracellular Metabolism		At1g78660		Gamma glutamyl hydrolase	718	Growth
Minimal *P*-value	Evidence	At1g78670		Gamma glutamyl hydrolase	746	Growth
1.45E−04	M	At1g78680		Gamma glutamyl hydrolase	928	Growth

(*Table continues on following page.*)

Table II. *(Continued from previous page.)*

Groups		AGI	Code	Annotation	Rank	Category
S-Adenosyl-Methionine - Homocysteine Cycle		At4g01850		*S*-Adenosylmethionine synthase 2	546	Sulfur
Minimal *P*-value	Evidence	At2g36880		Putative *S*-adenosylmethionine synthetase	656	Sulfur
1.87E−04	M (2x)	At4g13940		*S*-Adenosyl-L-homocysteinase	967	Sulfur
		At1g02500		*S*-Adenosylmethionine synthetase	1,206	Sulfur
		At5g57280		Protein carboxyl methylase-like	1,649	Sulfur
		At3g23810		*S*-Adenosyl-L-homocysteinase	2,868	Sulfur
		At3g17390		Putative *S*-adenosylmethionine synthetase	2,917	Sulfur
Biosynthesis of Glycosides						
Minimal *P*-value	Evidence	At5g28510		Glycosyl hydrolase family 1	6	
2.09E−04	M	At1g52400	BGL1	Beta-Glucosidase	9	
Indoleacetic Acid Biosynthesis		At2g20610	SUR1	SUPERROOT 1	1,018	
Minimal *P*-value	Evidence	At3g44300	NIT2	NITRILASE 2	1,128	
2.28E−04	G	At3g44310	NIT1	NITRILASE 1	1,350	
		At4g31500	SUR2	SUPERROOT 2 (CYP83B1)	1,367	
Oxidoreductase Activity, Acting on Sulfur Group of Donors						
Minimal *P*-value	Evidence	At4g21990	APR3	5-Adenylylsulfate reductase PRH26	80	Sulfur
2.47E−04	G	At4g04610	APR1	5-Adenylylsulfate reductase	163	Sulfur

current knowledge of these genes derives mostly from work on defense mechanisms during pathogenesis and wounding. Transcriptional changes of JA-related genes in response to the external K^+ supply were considerable in terms of both strength and statistical significance, thus pointing to a novel and exciting role of this plant hormone in mineral nutrition. Figure 3 gives an overview of K^+-responsive genes, which are directly or indirectly linked to JA. The most prominent expression profile within this category consisted in up-regulation during starvation and down-regulation upon K^+ resupply in shoots. Affected transcripts were related to (1) JA biosynthesis and known JA-downstream events, (2) glucosinolate synthesis and degradation, (3) polyamine metabolism, (4) defense mechanisms, and (5) production of secondary metabolites.

JA Biosynthesis and JA Markers

The first three steps of JA biosynthesis convert linolenic acid into 12-oxo-phytodienoic acid and are catalyzed by the enzymes lipoxygenase (LOX; Bell and Mullet, 1993), allene oxide synthase (AOS; Laudert and Weiler, 1998), and allene oxide cyclase (AOC; Weber, 2002). Genes for all three enzymes, i.e. *LOX2*, *AOS*, and *AOC*, were significantly up-regulated during K^+ starvation and down-regulated after K^+ resupply (the latter change was also observed for *OPR3*, encoding 12-oxo-phytodienoic acid reductase; Sanders et al., 2000). An increase of JA levels in response to K^+-starvation and a rapid decrease after K^+ resupply was also indicated by the concomitant regulation of well-known JA-responsive transcripts encoding vegetative storage protein (*VSP1*, *VSP2*; Utsugi et al., 1998), thionin (*THI2.1*; Epple et al., 1995), and chlorophyllase (*CORI1/CHL1*; Tsuchiya et al., 1999). These transcripts, as well as several plant defensin transcripts (*PDF1.2a*, *b*, *c*; *PDF1.3*) showed the highest average fold change (5–10) in shoots of K^+-starved plants (see supplemental material). Two transcripts encoding a Tyr aminotransferase are also included in this group as *TAT3* is annotated as JA-responsive (TAIR; Titarenko et al., 1997).

Glucosinolate Synthesis and Degradation

A second gene category reported to be regulated by JA contains genes involved in the glucosinolate-myrosinases system (for reviews, see Rask et al., 2000; Wittstock and Halkier, 2002). Transcript levels of the methylthioalkylmalate synthase-like gene (*MAM-L*, At5g23020) but not the methylthioalkylmalate synthase1 (*MAM1*, At5g23010; Kroymann et al., 2001) were reduced in shoots upon starvation. These enzymes are implicated in early synthesis steps of chain-elongated Met derived glucosinolates. Genes encoding cytochrome-P450-dependent mono-oxygenases of the CYP79 family are involved in the second step of glucosinolate synthesis by catalyzing the conversion of Trp to indole-3-acetaldoxime (Hull et al., 2000; Zhao et al., 2002). In our experiments, *CYP79B2* and *CYP79B3* were down-regulated upon K^+ resupply in roots and shoots. CYP79B2 and CYP79B3 are also involved in the Trp-dependent auxin biosynthesis (Wittstock and Halkier, 2002; Zhao et al., 2002; Mikkelsen et al., 2003). A possible involvement of auxin in the response to external K^+ supply was also pointed out by iGA with a minimal *P*-value of 2.28e−04 for a group of genes involved in auxin biosynthesis (*NIT1*, *NIT2*, *SUR1*, and *SUR2*; Table II). The counterpart to the synthesis of glucosinolates is

Table III. *Groups of functionally related genes down-regulated in roots after K^+ resupply*

K^+-responsive groups of functionally related genes were identified by iGA based on RP lists of root genes sorted for down-regulation over six experiments (6 h K^+ resupply, three replicates with two controls each). Significantly affected gene groups up to a FDR of 10% are shown. For rank and evidence details, see footnote of Table IV.

Groups		AGI	Code	Annotation	Rank	Category
Biosynthesis of Alkaloids; Fermentation; Linked; Oxidoreductase		At1g26420		FAD-linked oxidoreductase family	6	Stress
Minimal *P*-value	Evidence	At1g26390		FAD-linked oxidoreductase family	7	Stress
4.73E−11	M (2x), K(2x)	At1g26400		FAD-linked oxidoreductase family	8	Stress
		At1g26380		FAD-linked oxidoreductase family	15	Stress
		At4g20800		Putative protein	19	Stress
		At4g20830		FAD-linked oxidoreductase family	32	Stress
		At4g34900		Xanthine dehydrogenase	36	Stress
		At1g30730		FAD-linked oxidoreductase family	46	Stress
Electron Transfer Flavoprotein		At4g20800		Putative protein	19	Growth
Minimal *P*-value	Evidence	At2g46760		Hypothetical protein common family	79	Growth
1.39E−06	G	At2g46750		Unknown protein	99	Growth
		At5g64250		2-Nitropropane dioxygenase-like protein	129	Growth
P-Type Pump		At1g27770	ACA1/PEA1	Calcium-transporting ATPase 1, PLM-type	17	Transport
Minimal *P*-value	Evidence	At1g59820	ALA3	Chromaffin granule ATPase II homolog, putative	212	Transport
2.50E−05	T	At1g13210	ALA11	Potential phospholipid-transporting ATPase 11	216	Transport
		At4g29900	ACA10	Potential calcium-transporting ATPase 10, PLM-type	321	Transport
		At4g30190	AHA2	Putative H+-transporting ATPase	423	Transport
		At2g19110	HMA4	Putative cadmium-transporting ATPase	425	Transport
		At4g30120	HMA3	Cadmium-transporting ATPase-like protein	449	Transport
		At2g18960	AHA1	Plasma membrane proton ATPase (PMA)	549	Transport
		At3g22910	ACA13	Potential calcium-transporting ATPase 13, PLM-type	638	Transport
Tryptophan Catabolism						
Minimal *P*-value	Evidence	At4g39950	CYP79B2	Cytochrome P450-like protein	23	Stress
2.68E−05	G CYP79B	At2g22330	CYP79B3	Putative cytochrome P450	116	Stress
Minimal *P*-value	Evidence	At4g39950	CYP79B2	Cytochrome P450-like protein	23	Growth
8.72E−05	F	At2g22330	CYP79B3	Putative cytochrome P450	116	Growth
EF-Hand Containing Proteins:Group IV		At2g41100	CaM2	Calmodulin-like protein, TOUCH3	14	Calcium
Minimal *P*-value	Evidence	At1g18210	CML27	Calcium-binding protein, putative	60	Calcium
9.62E−05	F	At3g56800	CaM3	Calmodulin-3	97	Calcium
		At3g43810	CaM7	Calmodulin 7	188	Calcium
		At2g41090	CML10	Calcium-binding protein (CaBP-22;CAM10)	208	Calcium
		At4g03290	CML6	Putative calmodulin	243	Calcium
		At3g51920	CML9	Putative calmodulin (CAM9)	389	Calcium
Arginine Decarboxylase Activity; Polyamine Biosynthesis						
Minimal *P*-value	Evidence	At4g34710	ADC2	Arg decarboxylase SPE2	2	JA-Related
1.35E−04	G (2x)					
Harpin						
Minimal *P*-value	Evidence	At5g06320		Harpin-induced protein-like	13	Stress
1.44E−04	K	At2g35460		Similar to harpin-induced protein hin1 from tobacco	124	Stress
Stomach; Adrenal Gland; Catalase Reaction		At1g02920		Glutathione transferase, putative	56	Stress
Minimal *P*-value	Evidence	At1g02930		Glutathione transferase, putative	96	Stress
1.56E−04	M (3x)	At2g30860		Glutathione transferase, putative	853	Stress

(*Table continues on following page.*)

Table III. (*Continued from previous page.*)

Groups		AGI	Code	Annotation	Rank	Category
		At1g69920		Glutathione transferase, putative	1,105	Stress
Degradation of Met; S-Adenosyl-Methionine - Homocysteine Cycle; Transfer of Activated C-1 Groups		At2g36880		Putative *S*-adenosylmethionine Synthetase	292	Sulfur
Minimal *P*-value	Evidence	At4g01850		*S*-Adenosylmethionine synthase 2	358	Sulfur
1.74E−04	M (3x)	At1g02500		*S*-Adenosylmethionine synthetase	604	Sulfur
		At3g23810		*S*-Adenosyl-L-homocysteinas, putative	797	Sulfur
		At5g57280		Protein carboxyl methylase-like	1,390	Sulfur
		At3g17390		Putative *S*-adenosylmethionine synthetase	2,185	Sulfur
Phenylalanine Ammonia-Lyase Activity						
Minimal *P*-value	Evidence	At3g53260	PAL2	Phenylalanine ammonia-lyase	272	Stress
2.18E−04	G	At2g37040	PAL1	Phenylalanine ammonia-lyase	293	Stress
Peroxidase		At5g64120	AtP15	Peroxidase, putative	28	Cell Wall
Minimal *P*-value	Evidence	At2g41480		Peroxidase, putative	33	Cell Wall
2.20E−04	K	At5g39580	AtP24	Peroxidase, putative	38	Cell Wall
		At1g14540	AtP46	Anionic peroxidase, putative	83	Cell Wall
3′(2′),5′-Bisphosphate Nucleotidase Activity; Inositol/Phosphatidylinositol Phosphatase Activity		At5g64000	SAL2	3(2),5-Bisphosphate nucleotidase (emb| CAB05889.1)	146	Growth
Minimal *P*-value	Evidence	At5g63980	FIERY 1	3(2),5-Bisphosphate nucleotidase	326	Growth
2.51E−04	G (2x)					

their specific degradation by thioglucoside glucohydrolase also known as myrosinases. None of the three myrosinase genes characterized to date (*TGG1*, At5g26000; *TGG2*, At5g25980; pseudogene *TGG3*, At5g48375; Husebye et al., 2002) changed expression in our experiments. However, two myrosinase-like genes (*BGL1*, At1g52400 and At5g28510) were strongly up-regulated during K^+ starvation (average fold change of 8.6 and 7.6, respectively) and down-regulated upon K^+ resupply. Furthermore, several myrosinase-binding protein, homologs of which are induced by wounding and exogenous JA treatment in *Brassica napus* (Taipalensuu et al., 1997), showed the same expression profile.

Polyamine Metabolism

The third group of known JA-responsive genes showing the typical expression profile of up-regulation during K^+ starvation and down-regulation upon K^+ resupply concerned genes involved in polyamine synthesis. High levels of the polyamine putrescine are well known to be associated with K^+ deficiency in plants (Watson and Malmberg, 1996). The strongest response in terms of strength and significance was found for *AtADC2* (spe2, At4g34710), which encodes an Arg decarboxylase. *ADC2* induction has been reported for various stresses (Urano et al., 2003) and various growth regulators including JA (Perez-Amador et al., 2002). By contrast, transcript levels of *AtADC1* (spe1, At2g16500), which is not regulated by JA (Perez-Amador et al., 2002), remained unchanged in our treatments. Transcripts of the two arginase genes implicated in Orn production (usually a polyamine precursor) responded to K^+ treatments in Arabidopsis (compare Tables II and III).

Defense Mechanisms and Production of Secondary Metabolites

As JA is involved in signaling events related to pathogenesis and wounding (McConn et al., 1997; Leon et al., 2001), K^+-responsive transcripts implicated in defense and secondary metabolism were also included in Figure 3. Within theses categories, several different expression profiles emerged. Most genes showed the typical response of up-regulation during starvation and down-regulation upon resupply. Strikingly strong regulation was observed for genes encoding polygalacturonase inhibiting proteins (*PGIP1*, *PGIP2*; Ferrari et al., 2003), aspartic proteinases, protease-inhibitors, and several FAD-related oxidoreductases. A second pattern consisted of a strong up-regulation in roots and/or in shoots after K^+ resupply with weak or nondetectable change during long-term starvation. Transcripts presenting this profile encoded protease-inhibitors, germin-like proteins, and a harpin-induced protein.

In summary, a large number of K^+-responsive transcripts are linked to JA either as components of the JA biosynthesis pathway or through well-known JA-dependent processes. A clear relation with JA was evident in several cases where out of two closely related genes only the known JA-dependent isoform was affected by the K^+ treatment (e.g. *CHL1* versus

Table IV. *Groups of functionally related genes up-regulated in shoots after K^+ resupply*

K^+-responsive groups of functionally related genes were identified by iGA based on RP lists of shoot genes sorted for up-regulation over 12 experiments (2 and 6 h K^+ resupply, three replicates with two controls each). Significantly affected gene groups up to a FDR of 10% are shown.

Groups		AGL	Code	Annotation	Rank[a]	Category
Extensin		At2g43150		Putative extensin	21	Cell wall
Minimal *P*-value	Evidence[b]	At4g33970		Extensin-like protein	34	Cell wall
1.22E−07	K	At4g38080		Extensin related	90	Cell wall
		At4g08370		Extensin-like protein	96	Cell wall
		At3g54590		Extensin precursor-like protein	115	Cell wall
Aabinogalactan; Fasciclin		At5g44130	FLA13	Fasciclin-like arabinogalactan-protein, putative	127	Cell wall
Minimal *P*-value	Evidence	At3g61640	AGP20	Arabinogalactan-protein	133	Cell wall
1.10E−05	K (2x)	At4g12730	FLA2	Fasciclin-like arabinogalactan-protein	156	Cell wall
		At1g03870	FLA9	Fasciclin-like arabinogalactan-protein	170	Cell wall
		At5g10430	AGP4	Arabinogalactan-protein	182	Cell wall
Aquaporin		At3g16240	TIP2,1	Delta tonoplast integral protein	20	Transport
Minimal *P*-value	Evidence	At3g26520	TIP1,2	Gamma tonoplast intrinsic protein	89	Transport
1.31E−05	T	At4g35100	PIP2,7	Plasma membrane intrinsic protein	106	Transport
		At4g01470	TIP1,3	Putative water channel protein	513	Transport
		At1g52180	TIP	Aquaporin, putative	538	Transport
		At2g37170	PIP2,2	Aquaporin (plasma membrane intrinsic protein 2B)	544	Transport
		At2g36830	TIP1,1	Putative aquaporin (tonoplast intrinsic protein gamma)	703	Transport
		At3g04090	SIP1,1	Hypothetical protein	712	Transport
		At4g00430	PIP1,4	Probable plasma membrane intrinsic protein 1c	807	Transport
		At2g16850	PIP2,8	Putative plasma membrane intrinsic protein	995	Transport
		At3g53420	PIP2,1	Plasma membrane intrinsic protein 2a	1,217	Transport
		At2g25810	TIP4,1	Putative aquaporin (tonoplast intrinsic protein)	1,357	Transport
Cell Wall		At5g49080		Putative protein	28	Cell wall
Minimal *P*-value	Evidence	At4g33970		Extensin-like protein	34	Cell wall
2.31E−05	M	At2g06850	At-XTH4	Xyloglucan endotransglycosylase (ext/EXGT-A1)	50	Cell wall
		At5g06640		Putative protein	59	Cell wall
		At4g08370		Extensin-like protein	96	Cell wall
Germin-Like Protein Family						Growth
Minimal *P*-value	Evidence	At1g72610		Germin-like protein	31	Growth
5.68E−05	F	At5g20630		Germin-like protein	80	Growth
Plastidial Acyl Carrier Protein		At4g25050		Acyl carrier-like protein	58	Growth
Minimal *P*-value	Evidence	At1g54580	ACP2	Acyl-carrier protein	231	Growth
1.32E−04	F	At1g54630	ACP3	Expressed protein	486	Growth
Plasma Membrane		At4g12420	SKU5	Pectinesterase (pectin methylesterase) family	87	
Minimal *P*-value	Evidence	At4g02520		Glutathione transferase, putative	94	
2.12E−04	G	At4g35100	PIP2,7	Plasma membrane intrinsic protein (SIMIP)	106	
		At5g65430	GRF8	14-3-3 Protein GF14 kappa	123	
Cell Wall (sensu Magnoliophyta)						Cell wall
Minimal *P*-value	Evidence	At4g12420	SKU5	Pectinesterase (pectin methylesterase) family	87	Cell wall
2.14E−04	G	At5g65430	GRF8	14-3-3 Protein GF14 kappa	123	Cell wall

[a]Rank within all time point and control RP gene list ($n = 12$ for shoot data, $n = 6$ for root data). [b]Evidence: K, Key words from gene names; T, transporters; M, MAtDB functional classification; F, TAIR gene family; G, GeneOntology annotation.

Table V. *Groups of functionally related genes up-regulated in roots after K resupply*

K^+-responsive groups of functionally related genes were identified by iGA based on RP lists of root genes sorted for up-regulation over six experiments (6 h K^+resupply, three replicates with two controls each). Significantly affected gene groups up to a FDR of 10% are shown. For rank and evidence details, see footnote of Table IV.

Groups		AGI	Code	Annotation	Rank	Category
Nitrate Transporter Activity		At1g08090	NRT2.1	High-affinity nitrate transporter NRT2	1	Transport
Minimal *P*-value	Evidence	At3g45060	NRT2.6	High-affinity nitrate transporter-like protein	2	Transport
1.05E−10	G (2x), K(3x), F, M (3x), T	At5g60780	NRT2.3	High-affinity nitrate transporter protein-like	3	Transport
Aquaporin; Water Channel Activity		At2g37170	PIP2,2	Aquaporin (plasma membrane intrinsic protein 2B)	32	Transport
Minimal *P*-value	Evidence	At4g35100	PIP2,7	Plasma membrane intrinsic protein (SIMIP)	68	Transport
1.53E−07	T, G (3x), K, F	At3g26520	TIP1,2	Gamma tonoplast intrinsic protein	73	Transport
		At3g53420	PIP2,1	Plasma membrane intrinsic protein 2a	97	Transport
		At3g61430	PIP1,1	Plasma membrane intrinsic protein 1a	143	Transport
		At2g34390	NIP2,1	Putative aquaporin (plasma membrane intrinsic protein)	434	Transport
		At5g37820	NIP4,2	Membrane integral protein (MIP)-like	643	Transport
		At4g00430	PIP1,4	Probable plasma membrane intrinsic protein 1c	823	Transport
		At2g36830	TIP1,1	Putative aquaporin (tonoplast intrinsic protein gamma)	847	Transport
		At2g16850	PIP2,8	Putative plasma membrane intrinsic protein	951	Transport
		At4g01470	TIP1,3	Putative water channel protein	954	Transport
		At1g52180	TIP	Aquaporin, putative	956	Transport
		At2g37180	PIP2,3	Aquaporin (plasma membrane intrinsic protein 2C)	1,060	Transport
		At5g18290	SIP1,2	Putative protein	1,276	Transport
		At3g04090	SIP1,1	Hypothetical protein	1,313	Transport
Peroxidase		At4g11290	AtP19	Peroxidase, putative	10	Cell wall
Minimal *P*-value	Evidence	At5g19890	AtPN	Peroxidase, putative	21	Cell wall
8.75E−05	K	At3g01190	AtP12	Peroxidase, putative	25	Cell wall
		At4g30170	AtP8	Peroxidase, putative	111	Cell wall
		At4g08770	AtP38	Peroxidase, putative	151	Cell wall
		At5g66390	AtP6	Peroxidase, putative	174	Cell wall
Arabinogalactan		At4g26320	AGP13	Arabinogalactan-protein	6	Cell wall
Minimal *P*-value	Evidence	At5g10430	AGP4	Arabinogalactan-protein	11	Cell wall
9.37E−05	K	At5g53250	AGP22	Arabinogalactan-protein, putative	47	Cell wall

CHL2; Tsuchiya et al., 1999; *ADC2* versus *ADC1*; Perez-Amador et al., 2002).

Cell Wall Related Genes

Cell wall related transcripts constituted another large category of K^+-responsive genes pointed out by iGA. Within this category, transcripts for (1) extensins, (2) xyloglucan glucosyltransferases, (3) arabinogalactans (AGPs), and (4) peroxidases showed highly significant changes (Fig. 4). Their main expression profiles were characterized by up- or down-regulation upon K^+ resupply. Most cell wall related genes did not show a strong response to long-term starvation (compare supplemental material).

Extensins

Most extensin and extensin-like transcripts identified by our analysis were up-regulated upon resupply. These proteins are generally associated with cell wall reinforcement (for review, see Kieliszewski and Lamport, 1994; Cassab, 1998; Sommer-Knudsen et al., 1998). Up-regulation of extensin encoding transcripts was first observed in roots (within 2 h of K^+ resupply), whereas increase of transcript levels in shoots appeared with a delay. Extensin expression has also been shown to be increased in response to wounding, pathogen infection, and exogenous application of methyl JA and other hormones (Merkouropoulos and Shirsat, 2003).

Xyloglucan Glucosyltransferases

A second group of cell wall related transcripts showing a strong but delayed up-regulation in shoots encoded group-1 xyloglucan endotransglycosidases-hydrolases, namely At-XTH4 and At-XTH7. These enzymes are involved in the rearrangement of the network formed by association of xyloglucans with

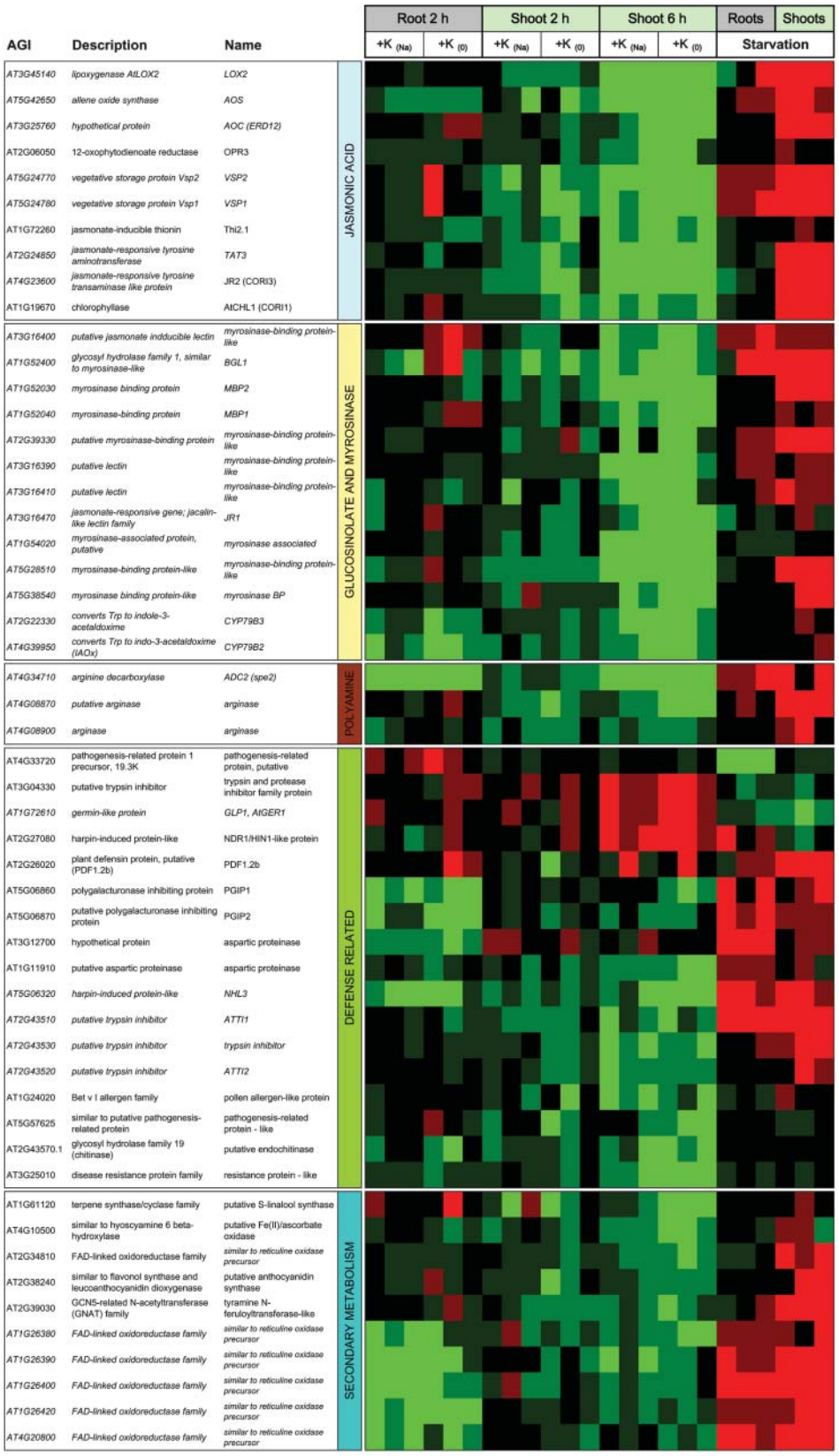


Figure 3. Expression profiles of highly significant K^+-responsive genes related to JA-related genes were extracted from RP lists for K^+ resupply experiments with FDR below 0.001%. On the left side AGI number, common name (if available) and a short description based on MIPS, TAIR, or TIGR are given for each gene. Genes identified by iGA are shown in italics. Functional super-categories extracted from iGA (Tables II–V) are given as vertical labels. On the right side expression profiles over all experimental conditions (compare Fig. 1C) are shown. Tissues and treatments are given on the top. Colors indicate change of transcript level in the treated samples with respect to the control samples (red for up-regulation, green for down-regulation, see color bar at the bottom of Fig. 4). For resupply treatments ($+K^+$) respective controls are given in brackets (Na for supply of Na^+ instead of K^+, 0 for supply of K^+-free medium). K^+-starved plants were compared to plants grown on K^+-sufficient medium. For each comparison data from three replicate experiments are shown.

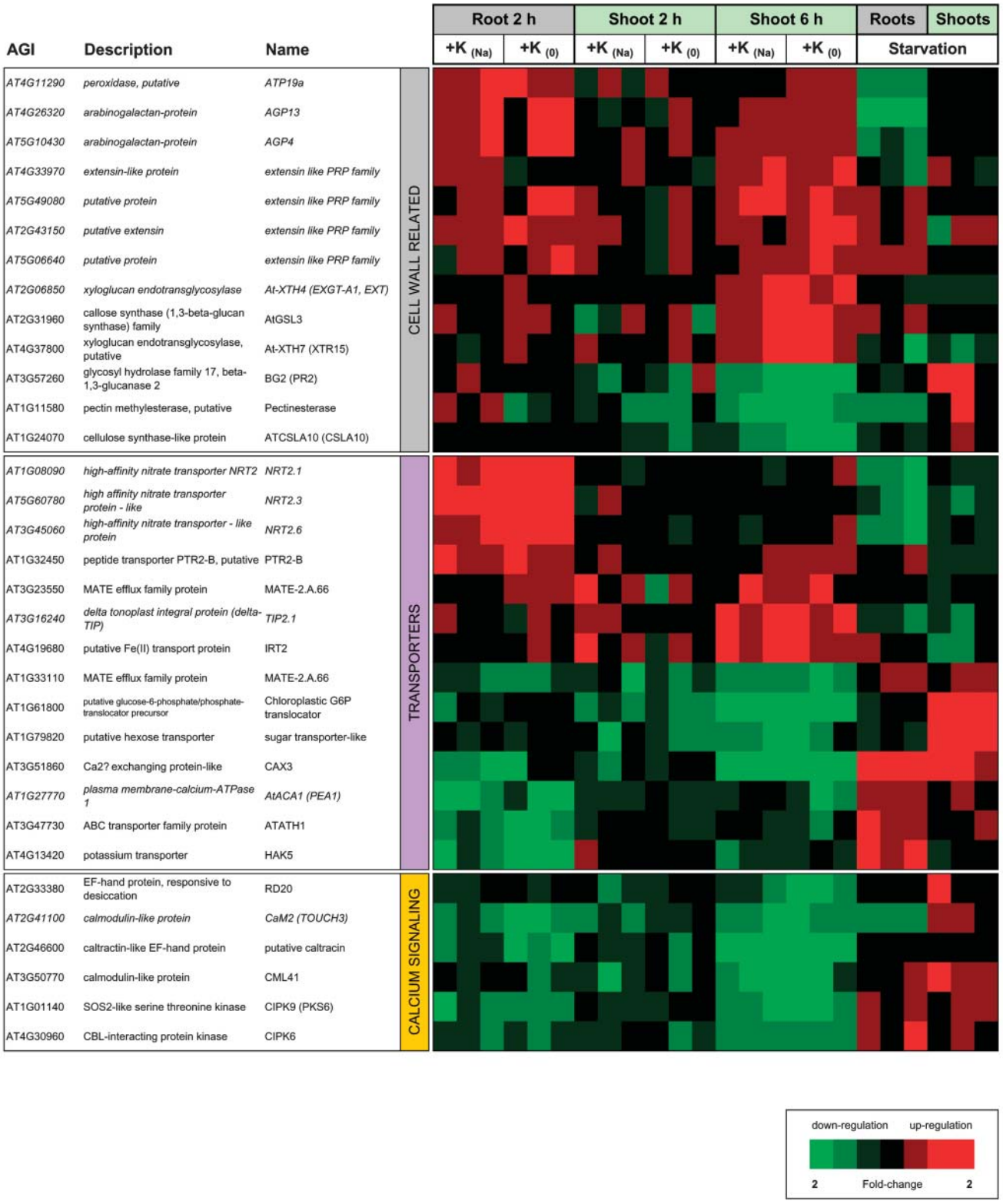


Figure 4. Expression profiles of highly significant K^+-responsive genes related to cell walls, transport, or calcium signaling. For explanation see Figure 3.

cellulose microfibrils (Cosgrove, 1997; Perrin et al., 1999). Although contrasting correlations between XTH and cell expansion have been reported, the overall available evidence argues for a functional role for XTH in primary cell wall enlargement (Rose et al., 2002). Besides its structural function, xyloglucans are also a source of growth-regulating oligosaccharides, which might play role in signaling (Perrin et al., 1999; Pilling and Hofte, 2003).

AGPs

Another well-represented group of K^+-responsive cell wall transcripts is the AGP family of highly glycosylated Hyp-rich glycoproteins (compare Tables IV and V). AGPs are localized in the cell wall and the plasma membrane and have been implicated in various processes of plant growth and development (Schultz et al., 2000; Gaspar et al., 2001; Showalter, 2001). Transcripts for *AGP4* and *AGP13* were down-regulated in roots during starvation and up-regulated upon K^+ reresupply. As most AGPs, all K^+-responsive AGPs contain a glycosylphosphatidylinositol-anchor, which provide an alternative to transmembrane domains for anchoring proteins to the cell surface (Udenfriend and Kodukula, 1995). As for animal cells, involvement of AGPs in signal transduction has been proposed for plants (Schultz et al., 1998).

Peroxidases

A member of the peroxidase gene family, *ATP19a*, known to be predominantly expressed in roots (Welinder et al., 2002), was strongly down-regulated in K^+-starved plants and derepressed upon K^+ resupply (Fig. 4). Many other members of this family were highlighted by iGA (Tables III and V). Peroxidases are involved in oxidative cross-linking of cell wall components (e.g. lignification) and in H_2O_2 detoxification. Secreted peroxidases are also involved in generating reactive oxygen species coupled to oxidation of hormones and defense compounds such as indole-3-acetic acid and salicylic acid (SA; Kawano, 2003).

Genes Encoding Membrane Transporters

K^+ Transporters. Changes in K^+ supply affected a large number of membrane transporters, but surprisingly only two of the transcripts belonged to a gene family with known function in K^+ transport. The *HAK5* gene, a member of the KUP/HAK family of K^+ transporters (13 members in Arabidopsis), was up-regulated during K^+ starvation and quickly down-regulated after K^+ resupply. The response occurred only in the roots. Similar results have been obtained with mature plants using real time PCR (Ahn et al., 2004). Another member of the same family, KUP12, was down-regulated in shoots after K^+ resupply (E-value 0.0079, FDR <0.004%; see supplemental material). None of the KUP/HAK genes that have been functionally characterized to date seem to be involved in K^+ nutrition although many of them transport K^+ when expressed in heterologous systems (Fu and Luan, 1998; Kim et al., 1998; Rubio et al., 2000), but some of them produce developmentally impaired phenotypes when knocked-out in Arabidopsis (Rigas et al., 2001; Elumalai et al., 2002).

Nitrate Transporters. Significant transcriptional changes were pointed out by iGA for nitrate transporters, aquaporins, and P-type pumps (Tables III–V). The strongest changes were observed for three members of the NRT2 family of high-affinity nitrate transporters. In contrast to *HAK5*, *NRT2.1*, *NRT2.3*, and *NRT2.6* were down-regulated during starvation and quickly up-regulated after K^+ resupply (Fig. 4). This response was specific for roots.

Aquaporins. Another group of genes that responded strongly to changes in external K^+ supply belonged to the family of aquaporins, which contains 38 genes in Arabidopsis. IGA identified subsets of 15 aquaporin genes in roots and 12 genes in shoots (9 common genes; Tables IV and V), which as a group were significantly up-regulated after resupply of K^+. Up-regulation of *TIP2.1* in shoots upon K^+ resupply was particularly significant and is shown in Figure 4. Aquaporins are membrane integral proteins that facilitate the transport of water across the plasma membrane and internal membranes (Chrispeels et al., 2001). Some aquaporins have been shown to transport small molecules such as urea, ammonia, and CO_2 in addition to water (Liu et al., 2003).

Pumps and Antiporters. A set of P-type pumps showed high group ranking among genes, which responded to K^+ resupply with a decrease of transcript level in roots (Table III), and included members of different functional classes such as plasma membrane proton pumps, known and putative Ca^{2+}-pumps, and heavy metal pumps (Axelsen and Palmgren, 2001). The strongest individual response was shown by the Ca^{2+} pump *ACA1*, which in addition to responding quickly to K^+ resupply was also up-regulated in roots during long-term K^+ starvation (Fig. 4). A possible link of K^+ nutrition and Ca^{2+} homeostasis was also evident in the transcriptional regulation of CAX3 by external K^+. CAX3, a tonoplast located Ca^{2+}/H^+ antiporter (Pittman et al., 2002), displayed strongly increased mRNA levels during K^+ starvation in both roots and shoots and was quickly down-regulated after K^+ resupply (Fig. 4).

Genes Related to Intracellular Ca^{2+} Signaling

A last category of K^+-responsive transcripts identified by iGA included genes encoding proteins with known or predicted Ca^{2+}-binding properties, such as calmodulins (Table III). Additional genes for calcium-binding proteins as well as interacting kinases appeared in the list of individual genes with highly

significant transcriptional response to K^+ resupply and are shown in Figure 4. Similarly to the group of genes identified by iGA, they were all down-regulated by K^+ resupply; in two cases the response was specific for shoots. Very few of the Ca^{2+}-related genes showed long-term responses during starvation. A transient response of these genes to changes in the environment is in accordance with their putative function in cellular signaling.

Other K^+-Responsive Genes

While iGA is a powerful tool to detect coherent groups of significantly affected genes, it may miss single genes with specialized functions or incomplete annotation. Thus, other genes that showed particularly strong significant changes (FDR <0.001%) to external K^+ supply are listed in the supplemental material. Among these are genes encoding transcription factors (bH2H, WRKY, NAM, AP2, E2F) as well as putative signal transduction components such as protein kinases (MAPK, receptor-like, and other types of protein kinases), ubiquitin-related proteins, and those containing putative F-box domains. Several transcript encoding stress responsive genes, GSH-dependent dehydroascorbate reductase (oxidative stress), *COR47*, *COR6.6* (cold stress), and *ERD1*, dehydrin (dehydration stress) were mostly down-regulated upon K^+ resupply and some of them also up-regulated during starvation. The same expression profile was also found for several transcripts encoding methyltransferase or methyltransferase-like proteins. Transfer of methyl groups through *S*-adenosylmethyltransferase occurs in several metabolic and hormonal pathways including the JA pathway. Transcripts such as *SYP81*, *COPII*, and *AtMEMB12* encoding trafficking components were up-regulated upon K^+ resupply. No such uniform expression profile was found for cytoskeleton-related transcripts. Finally a large number of K^+-responsive transcripts can be linked to primary metabolism. Among those, a particularly strong response in terms of fold change and reproducibility both during K^+ starvation (down) and after resupply (up) was found in a gene (At5g24420) with homology to 6-phosphogluconolactonase, the second enzyme of the pentose-phosphate pathway. Other primary-metabolism linked K^+-responsive transcripts are involved in sulfur assimilation (*APS1*, *APR3*), sugar metabolism (starch synthase, Glc-6-phosphate dehydrogenase), lipid synthesis (MGDG synthase *AtMGD3*, acyl-carrier proteins), and organic nitrogen metabolism (Glu dehydrogenase, Ala: glyoxylate aminotransferase 2).

DISCUSSION

As existing information on individual K^+-responsive genes has already been given in the Results section, the discussion focuses on three main questions: Which signaling pathways are involved in the perception of external K^+ and in the integration of responses at the whole plant level? Which membrane transporters are involved in nutrient allocation during K^+ deficiency? And, can we assign general physiological functions to K^+-responsive genes?

The K^+-Responsive Signaling Network

The response of JA to changes in external K^+ supply was the most conspicuous event observed in our study and therefore JA is likely to play a prominent role in plant responses to external K^+. Several transcripts for proteins involved in the biosynthesis of JA responded to K^+ starvation and resupply indicating that JA levels increase during starvation and rapidly decrease after K^+ resupply. The earliest steps of JA biosynthesis take place in chloroplasts (Schaller, 2001), and the transcriptional response of JA biosynthesis genes was indeed limited to the shoot with the exception of AOS, which was also regulated in the roots (Fig. 3). Since JA appears to be an important component of a K^+ signaling network, we compared our results with known JA responses, in particular those related to wounding and pathogen attack.

Signaling pathways leading to defense mechanisms are well characterized and involve positive and negative cross talks between JA, SA, and ethylene (ET; Devoto and Turner, 2003). This hormonal network has been progressively dissected using reporter genes for specific pathways as well as hormone signaling mutants and transcriptomic approaches (Berger, 2002; Van Zhong and Burns, 2003). ET production is unlikely to occur during long-term K^+ starvation since ET-biosynthesis and ET-responsive genes such as *ACO2* and *ERS1* (Gomez-Lim et al., 1993; Hua et al., 1998) were not changed in our treatments. Furthermore, *VSP2*, whose transcript level is strongly decreased in response to exogenous ET (Rojo et al., 1999; Van Zhong and Burns, 2003), was up-regulated during K^+ starvation. Similarly, searching our data for known SA-responsive genes such as *PR1-like* (At2g14610; Uknes et al., 1992; Laird et al., 2004), *PR5* (At1g75040), *NPR1* (At1g64280; Cao et al., 1997), and *WRKY18* (At4g31800; Yu et al., 2001) revealed no indication for increased SA production during K^+ starvation. Cross-talk between SA, JA, and ET has been modeled from gene expression data obtained from *Pseudomonas syringae* infected signaling-defective Arabidopsis mutants (using the Affymetrix 8K microarray; Glazebrook et al., 2003). The authors classified JA, SA, and ET signaling pathway on the basis of four specific responsive gene clusters (note that these clusters were only based on partial genome coverage). Comparing our results (restricted to <0.001% FDR gene list of all treatments and tissues) with these clusters revealed an overlap of K^+-responsive transcripts mainly with cluster D, representing COI1-mediated JA-dependent gene expression (37% overlap; Glazebrook et al., 2003). Very few K^+-

responsive transcripts were found in ET, SA, and combined clusters. We conclude that signaling pathways involved in plant responses to long-term starvation and short-term resupply of K^+ share signaling components with defense processes mainly through the JA pathway. This, however, does not preclude a role of ET in early responses to K^+ deprivation as recently proposed by Shin and Schachtman (2004).

A role of JA in nutrient signaling might not be restricted to potassium. Many JA-related genes including JA biosynthesis genes were also found to be induced in response to sulfur starvation (Hirai et al., 2003; Nikiforova et al., 2003) as well as in *sel1-10* mutants affected in the high-affinity sulfate transporter SULTR1;2 (Maruyama-Nakashita et al., 2003). By contrast, in nitrogen and phosphorus starved plants, no JA-responsive genes were identified by expression profiling of up to 8,000 genes, although differential regulation of several defense-related transcripts was observed (Wang et al., 2000, 2003; Hammond et al., 2003; Wu et al., 2003). To further assess this issue oxylipin profiling of plants exposed to various nutrient stresses is currently underway in our laboratory.

Whereas our microarray data revealed many downstream targets of JA signaling, identification of putative up-stream elements, in particular of those involved in early perception and signaling in roots, is more challenging. Several K^+-responsive peroxidases (Table III) and transcripts encoding enzymes related to oxidative stress were identified in our study supporting a role of reactive oxygen species in early K^+ signaling (Shin and Schachtman, 2004). H_2O_2 and other reactive oxygen species are also important signals in ion channel regulation (Demidchik et al., 2003; Kohler et al., 2003) as well as in early host-pathogen interactions (Lamb and Dixon, 1997).

Other candidates for early events in K^+-perception in roots include rapidly induced plasma membrane anchored cell wall proteins such as AGPs, known to participate in signaling events in animal cells (Tables IV and V; Fig. 4). Furthermore, transcriptional regulation of several Ca^{2+}-related proteins was detected in response to external K^+. This hints toward an involvement of cytoplasmic Ca^{2+} in K^+ signaling. Cytoplasmic Ca^{2+} was found to transiently increase after many abiotic stress treatments (Knight et al., 1997) but has not yet been assessed in response to changing nutrient supply.

Interestingly, many JA-related shoot genes responded to K^+ resupply before any measurable change of shoot K^+ had taken place (i.e. within 6 h, compare Fig. 2). It is therefore likely that they respond to an early root-shoot signal rather than to a rise of shoot K^+ content. The microarray data gave no obvious clues as to what might be the signal initiating the JA response. No genes involved in the synthesis of other hormones such as ABA, ET, or brassinosteroids responded to our K^+ treatments and the observed response of CYP79 genes with possible function in auxin biosynthesis is likely to be located downstream of JA rather than upstream.

K^+-Responsive Membrane Transporters

The finding that very few known K^+ transporters featured in the list of K^+ responsive genes is remarkable considering that K^+ homeostasis under varying K^+ supply requires reallocation of K^+ between different cellular compartments and tissues (Amtmann et al., 2004). The result is however in agreement with a recent microarray study on roots of mature, K^+-starved plants (Maathuis et al., 2003) as well as a northern-blot analysis of all Shaker K^+ channel genes (Pilot et al., 2003) and a real-time PCR analysis of all KUP/HAK/KT genes (Ahn et al., 2004). It appears that at the transcriptional level K^+ transporters are in general little responsive to external K^+. The strong transcriptional response to external K^+ of HAK5, a member of the KUP/HAK/KT family of putative high-affinity K^+ transporters, is an exception and might indicate a role in plant K^+ nutrition under low K^+ conditions. This will require further investigation using reverse genetic approaches.

The strong response of aquaporins to changing external K^+ supply is not surprising since K^+ is the main osmoticum and its uptake will be accompanied by an adjustment of water flux through aquaporins. Aquaporins might also contribute to ion homeostasis at the whole plant level, i.e. differential activity of tissue and membrane specific aquaporins will affect the ratio of apoplastic/symplastic water flow and thus direct solute flux through plant tissues.

A particularly strong response was found for three genes of the *NRT2* family of nitrate transporters, which were all down-regulated during starvation and up-regulated after K^+ resupply in roots. Furthermore, the nitrate inducible gene *NRT1.1* (*CHL1*, At1g12110), encoding a dual-affinity nitrate transporter, was up-regulated with a 3-time fold change in roots during starvation (E-value 0.0020; FDR $<0.013\%$; supplemental material). These results are interesting with respect to the well-known inter-dependence between nitrogen and potassium fertilization (Gething, 1993; Laegreid et al., 1999). Regulation of several nitrate transporter genes by changes in external K^+ was also reported for tomato (Wang et al., 2002). Although direct regulation of these genes by external K^+ cannot be excluded, this response might be related to changes in internal nitrate level or nitrogen metabolites (Forde, 2002). Changes in nitrogen assimilation during K^+ starvation were indicated by the up-regulation of characterized nitrogen assimilatory genes (Wang et al., 2003) such as *GS-GLN1-1* (At5g37600; E-value 0.0027; FDR $<0.01\%$) and *GS-GLN1-2* (At1g66200; E-value 0.0001; FDR $<0.005\%$) in roots and *AS-ASN1* (At3g47340; E-value 0.008; FDR $<0.027\%$) in shoots of K^+-starved plants (see supplemental material).

Finally, transcriptional regulation of genes encoding Ca^{2+} pumps and Ca^{2+} transporters (e.g. *ACA1* and

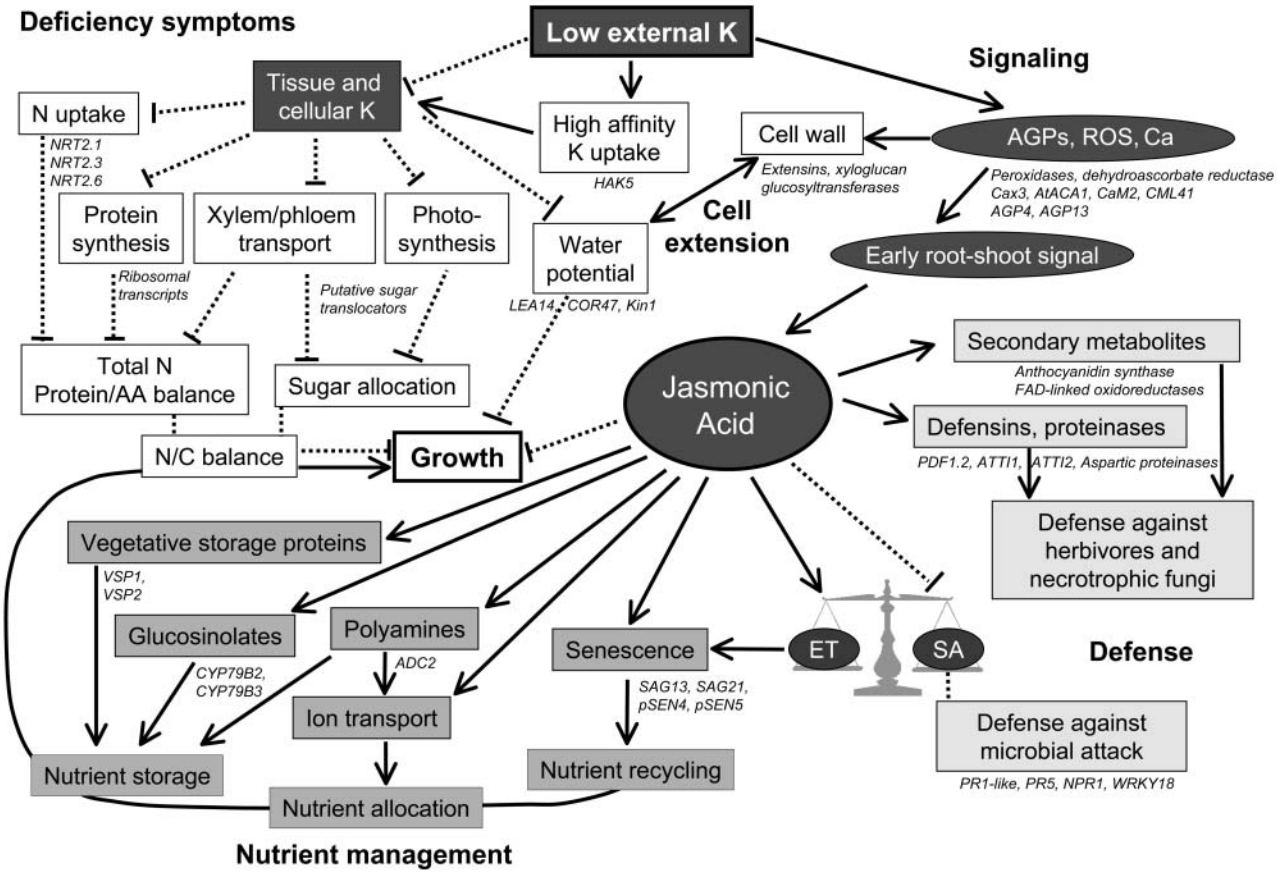


Figure 5. Model of molecular processes underlying plant adaptation to K^+ deficiency. Putative components of K^+ deficiency and adaptive responses are shown in boxes. Connecting lines are based on K^+-responsive genes identified in this study (shown in italics) and published information (see text). Black arrows indicate stimulation, dashed lines inhibition. Known K^+ deficiency symptoms are shown in white boxes. Putative components of signaling events are indicated in dark gray. Lighter gray shading marks different JA-dependent processes potentially leading to adaptive nutrient management and defense responses. For further discussion see text.

CAX3) was observed during K^+ starvation and resupply. Ca^{2+} can act as an osmoticum in fully expanded cells but due to its low mobility cannot replace K^+ in its osmotic function in fast growing tissues. Together with the elevated Ca^{2+} concentrations during K^+ starvation, up-regulation of Ca^{2+} transporters in K^+-starved plants could illustrate a preferential uptake of Ca^{2+} into the vacuoles of older tissues thus freeing up K^+ for transport to expanding tissues. The fast down-regulation of *ACA1* and *CAX3* upon K^+ resupply, which did not involve a change in external Ca^{2+} concentrations, suggests that active Ca^{2+} homeostasis is indeed a component of plant K^+ adaptation.

Physiological Role of K^+-Responsive Genes

This study has revealed a framework of physiological and metabolic processes occurring during plant adaptation to K^+ starvation. Based on K^+ regulated transcripts we developed a working model for future verification with other techniques such as reverse genetics and metabolic profiling (Fig. 5). Well-described symptoms of K^+ deficiency (Marschner, 1995) were reflected in many K^+-regulated genes, for example genes for nitrate transporters (reduced nitrogen levels), ribosomal proteins (inhibition of protein synthesis), and sugar translocators (impaired photosynthesis and long-distance transport). To compensate for decreased K^+ availability plants increase K^+ absorption through induction of high-affinity K^+ transport systems such as HAK5. However, during long-term starvation this strategy is not sufficient and the plant needs to engage in a proper acclimation response to complete its life cycle. This process requires perception and signaling of the plant K^+ status. Putative components of early signaling events emerging from our study include ROS, cytoplasmic Ca^{2+}, and cell wall proteins. JA was identified as the main player in the further integration of adaptive responses. Integrated nutrient management could involve storage of nutrients in energy-rich compounds, reallocation of nutrients via regulation of transporters, and recovery of nutrients from senescent leaves. K^+-regulated genes encoding vegetative storage proteins and enzymes

involved in the biosynthesis and degradation of glucosinolates and polyamines support the notion of altered storage strategies for nitrogen and carbon in K^+-deficient plants (Staswick, 1984; Rask et al., 2000; Kakkar and Sawhney, 2002). JA-dependent management of nitrogen allocation is in agreement with recent findings that JA treatment of tomato plants decreased nitrogen uptake and altered nitrogen partitioning toward root nitrogen storage (Meuriot et al., 2004). Regulation of K^+ channels by JA (Evans, 2003; Suhita et al., 2003) and polyamines (Brüggemann et al., 1998; Liu et al., 2000) has been reported and might contribute to K^+ reallocation between cellular compartments and tissues (Amtmann et al., 2004). Nutrient recycling is the purpose of the reversible stage of leaf senescence. Senescence leads to the induction of specific transcripts of so-called senescence-associated genes (Gepstein et al., 2003), several of which were reversibly up-regulated by K^+ starvation. Transfer of nutrients from senescing leaves to sink organs has been clearly demonstrated (Himelblau and Amasino, 2001; for review, see Quirino et al., 2000; Buchanan-Wollaston et al., 2003; Yoshida, 2003) and several lines of evidence support an involvement of JA (Park et al., 1998; Weaver et al., 1998; He et al., 2002). Finally, JA is well known for its role in plant defense responses against insect herbivores and (mainly necrotrophic) fungi, which are the most relevant enemies of K^+-starved plants (Perrenoud, 1990; Kessler and Baldwin, 2002; Kunkel and Brooks, 2002). One might hypothesize that the observed increase in JA serves to protect K^+-deficient plants against herbivore and fungal attack, while not entirely compensating for increased feeding and pathogen development. Conversely, constitutively increased JA levels in K^+-starved plants might interfere with inducible defense responses. JA, ET, and SA are all involved in pathogen responses, acting either synergistically or antagonistically on a partly overlapping range of pathogens and abiotic stresses (Turner et al., 2002). Elevated JA levels in K^+-starved plants might not only specifically inhibit some SA-dependent responses, but also generally interfere with the highly dynamic nature of an integrated stress response to pathogens.

CONCLUSIONS

Our study provides for the first time, to our knowledge, a comprehensive insight into molecular processes in growing seedlings induced by varying K^+ supply. The plant material used in the experiments was characterized with respect to morphology and tissue ion contents, and a thorough experimental design allowed us to assess both short-term and long-term effects and to compare them to several control treatments. We furthermore used new analysis tools for functional data mining that led to the discovery of four main super-categories of K^+-responsive genes namely genes related to the phytohormone JA, cell wall, ion transport, and Ca^{2+} signaling. On the basis of these findings, we propose a model of molecular mechanisms for nutrient stress adaptation. The large number of K^+-responsive genes identified here provides a platform for future analysis of their physiological roles as well as further dissection of signaling pathways involved in K^+ perception and nutrient homeostasis.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds were surface sterilized (2.5% sodium hypochlorite; 0.1% Tween 20) for 5 min, rinsed 5 times with sterile water, and placed in darkness at 4°C for 3 to 4 d to synchronize germination. Seeds were then sown in 120 × 120 mm square petri dishes (approximately 15 seeds/plate) containing 70 mL of nutrient medium with 3% Suc and 1% agar Type A (Sigma, Poole, UK) added. The control nutrient medium contained 1.25 mM KNO_3, 0.5 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 42.5 μM FeNaEDTA, 0.625 mM KH_2PO_4, 2 mM NaCl, and micronutrients (see Maathuis et al., 2003) at pH 5.6. In the K^+-free medium KNO_3 was replaced by $Ca(NO_3)_2$, KH_2PO_4 by NaH_2PO_4, and NaCl was lowered to 1.375 mM. Final ion concentrations in the two media, control (versus K^+-free), were 1.875 (0) mM K^+, 0.5 (1) mM Ca^{2+}, 1.25 (1) mM NO_3^- and 2 (1.375) mM Cl^- (all other ions unchanged). Petri dishes were sealed with parafilm and placed vertically under the light source (16 h at 100 μE) at 22°C. Resupply experiments were carried out with 2-week-old seedlings and consisted in replacing the condensed solution at the bottom of the petri dishes with liquid K^+-free medium supplemented or not ($-K^+$ control) with 10 mM KCl ($+K^+$ treatment) or 10 mM NaCl ($+Na^+$ control).

Ion Content Determination

For ion content analysis, seedlings from three to four petri dishes (approximately 50 seedlings) were pooled. A total of three seedling batches were grown and treated independently for replicates. Roots were briefly rinsed in ice-cold water and blotted dry before determination of fresh and dry weight. Ions were acid extracted in 2 M HCl overnight, diluted 50 times with distilled water, and analyzed by ICP-OES using an Optima 4300 DV Optical Emission spectrometer (Perkin Elmer Instruments, Wellesley, MA).

RNA Extraction, cDNA Synthesis, and Labeling

Shoot and root material were frozen and ground in liquid nitrogen. Total RNA was extracted using a guanidinium-based buffer and purified through a 5.7-M CsCl cushion as described by Chomczynski and Sacchi (1987). One hundred micrograms of total RNA were concentrated to a volume of 20 μL using a microcon column YM-30 (Millipore, Bedford, MA), denatured at 65°C for 10 min and cooled down to room temperature with 50 ng of a $polyT_{20}$ primer (MWG, Ebersberg, Germany). Labeling was carried out during reverse transcription adding 500 μM dATP, dGTP, dTTP, 200 μM dCTP, and 45 μM of either Cy3-dCTP or Cy5-dCTP (Amersham, Little Chalfont, UK) for synthesis of "control cDNA" and "treated cDNA," respectively, to the reaction mix, which also contained 500 units of Superscript II RT (INVITROGEN, Paisley, UK), 10 mM dithiothreitol, and 1 × Superscript II reverse transcriptase buffer (final volume of 40 μL). Labeled cDNA was purified using a PCR product purification kit (Qiagen, Crawley, UK) according to the manufacturer instructions.

Microarray Preparation and Hybridization

Microarrays spotted with the Arabidopsis Genome Oligo Set version 1.0. (Qiagen) were obtained from D. Galbraith (University of Arizona, http://www.ag.arizona.edu/microarray/). Slides were rehydrated and UV-cross linked according to the supplier's web page with an additional step consisting of a 45-min incubation in a blocking solution (5 × SSC, 0.1% SDS, 1% bovine serum albumin) followed by 5 rinses with double-distilled water. Excess water was drained using a 1,000 rpm spin for 5 min. Cy3- and Cy5-labeled cDNA

were speed-vacuum concentrated, resuspendend, and combined in a final volume of 36 μL formamide-based hybridization buffer (MWG, Ebersberg, Germany), deposited onto the array, and covered with hydrophobic coverslips (Sigma). Hybridization took place overnight at 42°C in a hybridization chamber. Arrays were then washes for 5 min at room temperature (2× SSC, 0.1%SDS; 1× SSC and 0.5× SSC solutions) and scanned immediately (ScanArray Express scanner and software suite; Perkin Elmer, Warrington, UK). Signal quantification was carried out using the fixed circle method as defined in QuantArray software.

Microarray Data Analysis

Normalization

For each chip, Cy3 and Cy5 hybridization signals were quantile normalized (Bolstad et al., 2003), so that the distribution of signal intensities in both channels became identical. Expression ratios were calculated directly from these data without background subtraction to obtain a minimal dependence of measurement variance on signal intensity. This procedure is a variation of the started-log procedure of Rocke and Durbin (2003) and led to excellent variance stabilization for our data (not shown).

Detection of Differentially Expressed Genes

Probes were sorted by their normalized expression ratio for each chip. Two sorted lists were produced for each chip, sorted in ascending and descending order, respectively. RPs were calculated for each gene according to Breitling et al., 2004b. RPs were compared to the RPs of 10,000 random permutations of the same data to assign E-values. To correct for the multiple testing problem inherent in microarray experiments we employed the FDR (Storey, 2003), i.e. we divided the E-value of each gene by its position in the list of changed transcripts. An FDR of $\leq$1% means that only 1% or less of the genes up to this position is expected to be observed by chance (false positives), the remaining 99% being genes that are indeed significantly affected (true positives).

Detection of Differentially Expressed Gene Groups

Classifications of genes into functional groups were obtained from various sources: gene ontology classifications and gene family assignments from TAIR (http://www.arabidopsis.org), functional categories from MAtDB (http://mips.gsf.de/proj/thal/db/index.html), and membrane transporter families from the annotation of the Arabidopsis Membrane Transporters microarrays (Maathuis et al., 2003). In addition, genes were classified according to the shared occurrence of keywords (strings of more than three nonnumerical characters, followed by any number of alphanumerical characters) in the gene names and functional annotation included in the microarray description files (http://www.ag.arizona.edu/microarray/). For each of these classification schemes we performed iGA (Breitling et al., 2004a), which determines the functional classes that are most enriched at the top of the gene lists sorted by rank products (separately for up- and down-regulation). The iGA procedure is based on calculating *P*-values using the hypergeometric distribution. For each functional class it iteratively finds the subset of members that minimizes this *P*-value. Only genes that were annotated in a specific classification scheme were considered for the calculations. As in the case of single genes, we face a multiple testing problem, because so many groups are examined simultaneously. To correct for this, we again used the FDR. An approximate FDR was estimated by comparing these results to those obtained for 100 randomly permuted lists for each type of classification.

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