Opinion

Using *Arabidopsis* to explore zinc tolerance and hyperaccumulation

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Identifying the particular gene or genes underlying a specific adaptation is a major challenge in modern biology. Currently, the study of naturally occurring variation in *Arabidopsis thaliana* provides a bridge between functional genetics and evolutionary analyses. Nevertheless, the use of *A. thaliana* to study adaptation is limited to those traits that have undergone selection. Therefore, to understand fully the genetics of adaptation, the vast arsenal of genetic resources developed in *A. thaliana* must be extended to other species that display traits absent in this model species. Here, we discuss how *A. thaliana* resources can significantly enhance the study of heavy-metal tolerance and hyperaccumulation in the wild species *Arabidopsis halleri*.

Using *Arabidopsis* to explore adaptation

Today, detecting the particular gene or genes that underlie a specific adaptation is a major challenge for a wide community of biologists. Analysing the genetics of adaptation has been greatly enhanced through the wealth of tools and resources developed for the study of model plants such as *Arabidopsis thaliana*. Such resources include the following: bacterial artificial chromosome (BAC) libraries, and BAC ends sequences; expressed sequence tag (EST) and cDNA collections; transcript profiling platforms; sophisticated technologies for the real-time non-destructive localization of proteins; sensors for and profiles of metabolite families; tools depending on RNA interference (RNAi); and collections of insertions lines mutant [1,2]. Owing to the use of *A. thaliana*, a significant increase in our understanding of the complex response of plants to stress was achieved [1,2]. However, research on stress biology, facilitated by the use of this model has, to some degree, lead to an under exploitation of non model species that are naturally tolerant to more extreme environments, but in which investigations are limited by the lacking of tools commonly available in model organisms [1,2]. Today, naturally occurring variation is considered as a necessary and valuable resource, complementing the use of *A. thaliana* mutants for the dissection of complex traits and the characterization of related genes [3,4]. The exploration of accessions of *A. thaliana* distributed worldwide in diverse environments revealed considerable variation for adaptive traits [3,4]. The development of high-throughput technologies enables exploitation of this naturally occurring variation. It also provides an opportunity to elucidate the evolution of complex traits by considering both the phenotypes in the natural environment and the molecular mechanisms underlying those phenotypes [3,4].

The study of variation identified in natural populations of *A. thaliana* currently provides the best opportunity for linking functional genetics and evolutionary analyses [5]. Nevertheless, studies of adaptation in *A. thaliana* are limited to those traits that have undergone selection in this model species. Moreover, crucial genes underlying important adaptive traits in other species might have evolved to function differently in the *A. thaliana* genome or might even be absent. Therefore, to understand fully the genetics of adaptation, the vast arsenal of resources developed in *A. thaliana* must be extended to its closest relatives that display the highest level of adaptation to extreme environments [2]. The species *Arabidopsis lyrata*, *Arabidopsis arenosa*, *Arabidopsis halleri*, and *Thellungiella halophila* were shown to display natural variation for complex traits, namely self-incompatibilty, hybridisation-polyploidization, heavy metal tolerance (see Glossary), and extreme osmotic stress tolerance that cannot be found in their close relative *A. thaliana* [2,6]. As a result of the knowledge and tools developed for *A. thaliana*, these wild species can now be used to address the genetics of stress adaptation [2,6].

Glossary

Heavy-metal tolerance: in plants, tolerance can defined as the ability to grow and reproduce in heavy-metal-polluted soils, which are toxic to most other plants of the same or different species.

Hyperaccumulation: ability of a plant to accumulate high metal concentrations in its aerial parts. The threshold values used to define hyperaccumulation depend on the metal considered and its concentration in the soil. For plants growing on native metalliferous sites, the following threshold have been proposed: >100 μg/g dry weight Cd, >1000 μg/g Ni, Co or Cu, >10 000 μg/g Zn or Mn. When growing in non-metalliferous soils, the definition will stress the capability of a hyperaccumulator to concentrate the metal in its aerial parts mainly as a result of an enhanced root-to-shoot translocation at amounts much higher than those observed for non-hyperaccumulating plants in the same conditions.

Metallicolous population: designates a population of pseudo-metallophyte species growing in a heavy-metal contaminated site.

Metalliferous soil: designates a soil characterized by high heavy-metal concentrations.

Metallophyte species: species growing in heavy-metal-contaminated soils. According to their occurrence on contaminated soils only, or on both contaminated and non-contaminated soils, they have been classified as being either absolute (strict or eumetallophytes) or facultative (pseudo-metallophytes) metallophytes.

Phytoremediation: the use of plants for environmental cleanup of heavy-metal-contaminated sites. Phytoremediation includes phytoextraction, in which plants are used to remove heavy metals from soils, and phytostabilization, in which plants are used to reduce the bioavailability of metals in the environment.

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1360-1385/$ – see front matter doi:10.1016/j.tplants.2008.02.006 Available online 11 April 2008

In this opinion article, we illustrate and discuss how recent knowledge and tools established in *A. thaliana* can stimulate and significantly enhance the study of heavy-metal tolerance and associated hyperaccumulation (see Glossary) in the wild metallophyte (see Glossary) species *A. halleri*.

A. halleri as a model to study zinc tolerance and hyperaccumulation

Zinc hyperaccumulators have attracted considerable interest for two reasons: Zn hyperaccumulation can potentially be used in phytoremediation (see Glossary) and biofortification [7–9]; and this specialized flora displays naturally selected tolerance to extremely high metal concentrations. Given that only a few species can survive and reproduce in this type of environment, this trait is established in the literature as one of the best examples to illustrate adaptation to extreme conditions [10]. Among the hypertolerant species, a rare class, named hyperaccumulator, is able to accumulate extremely high concentration of metals, in their leaves. Within the *Arabidopsis* genus, *A. halleri,* one of the closest relatives of *A. thaliana*, displays Zn hyperaccumulation and tolerance [6]. This species has colonized calamine soils, which are highly contaminated with Zn, Cd and Pb as a consequence of industrial activities. Many observations underline the dramatic alterations in metal homeostasis that occur in this species in comparison with *A. thaliana* or any close *Arabidopsis* relative. Under natural conditions, Zn concentrations ranging from 3000 to 22 000 μg/g dry biomass have been recorded in *A. halleri* leaves [11]. In addition, some populations have been reported to contain more than 100 μg/g dry biomass Cd in their leaves [12]. In hydroponic culture, *A. halleri* roots have been shown to tolerate at least 30-fold higher Zn and 10-fold higher Cd concentrations in their roots than *A. thaliana* can tolerate [13–15].

In addition to displaying traits of interest, *A. halleri* meets most of the criteria that define a model metal hyperaccumulating plant species (Box 1) [16]. Indeed, *A. halleri* is diploid ($2n = 16$) and its genome is only 40–60% larger than that of *A. thaliana* [6]. Both species display 94% nucleotide-sequence identity within coding regions [17]. This enabled the use of *A. thaliana* microarray chips to identify differentially expressed genes in *A. halleri* and *A. thaliana* [17–22]. With the aim of identifying the highly specialized genes underlying Zn and Cd tolerance and hyperaccumulation in *A. halleri*, several other tools have been developed for the study of this species. First, whereas segregating progenies cannot be obtained by crossing *A. halleri* and *A. thaliana*, *A. halleri* (*Ah*) can be efficiently crossed with its closest non-tolerant, non-hyperaccumulator relative, *Arabidopsis lyrata petraea* (*Alp*) [14]. Recently, this interspecific cross enabled the generation of *Ah* x *Alp* genetic linkage maps, and the identification of the quantitative trait loci (QTLs) regions for Zn and Cd tolerance [23–25], as well as for Zn hyperaccumulation [26]. Among the *A. thaliana* resources that can help to circumvent a significant part of the efforts required to understand Zn tolerance and hyperaccumulation in *A. halleri*, the high nucleotide-sequence identity and the good synteny with the known genome of *A. thaliana* [27,28] greatly help the positional-cloning gene strategies [25]. The genetic linkage maps available for *A. lyrata* [28] and the systematic sequencing of its genome, which was recently started (http://www.jgi.doe.gov/sequencing/why/CSP2006/AlyrataCrubella.html), also constitute precious additional resources. Second, a BAC library, generated on an individual metallicolous *A. halleri* plant, aids the isolation of promising gene candidates for Zn tolerance and hyperaccumulation (www.plant.univ-montp2.fr/stagem2/60.pdf; P. Berthomieu, personal communication). Third, a collection of 64 metallicolous and non-metallicolous *A. halleri* populations (see Glossary), widespread in central Europe and displaying constitutive Zn tolerance albeit with quantitative variations among populations [29,30], provides a useful tool for performing association mapping of Zn tolerance. Finally, the establishment of an efficient protocol for the transformation of *A. halleri* with *Agrobacterium tumefaciens* is in progress (I.N. Talke and U. Krämer, personal communication). This will, for the first time, enable the use of direct approaches, such as RNAi, to determine the functions of candidate genes. In this context, the self-incompatibility of *A. halleri* is a disadvantage, but it can be negated by treatment of the pistil with high CO_2 gas [31] (V. Llaurens, personal communication). Moreover, interesting transformants can be easily maintained as a result of the good clonal performance of *A. halleri* [32].

Box 1. Criteria for model metal hyperaccumulating plants

The following requirements can be used to evaluate whether a species or an accession could be a good model to study metal hyperaccumulation, according to Peer *et al.* [16].

Does the species hyperaccumulate metal in a defined soil media? Does it have a compact growth habit amenable to glasshouse propagation? Can it flower, self-pollinate, and set sufficient seeds on a reasonable time scale? Is it a diploid with a genome that is suitably compact for genetics studies? Can it be transformed with *A. tumefaciens* T-DNA and selected easily? Is it closely related to *A. thaliana*?

The model *A. halleri*

All the A. halleri populations characterized to date have been shown to tolerate and hyperaccumulate Zn in native metaliferous soil, as well as in controlled experiments, including defined soil experiments or hydroponics. In the greenhouse, *A. halleri* can grow, flower and be maintained by cloning in pots with a diameter of 17 cm. Its growth in hydroponics requires pots of 12-cm diameter with 1-L volume. *A. halleri* is self-incompatible and must consequently be outcrossed; interesting genotypes can be easily maintained by cloning, or the self-incompatibility can be destroyed with appropriate CO_2 treatment; it is able to flower after 24 weeks, including vernalization time; it produces usually 500–1000 seeds per plant. *A. halleri* is diploid ($2n = 16$) and its genome relatively compact, being 40–60% larger than that of *A. thaliana.* An efficient protocol for transformation of *A. halleri A. halleri* by *A. tumefaciens* is being developed. *A. halleri* is the closest Zn-tolerant hyperaccumulator relative of *A. thaliana*; the divergence between these two species is estimated to be 5.8 million years; both species display 94% nucleotide-sequence identity within coding regions.

The integration of *Arabidopsis* resources into our understanding of heavy-metal tolerance adaptation

Evidence indicates a major role for natural selection in the dynamic changes of transcriptomes [33,34]. The identification of genes differentially expressed in *A. halleri*

compared with non-tolerant, non-hyperaccumulator species, such as *A. thaliana* [17–22], or in progenies segregating for Zn hyperaccumulation [19], is thus a useful step in elucidating the genetic basis of Zn tolerance and hyperaccumulation. Comparing *A. halleri* and *A. thaliana* transcriptomes is a relatively easy method to quickly reveal candidate genes of *A. halleri*, but it might be a source of underestimation or overestimation of potential candidates. Underestimation might arise because some more highly expressed genes in *A. halleri* remain undetected for various reasons: first, these transcripts have only low levels of homology between the two species; second, *A. halleri* might contain genes missing in *A. thaliana*, as was suggested for the hyperaccumulator *Thlaspi caerulescens* [35]; or, finally, none of the microarrays platforms covers the entire *A. thaliana* genome. By contrast, overestimation might occur because differential expression of genes does not imply that they contribute to genetic metal tolerance or hyperaccumulation. It is difficult to distinguish between differentially expressed genes that are causative and those that show such an expression pattern as a consequence of the adaptive response, or simply as the result of divergence between species. A 'metal homeostasis function in *A. thaliana*' has therefore been considered as a supplementary criterion for identifying the most promising candidate genes for Zn tolerance and hyperaccumulation among the large number of differentially expressed genes revealed through transcriptomics [17,20,21,23].

The genes responsible for Zn tolerance are not necessarily more highly expressed in *A. halleri* than in *A. thaliana*, nor do they necessarily encode proteins directly involved in metal homeostasis (e.g. proteins such as metal transporters or metal chelators). Natural selection might act through structural mutations affecting protein function rather than transcript levels. Therefore, functional analyses, although not yet common with *A. halleri* genes [21–23,36,37], conducted on single genes and their corresponding proteins in heterologous systems represent another useful step in confirming or revealing novel candidates for Zn tolerance and accumulation. In this context, the plant defensins (PDFs), for instance, were reported to confer Zn tolerance and accumulation in yeast and might act as blockers of divalent metal cation channels [37]. Among the other types of genes that also require serious consideration are transcription factors that might alter the regulation of several genes overexpressed in *A. halleri,* and kinases, phosphatases and other regulatory proteins that might alter, for example, the activity, function or subcellular localization of a metal transport protein.

The most promising candidate genes, according to the present state of the art, emerging from microarray and/or isolated-gene studies, as well as the way in which they differ in *A. halleri* compared to the model species *A. thaliana* are given in Table 1.

QTL mapping is another powerful approach for dissecting complex adaptive traits, and for deciphering their genetic architecture [38,39]. In *A. halleri*, QTL analyses of Zn and Cd tolerance suggested only three QTLs for Zn tolerance and three QTLs for Cd tolerance, and these have a major additive effect in governing metal tolerance [23,24]. The QTL analysis of Zn accumulation, although not yet well defined, also implicated only a few QTLs in Zn hyperaccumulation [26]. The next challenge is to identify the genes underlying these QTLs. Unfortunately, this might be extremely difficult, if not impossible, because QTL regions can easily cover hundreds to thousands of genes, and the availability of genomic data on wild species such as *A. halleri* is limited. The high synteny, however, between *A. halleri* and *A. thaliana* enables the transfer of QTL regions from the *A. halleri* linkage map to the *A. thaliana* physical map. Making full use of the complete annotation of the *A. thaliana* genome [25], the transfer of QTL regions in *A. thaliana* will help to identify major candidates for Zn and Cd tolerance, as well as for Zn hyperaccumulation. Therefore, in combination with genome-wide transcriptome or single-gene studies, the transfer of QTL regions from *A. halleri* to *A. thaliana* provides an efficient means to reveal those genes that cause metal tolerance and/or hyperaccumulation among the large number of promising candidate genes currently reported. Indeed, on the basis of their position in the *A. thaliana* genome, the genes that co-localize with the QTLs of metal tolerance and/or hyperaccumulation after transfer to the *A. thaliana* physical map can be easily verified. A list of promising candidate genes for metal tolerance and hyperaccumulation has been generated through the integration of various *Arabidopsis* resources (Figure 1).

The origin of *A. halleri* heavy-metal tolerance adaptation

Recent studies have clearly established the occurrence of constitutive Zn tolerance in both metallicolous and non-metallicolous *A. halleri* populations [29], which strongly suggests that fixed Zn tolerance occurred only once, probably early in the species history [30]. In addition, evidence was provided for the occurrence of an *A. halleri* QTL involved in both Zn and Cd tolerance, as indicated by the co-localization of a Zn tolerance QTL with the major QTL for Cd tolerance, which explains almost half of the genetic variance [23,24]. Given that *A. halleri* is able to grow in calamine soils, with which Zn and Cd are generally associated, the most parsimonious hypothesis would be that metal tolerance initially evolved in *A. halleri* through the fixation of a QTL conferring tolerance to both metals. After the fixation of this QTL, specific tolerance to high Zn or Cd concentrations in the soil might have evolved through the fixation of additional QTLs that are particular to each population according to the specific geochemistry of its metalliferous growing site. The origin of metal tolerance in *A. halleri* would consequently follow the Orr exponential model of adaptation, which states that the distribution of factors during adaptation should be roughly exponential and that the first factors fixed often have larger effects than the next ones [40].

Because the QTL common for Zn and Cd tolerance is thought to be at the origin of the heavy-metal tolerance of the *A. halleri* species, we have decided to use this QTL as an example to illustrate how the integration of *Arabidopsis* resources helps in identifying the gene(s) at the origin of Zn and Cd tolerance in *A. halleri*. In *A. thaliana*, the QTL interval common to Zn and Cd tolerance covers a region comprising 739 genes. As previously described, natural

Table 1. Summary of microarray database on *A. thaliana* GeneChips hybridized with cDNA from *A. halleri* and *from A. thaliana* grown under normal conditions

Related function	AGI locus identifier	Name	Annotation	Higher expression in *A. halleri* than in *A. thaliana*	Microarray references	Functional references
Uptake into cells						
	At1g10970	ZIP4	ZIP family of metal transporters	Root	[20]	–
	At2g30080	ZIP6	ZIP family of metal transporters	Root–shoot	[17–20]	–
	At4g33020	ZIP9	ZIP family of metal transporters	Root	[19,21,22]	–
	At1g31260	ZIP10	ZIP family of metal transporters	Root	[20]	–
	At1g60960	IRT3	ZIP family of metal transporters	Root	[20]	–
Vacuolar sequestration						
	At2g46800	MTP1	Cation diffusion facilitator	Root–shoot	[17,18]	[36]
	At3g58060	MTP8	Cation diffusion facilitator	Shoot	[20]	–
	At2g39450	MTP11	Cation diffusion facilitator	Shoot	[20]	–
	At3g61940	MTPA1	Cation diffusion facilitator	Unknown	[18]	–
	At3g58810	MTPA2	Cation diffusion facilitator	Unknown	[18]	–
Remobilization from the vacuole						
	At2g23150	NRAMP3	Natural resistance associated macrophage	Root–shoot	[19,21]	–
	At4g18790	NRAMP5	Natural resistance associated macrophage	Root–shoot	[18]	–
Xylem loading						
	At4g30120	HMA3	P-type metal ATPase	Root–shoot	[17,18]	–
	At2g19110	HMA4	P-type metal ATPase	Root–shoot	[18,20]	[20,23]
	At3g08040	FRD3	Mutidrug and toxin efflux family transporter	Root–shoot	[20]	–
Chelation						
	At5g02380	MT2b	Metallothionein	Unknown	[18]	–
	At3g15353	MT3	Metallothionein	Unknown	[18]	–
	At5g56080	NAS2	Nicotiamine synthase	Root	[17,20,21]	–
	At1g09240	NAS3	Nicotiamine synthase,	Shoot	[20]	–
	At2g36880	SAMS3	*S*-adenosyl-Met synthetase, thiol biosynthesis pathway	Unknown	[20]	–
	At1g02500	SAMS1	*S*-adenosyl-Met synthetase, thiol biosynthesis pathway	Unknown	[20]	–
	At3g22460	OASA2	*O*-acetyl-Ser thiol lyase, thiol biosynthesis pathway	Shoot	[20]	–
Endomembrane transport						
	At3g12750	ZIP1	ZIP family of metal transporter	Unknown	[20]	–
	At2g32270	ZIP3	ZIP family of metal transporter	Root–shoot	[18,20]	–
	At5g62160	ZIP12	ZIP family of metal transporter	Unknown		–
Other metal handling and transport						
	At2g26020	PDF1.2b	Plant defensin	Unknown	[20]	[37]
	At3g27020	YSL6	Yellow-stripe-like transporter family (M-nicotiamine complex)	Shoot	[20]	–

Genes more highly expressed in *A. halleri* than in *A. thaliana* are selected as being good candidates according to the putative function related to Zn homeostasis of their corresponding protein in *A. thaliana*. The 'normal conditions' vary for the different microarrays experiments but are typically 1 μM Zn for *A. halleri* and *A. thaliana*.

selection might operate by modifying gene expression [17–22]. In addition, a 'metal homeostasis function in *A. thaliana*' can be considered as a supplementary criterion for identifying the most promising candidate. Among the 739 genes present in QTL, the present microarray analysis reveals that 11 are differentially expressed in *A. halleri* and *A. thaliana* (Box 2) [17–22]. Among these 11 genes, according to the function of their corresponding protein in *A. thaliana*, only one, namely the heavy metal transporting ATPases4 (*HMA4*) (At2g19110), is, with the present knowledge, a good candidate, because it belongs to the P-type ATPase family involved in the transport of transition metals. In *A. thaliana*, HMA4 is localized in the plasma membrane and is implicated in the root-to-shoot translocation of Zn, possibly by mediating xylem loading [41]. HMA4 isolated from *A. halleri* is, in a similar way to the *A. thaliana* homolog, more highly expressed in the root than in the shoot and is able to partially restore Zn tolerance in Zn-sensitive yeast and to confer Cd tolerance in yeast [20,23]. Therefore, it can be reasonably assumed that AhHMA4 performs a similar role to AtHMA4 and could actively participate both in hypertolerance - by transferring Zn and Cd from the cytoplasm to the xylem in the root cells - and in the hyperaccumulation - through its role in the root to shoot translocation of Zn. Moreover, recent research has demonstrated differences in copy number between *AhHMA4* and *AtHMA4* and has indicated that natural selection might have acted through structural mutations that also affect the protein function of AhHMA4 [20,23,41]. These additional modifications might, at least partially, contribute together with the increased expression of the root-to-shoot Zn and Cd translocation, the hyperaccumulation of metal in aerial parts, and the tolerance observed in *A. halleri* (Box 2). Furthermore, the genetic mapping of *AhHMA4* revealed its co-localization with the peak of the QTL involved in both Zn and Cd tolerance [23,24]. It has been shown that genes underlying QTLs are often positioned a maximum of 1 to 2 cM away from the position of the QTL peak [39]. Consequently, to date, *AhHMA4* is the strongest candidate gene for initiating the evolution of constitutive metal tolerance in *A. halleri* (Box 2). Therefore, further experimental tests to dissect its role in *A. halleri* must be one of the research targets although in a next future, the advance of the

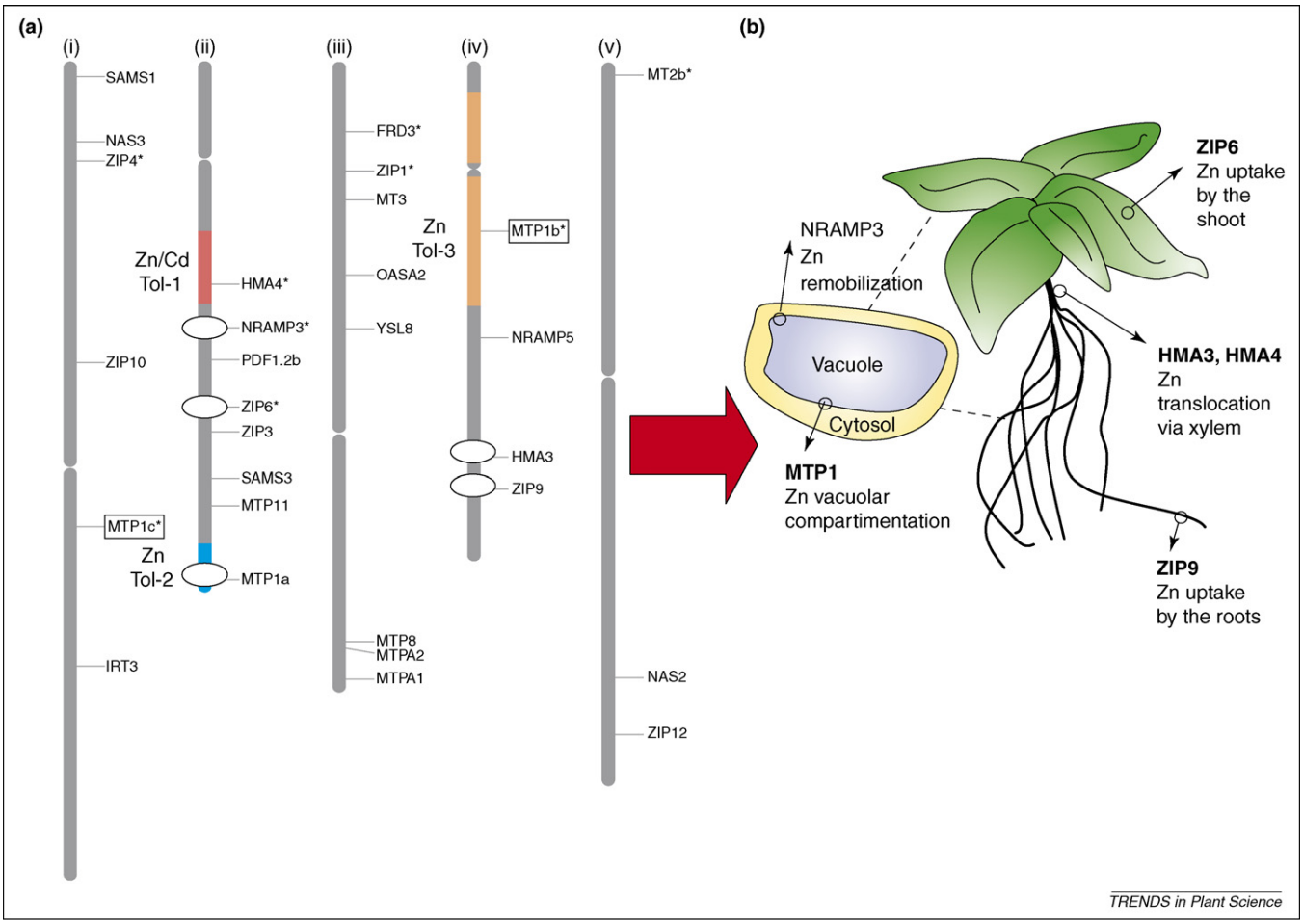

Figure 1. Integrating *Arabidopsis* resources to understand heavy-metal tolerance and hyperaccumulation *in A. halleri*. Making full use of the complete annotation of the *A. thaliana* genome [25], the transfer of QTL regions in *A. thaliana* can help in identifying major candidates for Zn and Cd tolerance, as well as for Zn hyperaccumulation. Therefore, in combination with genome-wide transcriptome or single-gene studies, the transfer of QTL regions from *A. halleri* to *A. thaliana* provides an efficient means to reveal those genes that cause metal tolerance and/or hyperaccumulation among the large number of promising candidate genes currently reported. **(a)** This can be done in three steps: (i) the high synteny between *A. halleri* and *A. thaliana* enables the transfer of the QTL for Zn tolerance (Zn Tol) [25], Cd tolerance (Cd Tol) [23] and hyperaccumulation [26] onto the physical map of *A. thaliana*; (ii) the candidates emerging from the microarray are screened for their presence within the QTL regions on the basis of their position in *A. thaliana*; (iii) the predicted presence of a gene in the QTL is verified by genetic mapping (*). At present, only the genes limiting the border of the Zn tolerance QTL and the QTLs common to Zn and Cd tolerance [23] are well anchored in *A. thaliana* and can therefore be exactly transferred onto the *A. thaliana* maps (dashed lines) [25]. The regions associated to a QTL for Zn hyperaccumulation [26] are indicated with ovals marks. Each oval mark on the *A. thaliana* map corresponds to the transfer of a gene present in this QTL region in *A. halleri* and anchored in *A. thaliana*. As previously reported, three copies [*MTP1a*, *MTP1b* and *MTP1c* (Metal Tolerance Protein 1)] were detected and mapped in *A. halleri*, whereas only one copy, corresponding to MTP1a, is present in *A. thaliana* [24,36]. The two supplementary copies (MTP1b and MTP1c) are annotated in boxes because they do not exist in *A. thaliana*. Their respective estimated localisation in *A. thaliana*, corresponds to the position of their closest marker on the *A. halleri* map and that is anchored in *A. thaliana* (At4g10180 for MTP1b; At1g46768 for MTP1c [25]. **(b)** Through the integration of various *Arabidopsis* resources, the promising candidate genes for metal tolerance and hyperaccumulation are thus: ZIP9, ZIP6, HMA3, HMA4, MTP1a, MTP1b and NRAMP3. The *A. thaliana* gene map in Figure 1a was generated using the Chromosome Map tool from the internet site http://www.Arabidopsis.org. Abbreviations: FRD, Ferric Reductase Defective; IRT, Iron-Regulated Transporter; MT, metallothionein; NRAMP, Natural Resistance-Associated Macrophage Protein-like metal transporter; NAS, NicotianAmine Synthase; OASA, O-AcetylSerine(thiol)lyase; PDF, Plant Defensin Fusion; SAMS, S-Adenosyl-L-Methionine Synthase; YSL, Yellow Stripe Like; ZIP, Zrt-(zinc-regulated transporter), Irt-like Proteins.

scientific knowledge in gene function like gene encoding transcriptional factors or regulatory proteins will probably reveals new candidates in this QTL region. Moreover, because the Zn tolerance tests sustaining the QTL analyses were only based on short-term root-elongation assays [24], the role of genes involved in processes of internal sequestration and/or detoxification in leaves could have been underestimated and/or missed. Therefore new phenotyping experiments based on a long-term assay in hydroponics have to be performed, and toxicity symptoms such as chlorosis, aerial biomass production or differential leaf size have to be considered, and these might reveal additional QTLs and candidate genes.

Using wild *Arabidopsis* relatives to elucidate adaptation to environmental stress

Most of the valid records for Zn hyperaccumulator species have been reported in the Brassicaceae family [42,43]. Within this family, only the species *A. halleri* and the genus *Thlaspi* unequivocally contain Zn hyperaccumulators [44,45]. Consequently, Zn hyperaccumulation has probably only arisen during two relatively recent evolutionary events within the Brassicaceae, and possibly on very few isolated occasions elsewhere in the angiosperms [43]. Therefore, concentrating research efforts on the study of the hyperaccumulators *A. halleri* and *T. caerulescens* will contribute to our understanding of the vast majority of Zn hyperaccumu-

Box 2. Identification of the gene(s) at the origin of heavy-metal tolerance in *A. halleri*

Because *A. halleri* is able to grow on calamine soils, in which Zn and Cd are generally associated, the most parsimonious hypothesis would be that metal tolerance initially evolved in *A. halleri* through the fixation of a QTL conferring tolerance to both metals. The strategy used to identify the most promising gene(s) at the origin of the heavy-metal tolerance in *A. halleri* combines transfer of the QTLs from *A. halleri* to *A. thaliana* (Figure I), and the identification of the candidates from microarray listed in Table I. The complete list of the most promising genes according to these criteria in the three QTL regions of Zn tolerance (Zn tol-1, Zn tol-2, and Zn tol-3) is provided as supplementary material (Supplementary Table 1). According to the function of their corresponding protein in *A. thaliana*, *HMA4* (*At2g19110*) is a good candidate for a heavy-metal tolerance gene, because it belongs to the P-type ATPase family involved in the transport of transition metals. Additional information provided from isolated genes on *AhHMA4* studies give further elements in favor of this candidate [20,23,41]: *AhHMA4* shows increased expression in roots (versus *A. thaliana*); *AhHMA4* expression is independent of external Zn concentration; AhHMA4 is encoded by more than one gene copy; the AhHMA4 cytosolic C-terminal domain differs highly from the AtHMA4 C-terminal domain.

In the near future, analysis of *A. halleri* HMA4 RNAi lines will greatly improve our knowledge about the contribution of HMA4 in the heavy-metal tolerance of *A. halleri*.

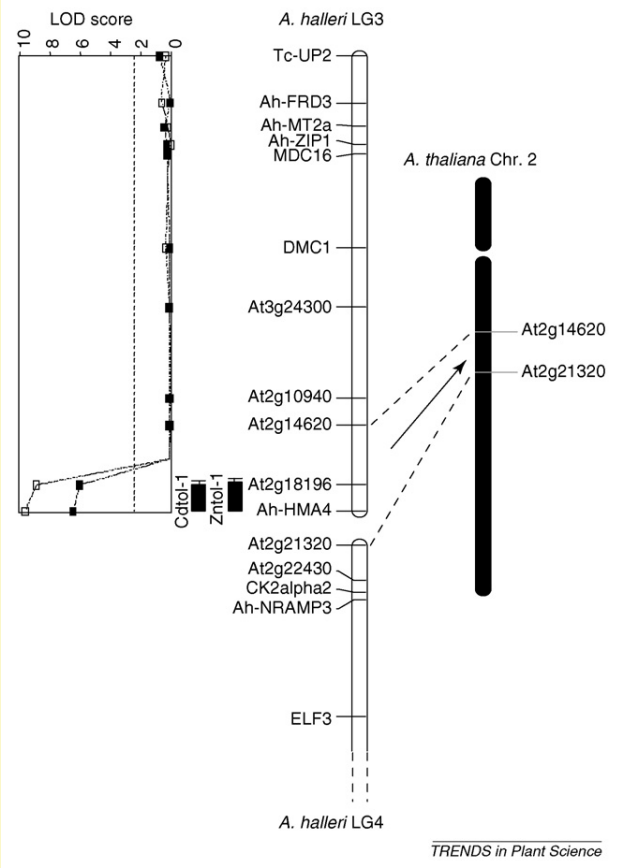

Figure I. Transfer of the QTL from *A. halleri* to *A. thaliana*. **(a)** The QTL involved in both Zn and Cd tolerance [23,24] is located on linkage group (LG) 3 of the *A. halleri* map. The LOD support intervals of the QTLs Zntol-1 and Cdtol-1 are indicated by black bars and whiskers (lines extending beyond the bars). LOD score profiles along LG3 are given for both Zn (black squares) and Cd tolerance (open squares). **(b)** Withinn the Zntol-1 and Cdtol-1 QTL regions, *AhHMA4* co-localizes with the QTL peaks **(c)** To identify the gene(s) underlying this QTL, the Zntol-1 QTL interval is transferred from *A. halleri* to *A. thaliana*. In *A. thaliana*, the Zntol-1 QTL interval is located on chromosome 2, and the markers *At2g14620* (on *A. halleri* LG3) and *At2g21320* (on *A. halleri* LG4) define the upper and lower limits of the Zntol-1 QTL interval in *A. thaliana*.

Table I. Identification of the candidates from microarrays

Candidate gene	Function	Refs
At2g16360	40S ribosomal protein S25	[17]
At2g16890	Putative glycosyltransferase	
At2g18720	Putative translation initiation factor	
At2g18480	Putative sugar transporter	
At2g16360	40S ribosomal protein S25	[21,22]
At2g16660	Nodulin like protein transporter	
At2g15830	Unknown protein	[22]
At2g19110	Heavy metal ATPase (HMA4)	[18,20]
At2g20730	Unknown protein	[19]
At2g20820	Unknown protein	
At2g20850	Putative LRR receptor protein	
At2g20860	Lipoic acid synthase	

lation phenomena in higher plants. Studies similar to those integrating *A. halleri* and *A. thaliana* resources have recently been initiated in *T. caerulescens* [35,46,47]. Indeed, QTLs for Zn and Cd hyperaccumulation in *T. caerulescens* were identified [48], although the *T. caerulescens* genetic map should now be anchored in the *A. thaliana* genome sequence to enable an integrated approach.

Another close relative of *A. thaliana* for which the research is well under way is *Thellungiella halophila*, which tolerates extreme cold, drought and salinity [1,2]. The results generated on physiology, gene expression and biochemistry that distinguish this extremophile from *A. thaliana*, and the tools developed for this species make it a valuable model to study tolerance to abiotic stress other than that caused by the heavy metals [49].

Furthermore, the Brassicaceae family comprises 3000 species, which are found in all types of natural environments colonized by plants [50]. Therefore, members of this

family display virtually all of the environmental stress adaptations occurring in plants. The integration of resources obtained from model and wild species of the Brassicaceae family becomes uniquely appropriate for interdisciplinary investigations of adaptation, bringing together fields as diverse as functional genomics, population genetics, ecology, molecular evolution and phylogenetics.

Acknowledgements
The authors thank Fabrice Roux, Pascal Touzet and four anonymous referees for their comments, which greatly helped to improve the manuscript. The authors also thank Lucy Moore for reviewing the manuscript. N. Roosens is supported by the Marie Curie intra-European Fellowship 'Metolevol' (contrat N°024683 MEIF-CT-2005–0224683), and G. Willems was supported by the European Research Training Network 'Metalhome' (HPRN-CT-2002–00243).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tplants.2008.02.006.

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