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Molecular Approach of Seagrasses Response Related to Tolerance Acquisition to Abiotic Stress

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1. Introduction

The debate surrounding climate change and its adverse effects on marine ecology is one of the most highly charged issues throughout the scientific community (e.g. Costanza et al., 1997; O'Neill, 1988). As far as seagrasses monitoring process is concerned, scientific data is needed that would contribute to the enhancement of marine environmental protection and their species conservation. Their use as biomarkers (Ferrat et al., 2003) is deemed as crucial due to the fact that they could be a reliable tool for researchers in the assessment of marine ecological status (transitional and coastal waters) in compliance with the Water Framework Directive (WFD, 2000/60/EC) and Marine Strategy (2008/56/EC) issued by the European Commission. Additionally, a challenge would be to deal with questions which arise from the underpinning tolerance mechanisms of seagrasses and whether they possess a sufficiently adjustable genetic background which in parallel can evolve in accordance with global warming.

Seagrasses play a critical role in the maintenance of marine environmental quality, creating complex, mosaic type habitats with high ecological and economic significance (Wiens et al., 1993; Hughes et al., 2003; Torre-Castro and Rönnbäck, 2004). The value of their contribution to the ecosystem is estimated at approximately 12,000€ per hectare/year, a part of which, concerns the support of commercial fish supplies (nurseries) and in general the conservation of marine biodiversity. Moreover, they contribute to coastal protection from sea waves, to the withholding of sediments and the recycling of nutritious substances (nutrient retention) (Cabaço et al., 2010), while they constitute important sources of carbon dioxide uptake from the atmosphere.

Seagrasses are highly productive submersed marine angiosperms that grow in shallow coastal and estuarine waters, providing key habitants of important ecological and financial value (Heck et al., 2003; Bloomfield and Gillanders, 2005; Heck et al., 2008). However, substantial

declines in such habitats have been reported worldwide, mostly attributed to light reduction from algal overgrowth, sediment loading and re-suspension, anthropogenic disturbance and global climate change (Duarte and Prairie, 2005; Duffy, 2006; Orth et al., 2006; Burkholder et al., 2007; Leoni et al., 2008). Changes in sea level, fluctuations in salinity and temperature can alter seagrass distribution, productivity, and community composition (Short and Neckles, 1999; Alberto et al., 2008).

Angiosperms are a unique group of plants comprising more than 50 species of monocotyledons which have returned to the sea, while retaining numerous physiological and morphological characteristics of terrestrial plants (Arnaud-Haond et al., 2007; Ito et al., 2011; Rubio et al., 2011). In doing so, they have evolved in a medium with a much higher salinity than that tolerated by their terrestrial counterparts. However, our knowledge on salinity tolerance mechanisms in these marine plants is limited compared with that concerning terrestrial plants and marine algae (e.g. Vermaat et al., 2000; Torquemada et al., 2005; Hartog and Kuo, 2006; Waycott et al., 2006; Touchette, 2007).

Evolutionary studies of seagrasses, which reconstruct the origin and development of salinity tolerance in a variety of plant lineages, may help us to understand why artificial breeding has failed to produce robust and productive salinity tolerant crops. Such studies may also help us develop new salinity-tolerant lines by revealing the order of components acquisition on salinity tolerance, or indicating favorable genetic background on which salinity tolerance may be developed. By examining the repeated evolution of this complex trait we may identify particular traits, or conditions that predispose species to evolve a complex, multifaceted trait such as salinity tolerance and give rise to halophyte lineages. More generally, this may shed light on the adaptation of angiosperm lineages to extreme environments (Dupont et al., 2007; Sharon et al., 2009). In order to achieve these hypotheses, more information is required on, at a minimum, the effects of salinity on the growth and ion relations of a wider range of plant species that may prove to be seagrasses (Flowers, 2004).

Therefore, important questions could be posed: (i) whether all seagrass species tolerate salinity in, fundamentally, the same way; (ii) whether specific mechanisms can be identified and, if so, whether these are linked taxonomically; and (iii) whether specific mechanisms have evolved to deal with interactions between salinity and other environmental variables (Vicente et al., 2004; Flowers and Colmer, 2008; Wissler et al., 2011). If so, are these common to different taxonomic groups and how often has salinity tolerance evolved?

2. Review of literature

Seagrasses are monocot plants which have evolved from terrestrial ancestors that returned to the sea approximately 100 million years ago and have adapted to growing on the sea bed (Touchette and Burkholder, 2000). They are exposed to an inexhaustible source of K^+ and conditions that vary slightly from 11 mM K^+, 470 mM Na^+, and pH 8.2. Although cells of seagrasses have a normal physiology and are energized, as other plants, by an H^+-pump

ATPase (Fukuhara et al., 1996; Garciadeblas et al., 2001), their K^+ transport system must be adapted to living permanently in a medium with a high K^+ content.

In living cells, potassium (K^+) is the most abundant cation whose contribution is considerable due to its ability to maintain the electrical and osmotic equilibrium of cell membrane. Since K^+ was selected for these functions very early in the evolution of life, the cellular processes evolved within a K^+ rich medium and many of them became K^+-dependent. Plant cells are not exceptions to these K^+ requirements, but with the peculiarity that, in the Cambric Era, plants evolved on the rocks emerging from the sea where they had to adapt to taking up K^+ from an extremely poor environment. In these conditions, plants developed complex mechanisms of K^+ uptake and distribution. At present, most soils are less K^+ deficient than cambric rocks, but still K^+ occurs at low concentrations and K^+ acquisition and distribution play key roles in the physiology of contemporary terrestrial plants (Rodríguez-Navarro and Rubio, 2006).

In terrestrial plants, trans-membrane K^+ movements are mediated by several types of non selective cation channels (NSC) (Fig. 1), and by transporters that belong to two families KcsA-TRK and Kup-HAK, present in prokaryotes and eukaryotes (Schachtman and Schroeder, 1994; Quintero and Blatt, 1997; Santa-María et al., 1997; Fu and Luan, 1998; Kim et al., 1998; Rubio et al., 2000; Rodríguez-Navarro and Rubio, 2006). The extensive expression of KT-HAK-KUP transporters in many organs of the plant suggests that they coexist with K^+ channels and that their functions may be redundant or perhaps complementary to these channels (Garciadeblás et al., 2002). Low-affinity K^+ uptake is thought to be mediated primarily by K^+ channels whereas, high-affinity K^+ uptake is dominated by transporters. However, it was found that K^+ transporters and channels may operate in parallel in the plasma membrane of root cells (Garciadeblás et al., 2002). Transporters would have their range of activity at micromolar K^+ concentrations, whereas transport at millimolar concentrations would be mediated by K^+ channels (Rodríguez-Navarro and Rubio, 2006). In contrast to this notion, it is now evident that some channels mediate the transport of K^+ at micromolar concentrations (Dennison et al., 2001), that some HKT transporters are Na^+ transporters (Fairbairn et al., 2000; Uozumi et al., 2000; Horie et al., 2001), and that some KT-HAK transporters may mediate exclusively low affinity K^+ uptake (Senn et al., 2001). Taking into account the above, the main key issue to be addressed concerning K^+ homeostasis mechanisms in seagrasses is whether HAK transporters are only involved in high-affinity K^+ uptake, whereas channels carry out the uptake at millimolar K^+ concentrations.

Maintenance of appropriate intracellular K^+/Na^+ balance is critical for metabolic function as Na^+ cytotoxicity is largely due to competition with K^+ for binding sites in enzymes essential for cellular functions (Flowers and Colmer, 2008; Pardo, 2010; Kronzucker and Britto, 2011; Pardo and Rubio, 2011). Another adverse effect of Na^+ cytotoxicity is the production of ROS (reactive oxygen species), which then in turn affect cellular structure and metabolism negatively (Bartels and Sunkar, 2005). Plant cells are much more intolerant to Na^+ than animal cells due to their lack of significant systems for regulating their Na^+ content. In the Na^+-abundant marine environment where early life evolved, the use of K^+ as a major cation for maintaining the osmotic and electrical equilibrium of cells (Rodríguez-Navarro, 2000; Rodríguez-Navarro and Rubio, 2006) evolved in parallel with mechanisms of K^+ uptake and Na^+ exclusion. Recently,
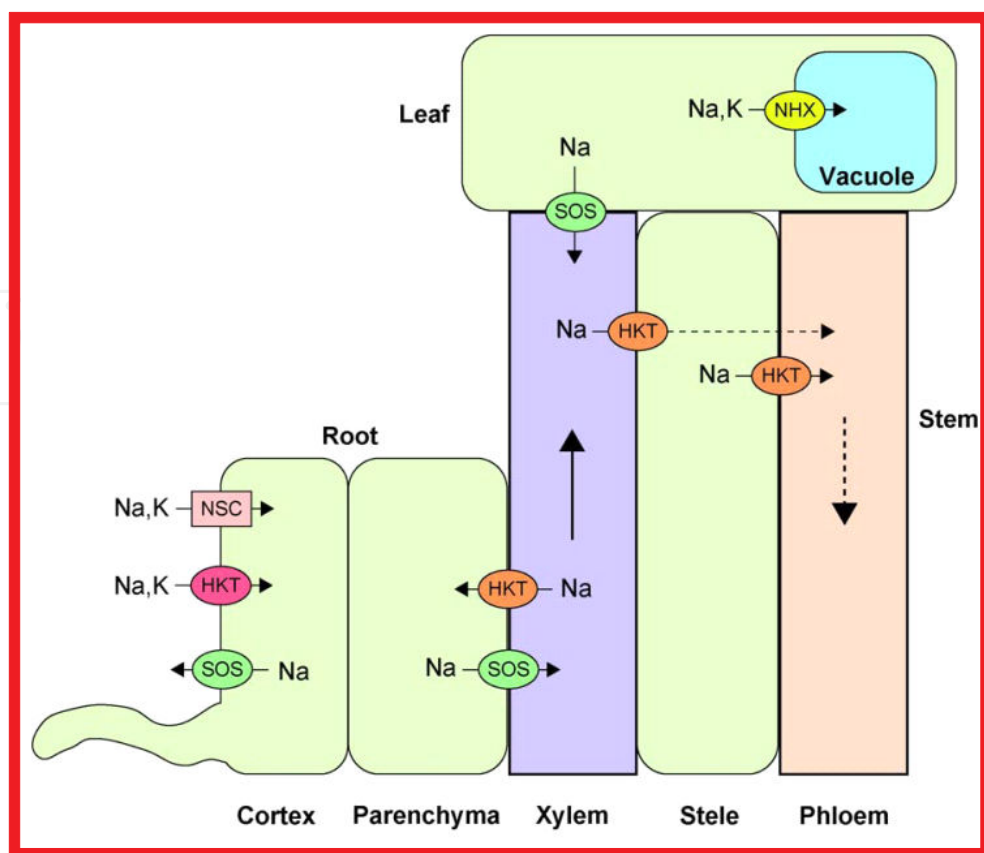
Adapted from Pardo, 2010

Figure 1. Schematic model for the function of SOS1, HKT proteins as well of nonselective cation channels (NSC) in achieving K+ uptake and Na+ exclusion in plants subjected to salinity stress.

it has been shown that a Na+-pump apparently does not exist in *Cymodocea nodosa*; on the contrary an electrogenic Na+/H+ antiporter seems to be the most likely mechanism that could mediate Na+ efflux in the epidermal cells of seagrasses (Apse and Blumwald, 2007; Garciadeblas et al., 2007; Touchette, 2007); however this activity has not yet been characterized. There is also evidence that H+-dependent systems are involved in the maintenance of a low cytosolic Na+ concentration in *Zostera marina* cells (Rubio et al., 2011).

The Na+/H+ antiporters in plants are electroneutral (Munns and Tester, 2008), which means they would not facilitate Na+ efflux at the alkaline pH values of seawater (Benito and Rodrí-guez-Navarro, 2003). However, seagrasses do presumably efflux Na+; their Na+/H+ antiporters function in this respect is unclear (Garciadeblás et al., 2007; Touchette, 2007; Flowers et al., 2010; Rubio et al., 2011). Recent molecular studies have demonstrated that genes encoding for Na+/H+ transporters (SOS1) are present in *C. nodosa* (Garciadeblás et al., 2007). SOS1 gene was initially cloned in the model plant *Arabidopsis thaliana*, encoding one of the plasma membrane Na+/H+ antiporter (Shi et al., 2000). Since then, SOS1-like genes have been detected in more than 30 terrestrial species, demonstrating its wide distribution in plants and its role in salinity tolerance as a sodium efflux mechanism (Rubio et al., 2011). The SOS1 system has been extensively investigated (Zhu, 2003) and it seems clear that it mediates Na+/H+ exchange (Shi

et al., 2000) under the regulation of a protein kinase, SOS2, and a Ca^{2+}-binding protein, SOS3 (Qiu et al., 2002). Recent evidence indicates that the Na^+-induced stability of AtSOS1 mRNA is mediated by ROS (Chung et al., 2008). Presence of SOS1 system in *C. nodosa* suggests that this antiporter also play an important role in seagrass adaptation to the marine environment. Furthermore, this transport may show different characteristics than that of terrestrial plants, as suggested for salinity tolerance mechanisms in seagrasses (Touchette, 2007).

On the other hand, it has been proved that osmotic stress causes disorganization of microtubules in cells of higher plants (Yancey, 2001). Accumulation in the cytoplasm of non toxic compounds (osmolytes such as amino acids and methylamines) regulates osmotic homeostasis. The efficiency of osmolytes to act kosmotropically and not chaotropically, permit marine phanerogams to function under adverse conditions. In the plasmolysed cells peculiar tubular structures of microtubules are formed that appear to be related to the mechanism of regulation of protoplast volume. Moreover, actin cytoskeleton undergoes intense changes and thick bundles of actin microfilaments are formed (Komis et al., 2002a, b, 2003). A pivotal role to the cellular compartmental model of salinity tolerance response is the accumulation of metabolically 'compatible' organic solutes (osmolytes) in the cytoplasm, in order to balance the osmotic potential of Na^+ and Cl^- accumulated in the vacuole. Although, accumulation of osmolytes is required for osmotic cell homeostasis these compounds do not affect cellular functions (Jones and Gorham, 2002). Among the previously described osmolytes are amino acids such as proline, glycine, taurine, and methylamines such as betaine and trimethylamine N-oxide (TMAO; Touchette, 2007). Osmolytes appear to have additional functions, such as stabilizing proteins and membranes under conditions of dehydration, or by removing ROS. Osmoprotectant properties of compatible solutes include reduced inhibitory effects of ions on enzymes, increased thermal stability of enzymes, and limited dissociation of enzyme complexes (including the oxygen-evolving complex of photosystem II; Touchette, 2007). Little is known of the signaling cascades regulating the synthesis of osmolytes in seagrasses, although the molecular basis of NaCl-enhanced accumulation of some organic solutes has been studied in a few halophytes (Flowers and Colmer, 2008). During salinity stress, carbohydrates are likely converted to other organic compounds that would better facilitate osmotic adjustment in these plants. This is further supported by observed decreases in sucrose-P synthase (a key enzyme involved in sucrose synthesis) activities in seagrasses exposed to higher salinities (Touchette, 2007), where in *Ruppia maritima*, total soluble carbohydrate content appeared to decrease with increasing salinities (Murphy et al., 2003).

3. Methodology

Methodology should implement an innovative "cross-curricular" approach combining different interrelated scientific fields such as ecology, physiology, microscopy on cellular level, molecular biology/genetics and analytical biochemistry. This "cross-curricular" dimension reflects the capability of such an approach to incorporate successfully various scientific fields articulating its benefits to tackle the key issues in a functional, flexible and practical way. The

selection and adoption policy should be based on the intention to support and to raise standards in marine ecology genetics research.

i.   Ecophysiology

Estimation of morphological and physiological parameters. Evaluation concerning the growth and photosynthetic (Fv/Fm και ΔF/Fm') response of seagrasses stress tolerance mechanisms on different levels of temperature, light intensity, PAR radiation and salinity. According to the literature review regarding seagrass species, it seems that the critical factors that affect their productivity and distribution in the Mediterranean Sea are temperature and PAR-radiation (Perez and Romero, 1992; Zharova et al., 2008). Particularly, at temperatures below 15°C and above 30°C flowering of species might be inhibited (i.e. Orfanidis et al., 2008; Sharon et al., 2009). Moreover, there is strong evidence to support the hypothesis that salinity, temperature and PAR-radiation fluctuations can critically affect seasonal distribution on a regional scale in certain phanerogam species (Gesti et al., 2005). Apart from the fact that seagrasses evolved by a common ancestor (high terrestrial plants) they seem to present relatively similar rapid growth rates with remarkable physiological plasticity, allowing them to respond and adapt to environmental stress, comprising them as ideal marine bio-indicators of environmental degradation.

ii.   Electronic microscopy on cellular level

The cellular structure (membranes, walls, organelles) mainly in the cytoskeleton organization and of the mechanism of the K^+/Na^+ pump function under various stress conditions using indirect fluorescent antibody (IFA) microscopy. The implementation of this technique allows successful spatial observation of cytoskeleton structures in cells. Otherwise, a Confocal Scanning Laser Microscope (CLSM) could be implemented. The main advantage of this method lies on the recombination of micro-slices in a three dimensional (3D) scale. The cellular mechanism of K^+-Na^+ pump function at different environmental stress conditions in means of plasma membrane vesicles would improve our knowledge on the adaptation mechanisms in terms of cell morphology.

iii.   Molecular biology/genetics

Isolation and characterization of HAK, SOS, HSPs and MT genes, which are putative gene-markers of the induced tolerance reactions under stress conditions. Whether any Na^+/H^+ antiporter activity is present at the plasma membrane of a leaf cell by *in situ* hybridization. In order to reveal homologous genes cloning of the corresponding cDNAs by using degenerated primers designed on highly conserved regions. Relative expression analysis by RT-qPCR evaluating the abundance of mRNAs in different tissues (indication of subcellular localization). The transcriptomic profiles (considering appropriate normalized libraries) in various abiotic stress conditions could be methodically determined. Enriched cDNA libraries could be thoroughly constructed by following the Illumina massively parallel sequencing technology (i.e. multiplex-based platform).

iv.   Analytical methods

Seagrasses under stressful conditions store in their vacuoles the toxic ions, such as Na^+. Therefore, the estimation of ions K^+, Na^+, Ca^{++}, Cl^- concentrations in different parts of the

seagrass (root, rhizome, leafage, sheath) would contribute to the comprehension of their adaptive response mechanisms. Hence, the identification and quantitation of osmolytes by RP-HPLC with OPA derivatization could be justfully applied to illustrate the topic.

Due to the fact that the lot of terrestrial cultivated species do not tolerate high concentrations of NaCl, it would be beneficial to implement genetic improvement upon them in order to become tolerant in salinity. Thus, a new prospect would be the appraisal of economical exploitation by successful cultivation in high salinity soils.

4. Results and discussion

The primary effect of increased global temperature on seagrasses is the alteration of growth rates and other physiological functions of the plants themselves (Gaines and Denny, 1993; Gambi et al., 2009); it is also predicted that distribution of seagrasses will shift as a result of increased temperature stress in accordance with changes in the patterns of sexual reproduction (Short and Neckles, 1999; Gesti et al., 2005; Zharova et al., 2008). Identifying differentially expressed genes under stress is very useful in order to understand plant defense mechanisms (Rose et al., 2004; Whitehead and Crawford, 2006; Ouborg and Vriezen, 2007). Powerful techniques such as microarray analysis provide a wealth of information about genes involved in environmental stress responses and adaptation (Feder and Mitchell-Olds, 2003; Kore-eda et al., 2004; Ruggiero et al., 2004; Vasemägi and Primmer, 2005; Procaccini et al., 2007; Reusch and Wood, 2007). Many studies have shown up-regulation of transcripts for heat shock proteins (HSPs) (Rizhsky et al., 2002; Simões-Araújo et al., 2002; Busch et al., 2005; Huang and Xu, 2008; Larkindale and Vierling, 2008). Likewise, some studies have identified other transcripts increased by heat treatment, including members of the DREB2 family of transcription factors, AsEXP1 encoding an expansin protein, genes encoding for galactinol synthase and enzymes in the raffinose oligosaccharide pathway and antioxidant enzymes (Rizhsky et al. 2002, 2004; Busch et al. 2005; Lim et al. 2006; Xu et al. 2007). The most abundant transcript indentified was a putative metallothionein (MT) gene with unknown pleiotropic function, rich in cysteine residues in *Z. marina* (Reusch et al., 2008). *In silico* investigation in GenBank reveals the existence of orthologous gene counterparts coding for proteins with similar function (Bouck and Vision, 2007). The same appears to happen in the case of MTs (e.g. Guo et al., 2003) and in members of HSPs (e.g. Waters et al., 1996). Moreover, abundance and distribution of seagrasses are strongly related to the intensity of light. It comprises a significant factor for fitness, while it is also related to their photosynthetic capacity. The chlorophyll activity is considered as an adequate indicator of the biochemical and physiological robustness of plants (Vangronsveld et al., 1998); whilst salinity stress can alter photosynthetic capacities in seagrasses (Murphy et al., 2003; Torquemada et al., 2005). While increased salinity stress can cause declines in chlorophyll content (Baek et al., 2005; Karimi et al., 2005), other inhibitory processes are also involved including inhibition of electron flow, decreased photosystem function, diminished rubisco abundance and activity, and changes in chloroplast ultrastructure (Kirst, 1989; Ziska et al., 1990; Stoynova-Bakalova and Toncheva-Panova, 2003). The

chlorophyll fluorescence is used in order to illustrate the stress degree due to an abiotic environmental factor, or combination of factors.

5. Conclusion

A comprehensive approach should consider the relative importance of each of the following components, thus providing a valuable insight on seagrasses multifunctional expression analyses.

i.   Marine angiosperms phenotype tolerance response from differential habitats to temperature, light and salinity fluctuations under controlled laboratory conditions.

    a.   Do factors such as space-time scales, environmental conditions, habitat type affect the variability of angiosperm species phenotype in representative coasts?

    b.   Estimation of morphological and physiological parameters. Measurements concerning the growth and photosynthetic (Fv/Fm, ΔF/Fm') response of seagrasess stress tolerance mechanisms on different levels of temperature, light intensity, PAR radiation and salinity.

ii.   Selective ion flux and ion portioning between cytoplasm and vacuole play an important role in establishing and maintaining different ion concentrations and ratios in seagrasses. However, the degree at which each mechanism is employed is not well understood. Exploration of the effects of various stress conditions on their cellular structure (membranes, walls, organelles) mainly in the cytoskeleton organization and of the mechanism of the K^+/Na^+ pump function with the implementation of electron microscopic techniques.

iii.   Comprehension of the molecular mechanisms involved at K^+ acquisition, Na^+ efflux and other pleiotropic responses.

iv.   Comparative genomic analysis of stress-specific cDNA libraries in order to evaluate the molecular homeostatic mechanisms that regulate tolerance reaction under various stress conditions.

    a.   Which are the genes of seagrasses that code for: 1) their HAK transporters, 2) their SOS antiporters that appear to intervene with Na^+ efflux, 3) their MT factors that pleiotropically intervene with the response at intense temperature fluctuations and 4) members of HSPs family that participate in the tolerance response induction under high temperatures.

    b.   Which is the transcriptomic profile for seagrass species that is induced in each stress factor?

v.   Identification and quantitation of osmolytes with osmoprotective activity. The estimation of ions K^+, Na^+, Ca^{++}, Cl^- concentrations in different parts of the seagrass

(root, rhizome, leafage and sheath) will contribute to the comprehension of their adaptive response mechanisms.

vi. Forecasting alterations in species distribution, abundance and diversity due to climate change.

vii. Potential use of seagrass species as bioindicators of coastal and transitional waters.

Physiological, cellular, molecular and biochemical mechanisms which regulate stress tolerance responses in different levels of salinity, intense temperature fluctuations and light regime are not sufficiently studied in marine seagrasses. Our understanding of salinity tolerance in terrestrial halophytes and marine algae has considerably progressed over the last decade. Our knowledge of their variability according to species and habitat type is minimal. Nevertheless, several stress-related genes have been isolated and characterized in seagrasses. Such genes code protein transporters and antiporters which are related to the distribution of K^+ and Na^+ efflux, respectively, as well as genes coding for metallothionines (MT) and members of heat shock proteins (HSPs) family, which participate in pleiotropic response related to the intense temperature fluctuations and photosynthetic ability. Focusing studies to transcriptomic profiles and their equivalent metabolic pathways that regulate them, in combination with the assessment of the respective phenotype and the relevant physiological aspects, one can comprehend important ecological traits, such as tolerance in abiotic stress. As hectares of salt-affected land increases around the globe, understanding the origins of the diversity of seagrasses should provide a basis for the use of novel cultivated species in bioremediation and conservation.

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