Merging methods in molecular and ecological genetics to study the adaptation of plants to anthropogenic metal-polluted sites: implications for phytoremediation

MAXIME PAUWELS,‡ GLENDA WILLEMS, NANCY ROOSENS, HÉLÈNE FRÉROT and
PIERRE SAUMITOU-LAPRADE
*Laboratoire de Génétique et Evolution des Populations Végétales, UMR CNRS 8016, Université des Sciences et Technologies de Lille,
Bâtiment SN2, F-59655 Villeneuve d'Ascq Cedex, France*

Abstract

Metallophyte species that occur naturally on metal-enriched soils represent major biological resources for the improvement of phytoremediation, a benign and cost-effective technology that uses plants to clean up anthropogenic metal-polluted soils. Within the last decade, molecular genetic studies carried out on several model organisms (including *Arabidopsis halleri*) have considerably enhanced our understanding of metal tolerance and hyperaccumulation in plants, but the identification of the genes of interest for phytoremediation purposes remains a challenge. To meet this challenge, we propose to combine '-omics' with molecular ecology methods. Using *A. halleri*, we confronted molecular genetic results with: (i) within-species polymorphism and large-scale population differentiation for zinc tolerance; (ii) the demographical context (e.g. migration pattern) of the species for zinc tolerance evolution; (iii) the Quantitative Trait Loci (QTL) analysis of the genetic architecture for zinc tolerance; and (iv) the fine-scale dissection of identified QTL regions, to discuss more precisely the nature of the genes potentially involved in the adaptation to zinc-polluted soils.

Keywords: *Arabidopsis halleri*, phylogeography, phytoremediation, pseudometallophyte, QTL mapping, zinc

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Introduction

Industrial and mining activities have left behind a legacy of ecosystems deeply impacted by a wide range of contaminants, including heavy metals (HMs). The amount of HMs in soils surrounding industrial or mining areas can indeed reach very high concentrations that are toxic for most biological organisms (Ernst 1990). HMs constitute a major threat to environmental health, disorganizing ecosystems through a drastic reduction of species richness (e.g. Banásová *et al.* 2006), but also to human health, directly or indirectly exposing humans to toxic doses of HMs (Jarup 2003). For these reasons, it is widely accepted that HM-contaminated soils have to be managed and rehabilitated as thoroughly as possible.

HMs are inorganic pollutants that cannot be degraded, but they can either be stabilized into less bioavailable forms in soils or extracted from soils (Pilon-Smits 2005). So far, numerous physical remediation methods have been developed and largely used (Mulligan *et al.* 2001; Bradl & Xenidis 2005). In the past decade, however, phytoremediation, i.e. the use of plants for environmental cleanup, has acquired an increasing popularity, mainly because it is cost-effective, safe for the environment and it benefits from the status of a 'green and clean' alternative to chemicals and bulldozers (Salt *et al.* 1998; Pilon-Smits 2005). Phytoremediation includes phytostabilization that uses plants to reduce the bioavailability of pollutants in the environment (Salt *et al.* 1998; Pilon-Smits 2005), but it is ideally considered as phytoextraction, i.e. the removal of HMs from soils (Lasat 2002; Whiting *et al.* 2004).

Correspondence: Pierre Saumitou-Laprade, Fax: +33 320436979;
E-mail: pierre.saumitou@univ-lille1.fr
‡Present address: Institut de Recherche sur la Biologie de l'Insecte,
UMR CNRS 6035, Faculté des Sciences, Bâtiment I, 31 Avenue
Monge, 37200 Tours, France

The occurrence of metal tolerance (Box 1) in plants that have naturally acquired the ability to survive in toxic metal-polluted soils (Antonovics *et al*. 1971; Macnair & Baker 1994) is an especially relevant biological resource for phytoremediation. These metallophyte plants are generally 'excluders', when their HM tolerance is related to the ability of the plant to restrict the entry of metals into the roots or their transport to the shoots, or '(hyper)accumulators', when the HMs can be concentrated in the shoots at sometimes more than 1% of their dry weight (Box 1, Baker 1981; Lasat *et al*. 1996; Baker *et al*. 2000). While excluders can be selected for phytostabilization and establishment of a vegetative cover on polluted sites, hyperaccumulators are considered for phytoextraction (Chaney *et al*. 2000; Whiting *et al*. 2004).

The tolerance of high HM levels in soils or tissues and the high rate of metal uptake are not sufficient to make a plant appropriate for phytoremediation. For practical and economical reasons, the selected plants must also produce a sufficient harvestable biomass, be responsive to agricultural practices and adapted to the wide range of environmental conditions that exist in contaminated soils (Chaney *et al*. 1997; Salt *et al*. 1998; Meagher 2000). Because phytoremediation is a long treatment, plants may also generate additional economically interesting by-products (Zayed *et al*. 2000). Unfortunately, hyperaccumulators are typically endemic to their native metalliferous soils (Baker *et al*. 2000; Whiting *et al*. 2004) and generally display low biomass production (Baker & Brooks 1989; Chaney *et al*. 1995). Therefore, the use of high-biomass crops (like *Zea mays*, or *Brassica juncea*) has also been given some thought (e.g. Murakami *et al*. 2007). The most promising alternative approach would consist in either selecting metallophyte genotypes with favourable growth characteristics (Salt *et al*. 1998; Chaney *et al*. 2000) or genetically engineering crop species to improve their metal tolerance and accumulation capacities (Whiting *et al*. 2004; Pilon-Smits 2005). Phytoremediation engineering could then involve either classical breeding or transgenic approaches (Zhu *et al*. 1999a, b; Wangeline *et al*. 2004; Singla-Pareek *et al*. 2006) or the exploitation of the available genetic variation for metal tolerance in crop species, through marker-assisted selection (Ghandilyan *et al*. 2006).

These approaches require the identification of the number and effect of the genes underlying the genetic variation between tolerant/nontolerant or accumulating/nonaccumulating species. In the past decade, extensive research efforts have been made to dissect the basic molecular mechanisms of metal tolerance in metallophytes (Clemens 2001). Physiological approaches, transcriptomics in particular, have allowed the identification of several candidates that seemed to result from modifications of the sequence or expression of genes belonging to the ubiquitous metal homeostasis network (Bernard *et al*. 2004; Dräger *et al*.

2004; Roosens *et al*. 2004, 2005; Hammond *et al*. 2006; Mirouze *et al*. 2006; Talke *et al*. 2006). However, none of these genes has been demonstrated to be at the origin of the trait so far and there is currently a need to distinguish, within the long list of candidate genes that have been identified, the gene(s) that play(s) a key role in the metal tolerance of plants and that could be genuinely useful for phytoremediation engineering.

It is currently accepted that the confirmation of the role of the candidate genes, that have initially been identified through '–omics' in molecular genetics studies, will require a more integrative approach including ecological genetics (Purugganan & Gibson 2003). Based on good ecological knowledge of the species studied and their character, such an approach should include an assessment of the association between the candidate genes and the phenotypes in controlled conditions, using either populations produced by controlled crosses [quantitative-trait-locus (QTL) mapping] or individuals isolated from wild populations that are structured by phenotypic differences (linkage-disequilibrium mapping). The approach should also involve molecular evolution studies and nucleotide profiling, to search for evidence of natural selection in the patterns of nucleotide variation observed in the candidate genes.

Integrative studies are necessarily organized around only a few model species that satisfy several criteria. Conventional model species, such as *Drosophila* and *Arabidopsis thaliana*, lack the stimulating characters needed for ecological studies, whereas many popular ecological models are genetically intractable, have poorly characterized genomes and have concerned smaller researcher communities (Feder & Mitchell-Olds 2003). Within the context of HM tolerance in plants, two pseudometallophyte species that both tolerate and hyperaccumulate very high concentrations of zinc (Zn) and cadmium (Cd) have recently emerged: *Arabidopsis halleri* (L) (O'Kane & Al-Shehbaz) [syn. *Cardaminopsis halleri* (L) Hayek] and *Thlaspi caerulescens* J. & C. Presl. Both are Brassicaceae species (Assunção *et al*. 2003). Because *A. halleri* is a very close relative of *Arabidopsis thaliana*, its genome is easily accessible and therefore this species is considered as one of the most promising models for the identification of the genes involved in metal tolerance and that could be used in phytoremediation engineering (Clauss & Koch 2006). *A. halleri* occurs on both metal-polluted and nonpolluted soils (Bert *et al*. 2000; Pauwels *et al*. 2006) and Zn and Cd tolerance and hyperaccumulation have been precisely described in the species (Bert *et al*. 2000; Bert *et al*. 2003; Pauwels *et al*. 2006). Thus, *A. halleri* has already been involved in many molecular genetics studies (Becher *et al*. 2004; Dräger *et al*. 2004; Weber *et al*. 2004; Filatov *et al*. 2006; Hammond *et al*. 2006). But although some candidate genes have already been proposed, the genetic architecture of metal tolerance and accumulation remains unsolved.

Box 1 Definition of heavy-metal tolerance: a complex plant–environment interaction

HEAVY-METAL TOLERANCE **is the capacity of a plant to survive and reproduce in a highly metal-polluted soil, toxic for most other plants (Antonovics *et al.* 1971; Macnair 1987).**

PHYSIOLOGICAL ASPECTS
How do metal-tolerant plants respond to high metal concentrations?
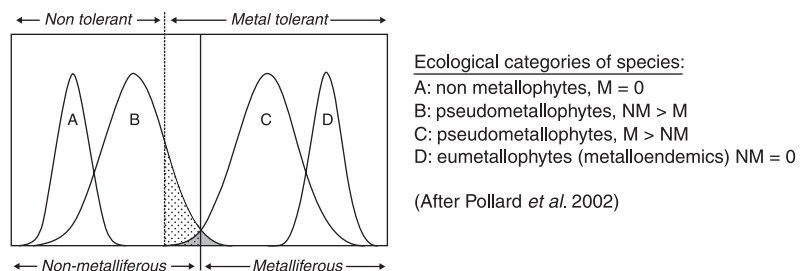

(After Baker, 1981)
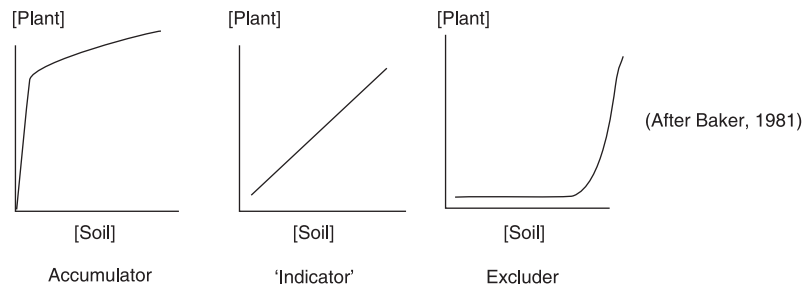
Accumulation and exclusion are the two main ways in which plants respond to increasing soil-metal levels, reflected by the metal concentrations in aerial plant parts. Excluders loose the control of metal translocation to aerial parts beyond a threshold of soil metal level. Hyperaccumulators are particular accumulators that show elevated metal concentrations in aerial parts mainly as a result of an enhanced root-to-shoot translocation (Lasat *et al.* 1996; Clemens 2001).

RESEARCH FOCUS: What are the **physiological pathways** responsible for heavy-metal tolerance? Are they different between excluders, indicators and (hyper) accumulators?

ECOLOGICAL ASPECTS
Is metal tolerance specific of plants from polluted soils?

Ecological categories of species:
A: non metallophytes, M = 0
B: pseudometallophytes, NM > M
C: pseudometallophytes, M > NM
D: eumetallophytes (metalloendemics) NM = 0

(After Pollard *et al.* 2002)

'Non-tolerant,' or 'metal-tolerant' refers to the plant genotype while 'non-metalliferous' or 'metalliferous' refers to the soil type. Non-metallicolous populations (NM) develop on non-metalliferous soils and metallicolous populations (M) develop on metalliferous soils. Non metallophytes (A category) never occur on metalliferous soils (no M populations). Pseudometallophyte (B and C categories) have metallicolous and non-metallicolous populations in various relative proportions (NM > M or NM < M). Eumetallophytes (D category) always occur on metalliferous soils (metalloendemics, no NM population).

RESEARCH FOCUS: What is the origin of the **genetic variability** of heavy-metal tolerance? How do metal tolerant genotypes maintain in non-metalliferous soils?
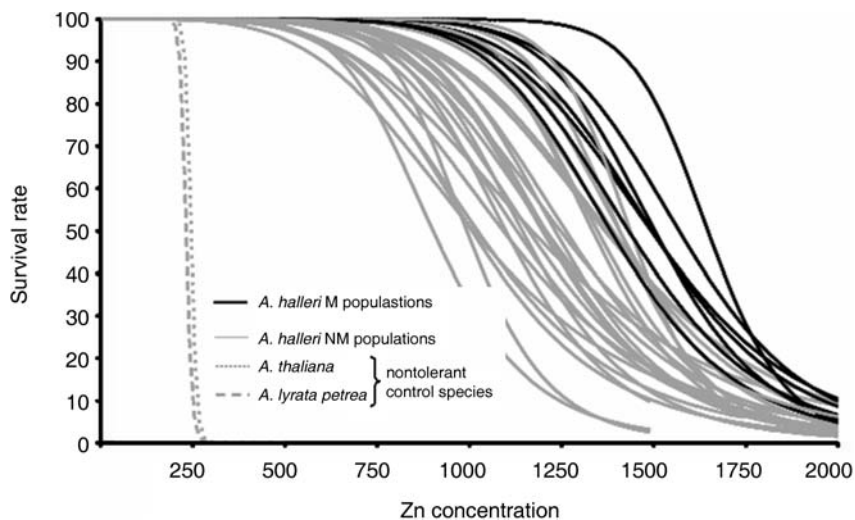
Fig. 1 Survival curves of *Arabidopsis halleri* populations (adapted from Fig. 3 in Pauwels *et al.* 2006). The population samples were exposed to increasing concentrations of zinc (Zn) in hydroponics (Pauwels *et al.* 2006). At each experimental dose, the survival rate of populations was recorded and survival curves were drawn. The survival curves of *A. thaliana* and *A. lyrata petrea,* used as nontolerant control species, severely dropped in the lowest exposure doses. Conversely, the survival rates of all *A. halleri* populations remained high at higher Zn concentrations, attesting constitutive Zn tolerance. The fact that mortality events generally occurred at higher Zn concentration in M populations than in NM ones was interpreted as a higher mean tolerance level of M populations.

Within this context, in order to contribute to an interdisciplinary study of the adaptation of plants to HM-polluted environments, we applied modern molecular ecology methods to study the Zn tolerance of the *A. halleri* model. Using *A. halleri,* we analysed: (i) the large-scale population differentiation for Zn tolerance, in order to assess the within-species polymorphism and population structure of the trait; (ii) the neutral genetic population structure, in order to provide a demographical context (e.g. migration pattern) for the within-species Zn-tolerance evolution; (iii) the genetic architecture for Zn tolerance, in order to determine the number and effects of the genomic regions involved in the character; and (iv) the fine-scale dissection of identified QTL regions, to more precisely discuss the nature of the genes that could be involved in Zn tolerance. In the present paper, we present our main results in a synthetic way to highlight the specific knowledge brought by such an integrative approach.

Tolerance is constitutive but varies quantitatively in *Arabidopsis halleri*

A faithful description of the distribution of Zn tolerance abilities in *Arabidopsis halleri* populations is a fundamental step in the integrative study of character. It provides an assessment of the overall genetically determined phenotypic polymorphism for metal tolerance in the species, on which any evolutionary change is based. This also allows identifying the genotypes of interest for genetic studies or phytoremediation purposes.

In the past years, we assessed the tolerance abilities of scattered populations of *A. halleri.* Our field work confirmed that the ecological range of *A. halleri* matched that of the typical pseudometallophyte species, with most populations being metallicolous (M) (Pollard *et al.* 2002). Consequently, only six out of 31 sampled populations were actual non

metallicolous (NM) populations; they were peripheral to the main species range, in three anthropogenic polluted areas, in northern France, Silesia (Poland) and Harz (Germany) (for details, see Bert *et al.* 2000; Pauwels *et al.* 2006). We disclosed that Zn tolerance in *A. halleri* was constitutive, i.e. concerned all genotypes, whatever their edaphic origin (Fig. 1, Pauwels *et al.* 2006). Significant differences in the Zn-tolerance abilities of individuals and populations were nevertheless observed. We brought out that there exists a quantitative polymorphism for tolerance in *A. halleri,* with a continuum from 'little-' to 'very tolerant' individuals (Fig. 1, Pauwels *et al.* 2006). The distribution of the polymorphism was heterogeneous and population differentiation was observed. Differences in the tolerance levels of populations was related not to available geographical variables (altitude, longitude, latitude), but to the HM concentrations in soils, with the highest tolerance observed in M populations (Pauwels *et al.* 2006).

Historical contingency of tolerance evolution in *Arabidopsis halleri*

Recent phylogeographical studies have revealed that biological species are not homogeneous genetic assemblages but rather subdivided into distinct genetic subgroups. They have revealed that both historical factors and population-genetic processes can act to shape the distribution of allelic variation in genes underlying ecologically important traits (Staton *et al.* 2001). They have highlighted that the identification of the population-genetic structure using neutral markers is a prerequisite for the global understanding of the evolution of adaptive traits (Feder & Mitchell-Olds 2003; Storz 2005; Wright & Gaut 2005).

In order to address the issues of the origin of the more tolerant M populations and of the genealogical relationships between those populations, we performed

a phylogeographical study of *Arabidopsis halleri* using 64 populations (including the populations phenotyped for Zn tolerance, mean sample size = 19.32 ± 12 individuals per population). Attention was paid to include populations that were representative of the European species range and from all known polluted sites where the species was reported. The chloroplast genome was used for genotyping. Genetic polymorphism was revealed using either PCR-RFLP directly, as presented in Pauwels *et al*. (2005), or a conversion of polymorphic sites revealed by polymerase chain reaction restriction (PCR)–fragment length polymorphism (RFLP) into SNPs detected by ABI PRISM® SNaPshot™ Multiplex system, a high-throughput SNP technology well adapted to large sample sizes [this concerned the genotyping of the K1K2 and CS chloroplast DNA (cpDNA) fragments of populations that are presented for the first time in Pauwels *et al*. (2005)].

Despite a strong population structure ($F_{ST} = 0.727, P < 0.001$), no significant phylogeographical signal was detected [$N_{ST} = 0.766$, $N_{ST} > F_{ST}$ with $P = 0.074$ (see Pauwels *et al*. 2005)]. Nevertheless there was a clear distinction between two groups of populations schematically separated by the 47° north parallel, corresponding to the Alps in the western part of the species distribution (Fig. 2). 'Northern' populations were essentially composed of one genetic lineage (including chlorotypes A to F) whereas 'southern' samples were essentially composed of another lineage (including chlorotypes G to O). This clear cleavage of the *A. halleri* populations into two divergent genetic assemblages corresponding to two chlorotype lineages likely indicates the occurrence of population differentiation through vicariance (Avise 2000). It supports the hypothesis that populations might have been lastingly separated in at least two distinct refugia (north and south of the Alps) during the last glacial age.

All M populations we sampled belonged to the northern group. Within this group, the population structure was the result of isolation by distance rather than of zinc exposure. The M populations from one geographical group were thus more genetically related to the geographically closest NM ones than to the M populations from an other group (see Fig. 2; for further details, see Pauwels *et al*. 2005). This indicates that M populations might have been founded independently in distinct polluted areas. This sustains the assumption that the enhanced tolerance of these populations might have evolved repeatedly, in parallel rather than through the migration of a single adaptation event.

Genetic architecture of the adaptation to polluted sites resolved by interspecif crosses and QTL mapping

The resolution of quantitative traits into discrete Mendelian locus analysis has made substantial progress since the development of genetic linkage maps. QTL mapping has proved to be very powerful for the analysis of complex adaptive traits (Doebley *et al*. 1997; Ungerer *et al*. 2002); it provides an efficient means to determine the number of genes implicated in a trait as well as their effects and interactions, which are important for understanding the evolutionary history of a trait (Mackay 2001; Erickson *et al*. 2004).

Until recently, the constitutive nature of Zn tolerance in *Arabidopsis halleri* (Pauwels *et al*. 2006) made its genetic analysis impossible. Macnair *et al*. (1999) were the first to bypass this major handicap by analysing the segregation of Zn tolerance in interspecific crosses performed between *A. halleri* and its closest nontolerant relative *A. lyrata petraea*. Based on the segregation analysis of one F2 progeny, the authors hypothesized that a single major gene determines Zn tolerance in *A. halleri*, as already described for other metals and species (Schat *et al*. 1993; Smith & Macnair 1998). In order to identify the QTL of Zn tolerance, we selected an *A. halleri* ssp. *halleri* individual from a highly contaminated industrial site in northern France which was crossed and backcrossed with *A. lyrata* ssp. *petraea*. The backcross (BC) progeny was used to construct a genetic map. The Zn tolerance of the BC progeny was measured by a sequential test established by Schat & Ten Bookum (1992). At least three QTL regions of comparable magnitude with additive effect (so-called Zntol1, Zntol2 and Zntol3), located on three different chromosomes (Ah3, Ah4 and Ah6) are involved in the adaptation of *A. halleri* to high Zn concentrations (Willems *et al*. 2007). These three QTLs explained 42% of the genetic variance of Zn tolerance.

Because of the metallicolous origin of the *A. halleri* parent of the BC1, it remains unclear which QTL region reflects the constitutive tolerance of the *A. halleri* species, and which one reflects the enhanced tolerance specifically observed in the metallicolous populations compared to the nonmetallicolous ones. The QTL mapping in recombinant populations produced either by interspecific crosses with *A. halleri* from NM populations or by intraspecific crosses between independently founded M and NM populations should allow to make the distinction between these different QTL origins (Pauwels *et al*. 2005).

From QTL regions to candidate genes identification: synteny among *Arabidopsis* species enables dissecting metal tolerance in plants

The isolation of the QTL regions conferring Zn tolerance (Zntol1, Zntol2, Zntol3) holds great promise for improving the identification of the main gene(s) responsible for the tolerance, as it restricts to about 40cM the genomic regions responsible for the trait (the two-LOD intervals associated with these QTLs cover 24 cm, 4 cm, and 13 cm, respectively). These regions still represent thousands of genes that are difficult to identify in species for which a dense physical map
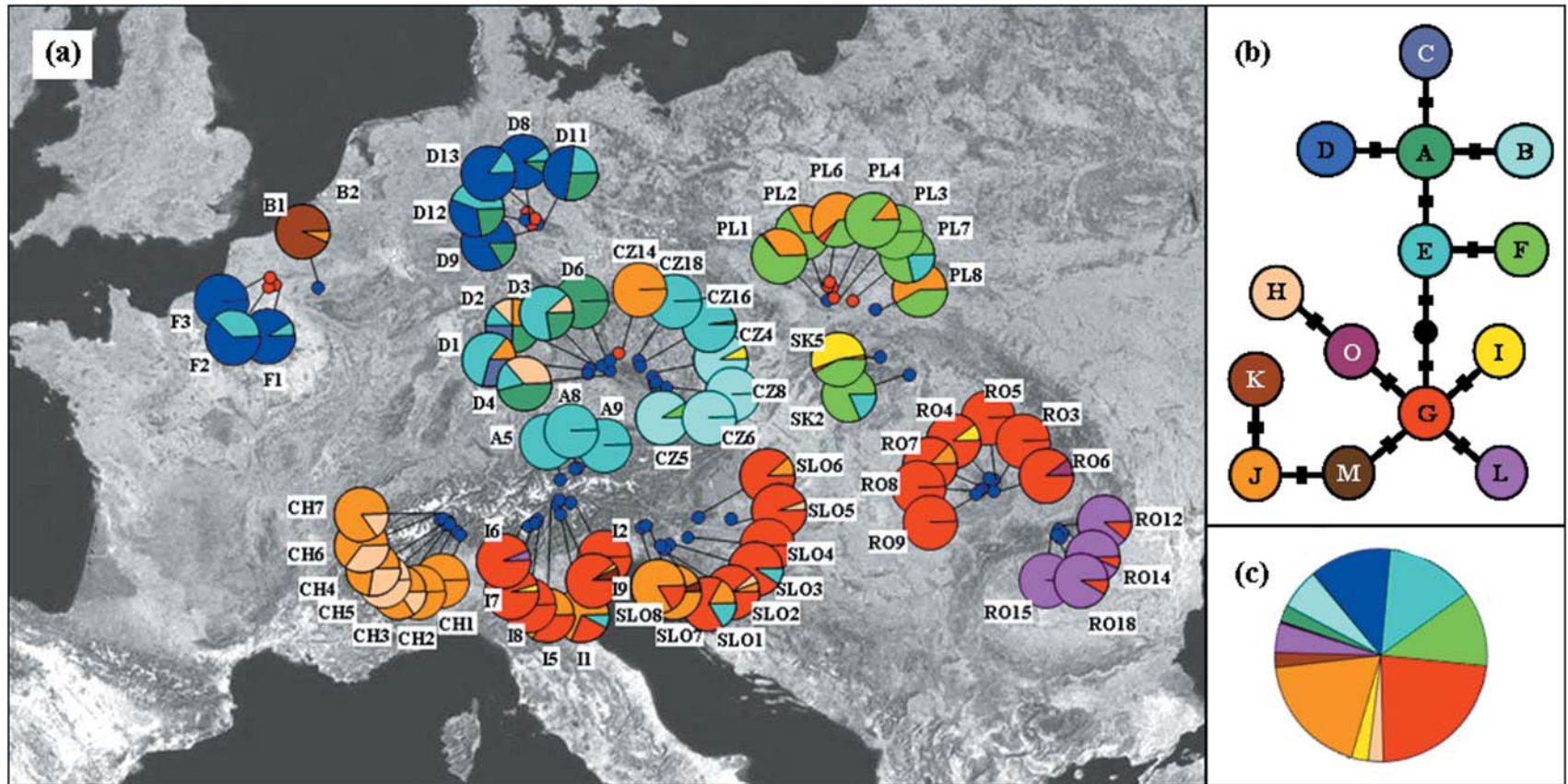
Fig. 2 (a) Distribution of *Arabidopsis halleri* chlorotypes in populations; (b) minimum spanning tree representing the phylogenetic relationships between haplotypes; (c) overall relative frequencies of chlorotypes (from Figs 1 and 2, Pauwels *et al.* 2005). Note that, in comparison to Pauwels *et al.* (2005), 36 populations were added and three additional chlorotypes were identified, thus totalling 14 the number of chlorotypes, labelled from A to O. However, no new mutation was identified. Chlorotypes M and O were indeed 'missing chlorotypes' in Pauwels *et al.* (2005). They were confirmed to display very low frequencies in the overall sampling (0.32% and 0.08%, respectively). Chlorotype L derived from chlorotype G by the same mutation that linked chlorotypes A and D. The homoplasic nature of the mutation was confirmed by sequencing (unpublished). Red circles represent metallicolous populations; blue circles represent NM ones. A, Austria; B, Belgium; CH, Switzerland; D, Germany; F, France; I, Italy; PL, Poland; RO, Romania; SK, Slovakia; SLO, Slovenia.

or complete sequencing are not yet available. Nevertheless, the close relatedness between *Arabidopsis thaliana* and *A. halleri* has enabled constructing the *A. halleri* genetic map using markers that were anchored in *A. thaliana* (Willems *et al.* 2007). The comparison of the *Arabidopsis* genetic maps revealed a very high synteny which allows us to transfer the QTL regions localized on the *A. halleri* genetic map to the physical map of *A. thaliana* (Fig. 3) and to use the complete annotation of its genomic sequence to shortcut the extensive cartography of the QTL regions.

The markers flanking the QTL regions Zntol1, Zntol2 and Zntol3 in the *Ah × Alp* map correspond, respectively, to coordinates 2763 kb and 8744 kb of Chromosome 2; 19049 kb and 19246 kb of Chromosome 2; and 1123kb and 9207 kb of Chromosome 4 in *A. thaliana*. These regions included 1529 genes, 78 genes and 302 genes, respectively, in *A. thaliana,* and the issue is now to select those that are liable to play major roles in the Zn tolerance and accumulation of *A. halleri*. Data coming from the literature in transcriptomics and functional genetics may help us with this task (Borevitz & Chory 2004).

During the past years, single-gene and transcriptomic approaches have been implemented to study Zn tolerance in hyperaccumulators such as *A. halleri* (Becher *et al.* 2004; Bernard *et al.* 2004; Dräger *et al.* 2004; Roosens *et al.* 2004, 2005; Weber *et al.* 2004; Hammond *et al.* 2006; Mirouze *et al.* 2006; Talke *et al.* 2006). One of the results highlighted by trancriptomic studies was the constitutive higher transcript levels of many metal homeostasis genes in metallophyte species compared to that of *A. thaliana*. In a first approach, we considered genes that have been shown to be differentially expressed in metal-tolerant species. These genes may logically be the candidate genes potentially involved in metal tolerance, and we used synteny to detect which ones among them colocalize with QTL regions. Only the transcript At2g46800, corresponding to the MTP1 gene in *A. thaliana,* is localized in the Zntol-2 region (Fig. 3). It is a vacuolar transporter and many data indicate that it could be involved in Zn detoxification in the hyperaccumulators *A. halleri* and *Thlaspi caerulescens*, through sequestration of Zn in the vacuole (Assunção *et al.* 2001; Dräger *et al.* 2004). Other good candidate genes emerging from microarrays like At4g30120 encoding HMA3, a putative P-type metal ATPase, were not localized within identified QTL regions. They may be disregarded with respect to having a major effect on the Zn tolerance of *A. halleri*. In a second approach, we considered the genes described in the literature as potential good candidates on the basis of their predicted function, which are localized in the regions of the *A. thaliana* genome corresponding to the QTL regions we identified, but which were not detected by transcriptomic studies. So far, we identified in the Zntol1-QTL region the metal homeostasis gene At2g19110 encoding HMA4, a P-type ATPase. This transporter is probably involved in Zn trans-

location in both *A. halleri* and *T. caerulescens* (Bernard *et al.* 2004; Papoyan & Kochian 2004; Talke *et al.* 2006; Courbot *et al.* 2007).
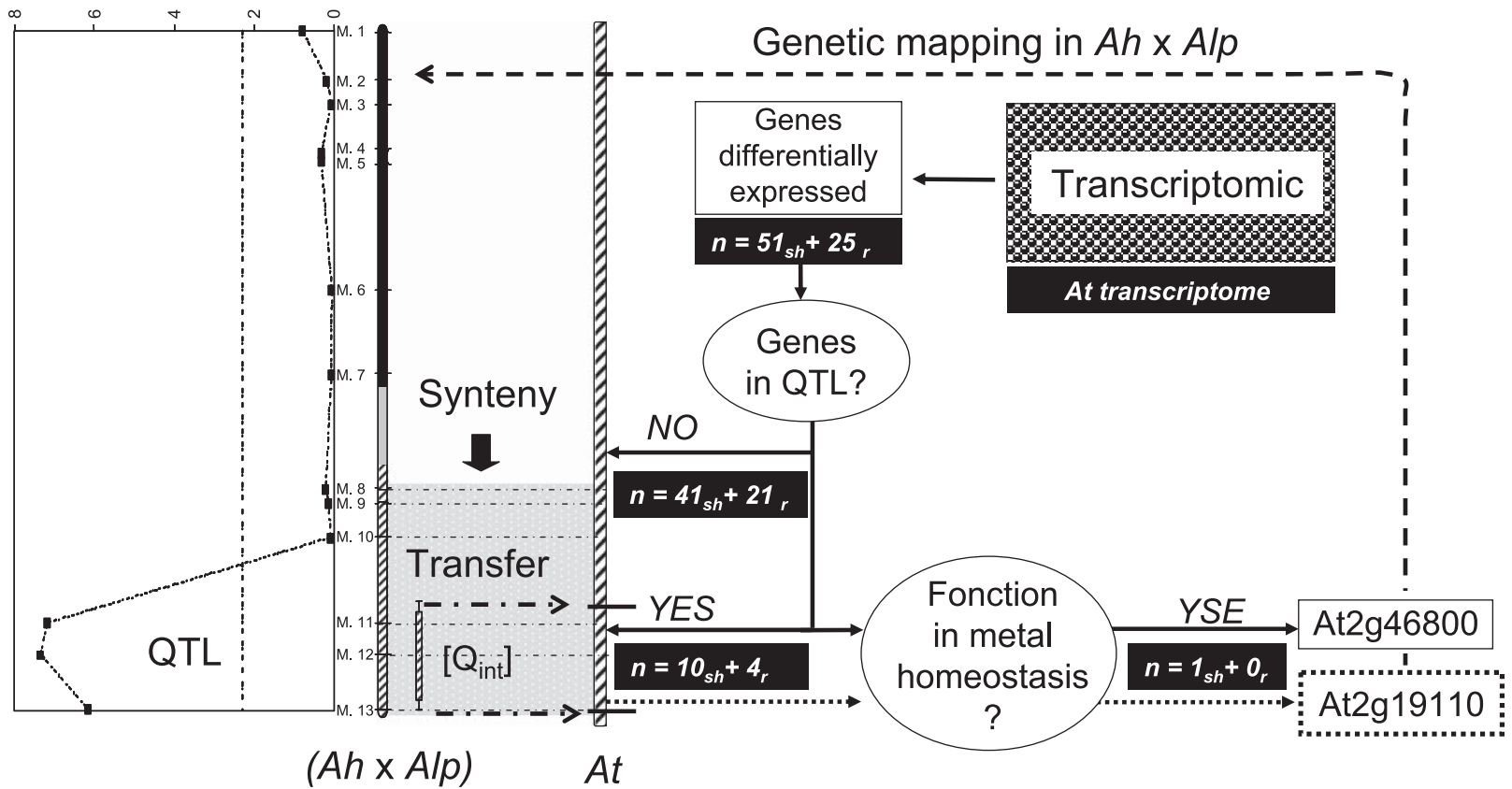
The position of the selected genes was confirmed by mapping on the *Ah × Alp* map using primers defined on the sequence of *A. thaliana*. Whereas HMA4 mapped exactly at the expected position on linkage group 3 (Ah3), two duplication events of the *MTP1* gene were detected in *A. halleri*. The *A. halleri* MTP1-A copy, localized on Ah4, is orthologous to the unique *A. thaliana* copy and the two paralogous copies identified in *A. halleri* (*MTP1-B* and *MTP1-C*) (Dräger *et al.* 2004) localized on two different linkage groups (Ah6 and Ah11, respectively). As expected, *MTP1-A* colocalizes with the QTL region Zntol2, but interestingly, *MTP1-B* colocalizes with the QTL region Zntol3. This last result shows the limits of using only the synteny to identify genes present in the QTL region of nonmodel organisms as the presence of a duplicated gene (or any other specific rearrangement) cannot be predicted.

Finally, confronting the genomic and transcriptomic data available with our QTL results allowed us to target *AhMTP1-A*, *AhMTP1-B* and *AhHMA4*. Consequently, these genes can be considered as the most relevant candidates for Zn tolerance. They are currently submitted to further investigations.

Conclusion: implications of the molecular ecology results for phytoremediation policy

In the last few decades, the tremendous developments in molecular biology and the success of genomics have highly encouraged studies in molecular genetics, mainly transcriptomics, for the identification of the functional genes implied in metal tolerance in plants. These studies have already succeeded in the identification of hundreds of genes that largely belong to the metal-homeostasis network. These genes are differentially expressed and/or regulated in metallophyte species with respect to *Arabidopsis thaliana* for various Zn concentrations (Becher *et al.* 2004; Weber *et al.* 2004). However, until today, the differential expression and/or regulation may be seen as either the primary cause of HM tolerance, its consequence or simply the result of the divergence time separating the species from *A. thaliana*. Nonetheless, pinpointing the few genes that are of major ecological importance for the colonization of metal-polluted sites and the hyperaccumulation of metal among all candidate genes is a prerequisite for their application in phytoremediation engineering.

Important information has already been produced by QTL studies that have been carried out during the last five years to investigate the genetic bases of the adaptation of metallophytes to metal-polluted soils. These studies have given a more precise description of the number of genes underlying metal tolerance as well as indications on the

Genetic mapping in *Ah* x *Alp*

Genes differentially expressed

$n = 51_{sh} + 25_r$

Transcriptomic

At transcriptome

Genes in QTL?

Synteny

NO

$n = 41_{sh} + 21_r$

Transfer

$[Q_{int}]$

YES

$n = 10_{sh} + 4_r$

Fonction in metal homeostasis ?

YSE

At2g46800

$n = 1_{sh} + 0_r$

At2g19110

QTL

M. 1
M. 2
M. 3
M. 4
M. 5
M. 6
M. 7
M. 8
M. 9
M. 10
M. 11
M. 12
M. 13

8 6 4 2 0

(*Ah* x *Alp*)   *At*

Fig. 3 The synteny between interspecific (*Ah x Alp*) and *Arabidopsis thaliana* (*A. t*) maps allows the transfer to *A. thaliana* of a *A. halleri* genomic region which includes the QTL interval ([Qint]). Transcriptomic method combined with QTL mapping. The comparative trancriptomic analyses reveal 51 genes in the shoot (sh) and 25 genes in the roots (r) that are differentially expressed between *A. thaliana* and *A. halleri* (Becher *et al*. 2004; Weber *et al*. 2004). Ten genes for the shoot and four genes for the roots are localized in QTL. Among these genes, only one transcript (At2g46800) has a function in metal homeostasis. The synteny also allows detecting one candidate gene (At2g19110) on the basis of its function in metal homeostasis. These two candidates have been mapped in the BC1 (*Ah × Alp*) to validate their position in *A. halleri*.

epistatic relationships that exist between those genes. Interestingly, all recent studies have confirmed that, as we demonstrated for *A. halleri*, metal tolerance is generally controlled by only a few genes with major effects that might have evolved under consistent directional selection (Bratteler *et al*. 2006). In *Thlaspi caerulescens*, using intraspecific crosses between plants from Pb/Cd/Zn-contaminated sites in Belgium and France, three QTLs and one QTL were mapped for Zn and Cd concentration in shoots, respectively. These QTLs explain 23.8–60.4% of the total variance of the traits (Deniau *et al*. 2006). In *Silene vulgaris* using intraspecific crosses between a serpentine and a nonserpentine ecotype, the genetic architecture of seven morphological, physiological and life-history traits was explored by QTL analysis and revealed a number of QTLs per trait of the same order (1–3) (Bratteler *et al*. 2006).

How and why is Arabidopsis halleri *a powerful model to study metal tolerance in general?*

As long as physical maps are unavailable, QTL studies will only describe the genetic architecture and relationships between QTLs. They will not allow us to determine the precise nature of the corresponding genes. However, this challenge could be overcome using synteny with a relative that has a sequenced genome to identify the candidate genes in the regions of the described QTLs (Borevitz & Chory 2004). Thanks to the many massive genomics projects involving *A. thaliana* and the sequencing project in progress on *A. l. lyrata* (http://www.jgi.doe.gov/sequencing/why/CSP2006/Alyrata Crubella.html), *A. halleri* may be the pseudometallophyte in which conditions are best met.

The very high synteny observed between *A. halleri* and *A. thaliana* has already greatly helped us to orientate the selection of the candidate genes according to their predicted functions. This synteny offered a unique opportunity for pinpointing among all the candidate genes those that actually matter for metal tolerance. Reciprocally, it enabled us to exclude the genes that could have been involved in metal tolerance but that were outside QTL regions. Finally, confronting QTL data to transcriptomic and genomic available data significantly reduced the number of genes that require further studies.

How can we further improve the understanding *of metal tolerance?*

It is currently well established that the candidate genes, revealed either using transcriptomics or synteny, should also display several additional features in order to be definitively related to adaptive characters (Feder & Walser 2005). In particular, corresponding proteins should have phenotypes relevant to the character under study (metal tolerance), and their encoding genes should bear the imprint of positive selection (Feder & Walser 2005). Thus, the understanding of the basic mechanisms of metal tolerance in plants necessarily requires that we take into account the natural variation in organisms' fitness as well as evolutionary processes shaping the patterns of genetic variation.

Analyzing population differentiation for metal tolerance emphasized that the tolerance levels of sampled genotypes could greatly differ according to their edaphic/geographical origin. The tolerance level can be particularly low for some NM genotypes or very high for some M ones. The occurrence of higher tolerance levels in populations that live on soils with higher metal concentrations is not restricted to *A. halleri* but is frequently observed in pseudometallophytes (Macnair 1983; Schat & Ten Bookum 1992; Schat *et al*. 1993; Smith & Macnair 1998; van Hoof *et al*. 2001). Phenotypic variation among populations is generally related to the occurrence of secondary selected hypostatic modifier genes that enhance the fitness of individuals after the colonization of metal polluted sites (Macnair 1993). Thus, the genetic mechanisms underlying metal tolerance may differ between individuals; it could be more complex in M genotypes because adaptation to the recent anthropogenic highly polluted sites could involve additional genes compared to NM genotypes with basal tolerance. Comparing mRNA variation among conspecific genotypes (i.e. that share recent common genetic background) showing different tolerance levels should help identify the genes for which a variation in expression reflects adaptive processes (Crawford & Oleksiak 2007). Moreover, whereas comparisons among conspecific individuals and populations are barely taken into account by transcriptomics studies that usually perform interspecific comparisons, they could greatly help distinguishing the basal mechanisms from the secondary mechanisms that are neither essential for metal tolerance nor interesting for phytoremediation engineering.

Our phylogeographical survey showed that geographically distant M populations might have been founded independently in distinct polluted areas. This is generally assumed for pseudometallophytes whose M populations are generally discontinuously distributed (Schat *et al*. 1996; Mengoni *et al*. 2003). To our knowledge, however, this is the first time that it is demonstrated using gene genealogy at the species-range scale. There are two antagonistic ways by which conspecific populations can independently converge towards equivalent levels of tolerance: the modified structures may either be homologous or nonhomologous. In the later case, the adaptive improvement of tolerance abilities may involve distinct mechanisms in distinct M populations. Therefore, simultaneously carrying out a phenotypic survey and a phylogeographical study in a metallophyte species highlights: (i) the need for studies to identify distinct tolerance mechanisms selected in independently founded M populations; and (ii) the genotypes that have to be included

in these comparisons. The identification of distinct tolerance mechanisms will be possible through a multiple QTL approach including M and NM genotypes from different geographical origins that have been demonstrated to be genetically differentiated. The discovery of distinct tolerance mechanisms could be of great interest in a perspective of phytoremediation: hybrids could be obtained that would combine all genetic adaptations. At the moment, dealing with such considerations is inevitably speculative since molecular studies comparing genetic mechanisms of tolerance in multiple genotypes, selected in independently colonized polluted sites, are still lacking. Our results provide evidence for the need to carry out such studies.

Why is the management of polluted sites important?

The interest of metallophytes for the development of phytoremediation technologies has already been largely demonstrated and is now consensually recognized by ecological scientists (Whiting *et al.* 2004). In particular because they have naturally acquired the ability to tolerate extreme metal concentrations in soils, metallophytes are considered to be 'the optimal choice for site restoration at mine closure, for the rehabilitation of metal-contaminated land [. . .] and the underpinnings for the development of environmental technologies' (Whiting *et al.* 2004). Our results further highlight the importance of including multiple M accessions in metallophyte studies to consider the putative intraspecific polymorphism of metal tolerance. Geographically distant metalliferous genotypes may have been independently selected, and tolerance mechanisms could differ between these genotypes. Combining different tolerance mechanisms could, for example, increase the ability to accumulate metals in hybrid lines that could be used for phytoextraction. Moreover, geographically distant M genotypes may be differentially adapted to local conditions, and such a differentiation could enable a fine-tuning of selected genotypes to match the ecological conditions of sites in need of remediation.

In summary, merging molecular and ecological genetics to the study of plant metal tolerance greatly improves the overall knowledge of metal tolerance mechanisms and provides a currently unreleased context for the exploitation of metal tolerance genes. This approach should quickly allow scientists to identify the metal tolerance genes that, in return, could be investigated in other pseudometallophyte or crop species and finally be used in phytoremediation engineering. Nevertheless, the study of metal tolerance is highly dependent on the occurrence of metallophytes in the wild, most of which have never been investigated by molecular studies. Metallophytes are commonly restricted to metal-polluted sites (Box 1, Whiting *et al.* 2004). Interestingly, the maintenance of these sites is also crucial for phytoremediation because they harbour the genetic resources necessary

for the discovery of genes of interest that will help develop phytoremediation techniques in the near future. Additionally, M populations of metallophytes have to be protected from the high risk of extinction associated to their restricted and dispersed habitat (Whiting *et al.* 2004). Therefore, although the remediation of polluted sites is necessary for environmental and human health reasons, we agree with Whiting *et al.* (2004) who insist that the management of polluted sites must also include the conservation of the biological resources they provide.

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The authors are or have been working at the laboratory Génétique et Evolution des Populations Végétales (GEPV). They are members of the team 'Genetics and evolution of plant adaptation to sites polluted by heavy metal' headed by Pierre Saumitou-Laprade. They are experts in population genetics of plant species and QTL mapping or functional genetics with a special attention given to the genetics and evolution of metal tolerance and hyperaccumulation of zinc and cadmium in *Arabidopsis halleri*. M. Pauwels's research interests focuses on the evolution of adaptive traits and its consequences in terms of population differentiation and speciation; G. Willems interest is focused on QTL mapping and candidate genes identification for stress tolerance; H. Frêrot's research topics concern the ecophysiological response of plants to metal pressure and the genetic architecture of metal tolerance and hyperaccumulation, she also has experience and practice in phytostabilization; N. Roosens has an expertise in the molecular identification and functional analysis of candidate genes for stress tolerance.