

Technical Report

Gene mining in halophytes: functional identification of stress tolerance genes in *Lepidium crassifolium*

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ABSTRACT

Extremophile plants are valuable sources of genes conferring tolerance traits, which can be explored to improve stress tolerance of crops. *Lepidium crassifolium* is a halophytic relative of the model plant *Arabidopsis thaliana*, and displays tolerance to salt, osmotic and oxidative stresses. We have employed the modified Conditional cDNA Overexpression System to transfer a cDNA library from *L. crassifolium* to the glycophyte *A. thaliana*. By screening for salt, osmotic and oxidative stress tolerance through *in vitro* growth assays and non-destructive chlorophyll fluorescence imaging, 20 *Arabidopsis* lines were identified with superior performance under restrictive conditions. Several cDNA inserts were cloned and confirmed to be responsible for the enhanced tolerance by analysing independent transgenic lines. Examples include full-length cDNAs encoding proteins with high homologies to GDSL-lipase/esterase or acyl CoA-binding protein or proteins without known function, which could confer tolerance to one or several stress conditions. Our results confirm that random gene transfer from stress tolerant to sensitive plant species is a valuable tool to discover novel genes with potential for biotechnological applications.

Key-words: cDNA library; COS system; drought tolerance; gene identification; halophyte; *Lepidium crassifolium*; salt tolerance.

INTRODUCTION

Extreme environmental conditions limit plant growth and impose abiotic stress to plants. Land degradation, including desertification, drought and salinity affects around one third of the global land surface (Jarraud 2005). Climate change is predicted to increase environmental problems in the coming decades, enhancing land degradation and putting food security at risk (Gregory *et al.* 2005; Kintisch 2009; Reynolds *et al.* 2007). Adaptation of plants to suboptimal conditions requires extensive physiological and molecular reprogramming, leading to major changes in metabolic, proteomic and transcript profiles. Research on model organisms such as *Arabidopsis thaliana*

and application of system biology approaches has identified a number of genes and regulatory hubs which control the networks linking stress perception and metabolic or developmental responses (Ahuja *et al.* 2010; Cramer *et al.* 2011). However, study of a stress sensitive model has limitations in understanding tolerance to harsh environments. Extremophile plants, such as xerophytes and halophytes can grow in arid regions or on saline soils, which are otherwise lethal to non-adapted species. Halophytes represent 1% of all plant species; can optimally thrive in the presence of 50–250 mM NaCl, whilst some withstand salt concentrations up to 600 mM NaCl (Flowers & Colmer 2008). Features which influence tolerance are transport and sequestration of toxic ions (mainly Na⁺), regulation of cytosolic K⁺ retention, optimization of water use, control of stomata aperture, regulation of osmotic adjustment via osmoprotectants and control of oxidative damage through detoxification of reactive oxygen species (Flowers & Colmer 2008; Shabala 2013). While the physiology of halophytes has been extensively studied, molecular regulation of the extremophile character still remains to be understood. *Thellungiella salsuginea* is a salt tolerant relative of *Arabidopsis*, which has been used in a number of comparative studies to reveal the genetic and molecular basis of halophytism (Amtmann 2009; Gong *et al.* 2005). Other extremophile relatives of *Arabidopsis* possess different degrees of tolerance not only to salt, but also to other stresses such as drought, cold, waterlogging or nutrient limitations (Amtmann 2009; Colmer & Flowers 2008; Orsini *et al.* 2010). Genome sequences of several such species have been determined, including *Arabis lyrata* (Hu *et al.* 2011), *T. salsuginea* (Wu *et al.* 2012) and *T. parvula* (Dassanayake *et al.* 2011), facilitating the identification of genes implicated in stress tolerance (Dassanayake *et al.* 2011; Oh *et al.* 2014).

Natural genetic variability of extremophiles is an attractive genetic resource to improve tolerance of crops to adverse environments (Nevo & Chen 2010). Transfer of tolerance traits to other species is however usually hampered by incompatibility. Transformation of genomic or cDNA libraries can facilitate random gene transfer between different species. Examples include a cDNA library of *T. salsuginea*, expressed in *Arabidopsis*, leading to the identification of several *Thellungiella* genes which improved salt tolerance (Du *et al.* 2008). A binary bacterial artificial chromosome library was used to transfer large genomic fragments of *T. salsuginea* to *Arabidopsis* and screen for salt tolerance (Wang *et al.* 2010).

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The Full-length cDNA Overexpressing gene system was used to identify the Thellungiella heat shock factor TsHsfA1d, which enhanced heat tolerance in Arabidopsis (Higashi et al. 2013).

Here, we describe the novel version of the Conditional cDNA Overexpressing System (COS), which was developed to randomly transfer and express cDNA clones in Arabidopsis under the control of a chemically inducible promoter system (Papdi et al. 2008; Rigo et al. 2012). The cDNA library was derived from the less-known halophyte of the Brassicaceae family *Lepidium crassifolium*, which naturally grows on salty-sodic soils in Central Europe and Asia. In saline environment *L. crassifolium* accumulates high levels of proline and soluble carbohydrates (Murakeozy et al. 2003). Random transfer and overexpression of *L. crassifolium* cDNA in Arabidopsis could facilitate the identification of novel tolerance genes. Here, we demonstrate that regulated expression of several *L. crassifolium* cDNA could enhance salt, osmotic or oxidative stress tolerance of Arabidopsis. The COS system is therefore suitable for interspecific gene transfer and can be employed to identify valuable genes from less-known wild species.

MATERIALS AND METHODS

cDNA library construction

A cDNA library of *L. crassifolium* was prepared in principle as described (Papdi et al. 2008; Rigo et al. 2012). RNA was isolated from leaf and root samples of *in vitro* and greenhouse-grown plants. *In vitro* germinated seedlings were treated with 200 mM NaCl for 30 min, for 5 and 72 h, or subjected to desiccation by opening the lid of the Petri dish for 3 h. Greenhouse-grown 6 weeks-old plants were stressed either by salt irrigation (250 mM NaCl) or drought by withdrawing water for 10 and 20 days. Leaves and roots of the plants were collected and used for RNA isolation separately. Total RNA was isolated with [Q1] RNeasy kit (Qiagen, Valencia, CA, USA).

The cDNA library was prepared with SuperScript Full Length cDNA Library Construction Kit (Invitrogen, Cat.No.: [Q2] A11406, Carlsbad, CA, USA), which uses Gateway technology for cloning. Primary library was constructed in the pDONR222 vector resulting in 1.7×10^6 independent colonies. cDNA inserts [Q3] were transferred into the pTCES vector using the Gateway LR reaction. The pTCES vector was newly constructed based on a pGSC1700-based backbone (Cornelissen & Vandewiele 1989) with bar gene as a selectable marker and our previously reported estradiol inducible system (Papdi et al. 2008) with codon-optimized XVE fragment (Fig. S2). Plasmid DNA was purified from the recovered colonies (1.3×10^6), and transformed into the GV3101/pMP90 Agrobacterium strain (Koncz et al. 1994). 1.7×10^6 colonies were recovered, resuspended in culture medium containing 30% glycerol and stored in 2 ml aliquots at -80°C until use.

Plant transformation

The *Lepidium* cDNA library and cloned *Lepidium* genes were introduced into wild type Arabidopsis (Col-0 ecotype) by *in*

planta transformation (Clough & Bent 1998; Rigo et al. 2012). In a typical library transformation experiment 100–150 pots, each containing 10–20 flowering Arabidopsis plants, were infiltrated with the Agrobacterium culture containing the cDNA library. Infiltration was repeated twice with 1 week difference. Plants were then allowed to flower and set seeds. Seeds were germinated in soil and transgenic plants were selected by spraying them three times with BASTA herbicide, [Q4] which contains 300 mg/L glufosinate-ammonium (Finale 14 SL, [Q5] Bayer). BASTA resistant plants were transferred to pots, and allowed to flower and set seeds. Seeds were pooled from 25 transgenic plants and were used for subsequent screening procedures.

Genetic screens

For screening pooled T2 generation seeds were germinated on $\frac{1}{2}$ Murashige and Skoog (MS) medium containing 0.5% [Q6] saccharose, supplemented by $5 \mu\text{M}$ estradiol and one of the selective agents: paraquat ($0.2 \mu\text{M}$), sorbitol (200 mM) or NaCl (150 mM). Germination efficiencies were tested on standard $\frac{1}{2}$ MS culture medium. Growth conditions were the following: temperature: 22°C , light: $250 \mu\text{Einstein}$, 12 h illumination cycle, 50% humidity. Growth of seedlings was monitored for 3–4 weeks after germination. Seedlings with superior growth were transferred to standard $\frac{1}{2}$ MS culture medium, and plantlets with healthy roots were subsequently transferred to soil to flower and set seeds.

To screen for superior photosynthetic activity in stress conditions, 7 days-old seedlings, grown on nylon mesh on $\frac{1}{2}$ MS medium, were sprayed with $5 \mu\text{M}$ estradiol dissolved in 0.01% Silwet L-77 solution and kept for 2 days. Plantlets were transferred with mesh to sugar free high osmotic medium containing 600 mM sorbitol and $5 \mu\text{M}$ estradiol and were kept for further 48 h. Chlorophyll a fluorescence images were recorded with Imaging-PAM M-Series, Maxi version (Heinz Walz GmbH, Effeltrich, Germany). Plants were adapted to [Q7] dark for 30 min before imaging. The kinetics of fluorescence was measured with the ‘induction and recovery’ mode of the equipment. The parameters were the following: actinic light intensity: $145 \mu\text{mol}^{-2} \text{s}^{-1}$, saturation pulse intensity: $3000 \mu\text{mol}^{-2} \text{s}^{-1}$. The maximal photosystem II (PS II) quantum yield [variable fluorescence/maximum fluorescence (Fv/Fm)] and the effective PS II quantum yield (Φ_{PSII}) (Genty et al. 1988) were used to select tolerant plants. Col-0 wild type seedlings were used as control. Plantlets with altered Fv/Fm or Φ_{PSII} values were transferred to $\frac{1}{2}$ MS medium for recovery and later to greenhouse for further growth, flowering and seed production.

Gene cloning

Genomic DNA was isolated from transgenic plants with Aquagenomic (<http://www.aquaplasmid.com/AquaGenomic.html>) DNA isolation kit. cDNA inserts were PCR amplified using vector specific primers pTCRE8A5' and pTCLEXA3' flanking the inserts (Table S1) and employing Phusion High fidelity Polymerase ([Q8] Thermo Scientific). The PCR product [Q10]

was separated on 0.8% Agarose gel, and the fragment was purified using GeneJet Gel Extraction Kit (Thermo Scientific). Nucleotide sequence of the isolated DNA fragment was determined using the p35S2 primer (Papdi *et al.* 2008) (Table S1). Identity of the encoded protein was determined by Blast sequence homology search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Sequence alignment was made with Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>).

The isolated cDNA fragment was cloned in pDONR222 vector using Gateway BP Clonase II enzyme (Life Technologies, Rockville, MD, USA) and were verified by sequencing. Cloned cDNAs were subsequently inserted into pTCO272RD29 or pTCO27235S binary plant transformation vectors, using the Gateway LR Clonase Enzyme (Life Technologies). The pTCO272RD29 and pTCO27235S vectors are based on the pK7WG2 Agrobacterium binary vector (Karimi *et al.* 2002) and carry the expression cassettes with stress-induced RD29A or constitutive CaMV35S promoters, respectively. DNA inserts in the plant expression vectors were sequenced using the RD29B5' and the pTCO35SNEW5' primers (Table S1).

Growth assays

To evaluate stress tolerance, seeds were germinated on ½MS growth medium supplemented with 5 µM estradiol and one of the following additives: 100 mM, 125 mM or 150 mM NaCl, 200 mM sorbitol or 0.2 µM paraquat. Plates with growing seedlings were regularly photographed on a trans-illuminator and rosette sizes were measured with IMAGEJ 1.48v software (imagej.nih.gov/ij/). Images were processed to subtract background and invert image. Densities of 300 × 300 pixel areas were measured, and the actual data were normalized to the parallel wild type control (processed with Microsoft Excel).

Tolerance to salt or osmotic stress in greenhouse conditions was measured by growing plants in plastic trays, each containing three wild type and three transgenic plants. Osmotic and salt stress was applied by irrigation in weekly intervals with water containing 10% polyethylene glycol 6000 or 250 mM NaCl, respectively. Plant survival, rosette diameter and chlorophyll fluorescence was monitored.

Analysis of gene expression

To determine transcript levels of selected genes, Northern hybridization or RT-PCR was employed. Total RNA was isolated with the RNeasy Plant Mini kit (Qiagen). For Northern hybridization, equal amounts of RNA (20 µM) were separated in denaturing formaldehyde-containing agarose gel and were transferred to nylon hybridization membrane (Hybond). Northern hybridization was performed as described (Sambrook *et al.* 2001), using cDNA fragments as hybridization probes, radiolabeled with DecaLabel DNA Labeling Kit (Thermo Scientific). When transcript levels were determined with RT-PCR, the Verso 1-Step RT-PCR Kit was employed (Thermo Scientific).

RESULTS

Stress tolerance of *Lepidium crassifolium*

Stress tolerance of *L. crassifolium* was evaluated by comparing salt, osmotic and oxidative stress responses with known glycophytic and halophytic species of Brassicaceae family (*A. thaliana* and *T. salsuginea*, respectively) in controlled conditions (Fig. S1). When compared with Arabidopsis, both halophytes showed remarkable salt tolerance in both greenhouse and *in vitro* conditions as recorded by plant growth and survival, root growth and chlorophyll fluorescence (Fig. S1A-C). Drought tolerance of *L. crassifolium* was also higher than Arabidopsis and *T. salsuginea* when tested by withholding water (Fig. S1D). The effect of osmotic and oxidative stress on chlorophyll fluorescence was more dramatic in Arabidopsis than in the two halophytic species (Fig. S1E-F). These data showed that *L. crassifolium* has remarkable salt and drought tolerance, comparable to or higher than that of *T. salsuginea*.

The *Lepidium* cDNA library

To identify genes in *L. crassifolium* which can confer stress tolerance to *A. thaliana*, the COS was adapted to this species (Fig. 1) (Papdi *et al.* 2008; Rigo *et al.* 2012). The cDNA library of *L. crassifolium* was constructed in the plant expression vector pTCES (Fig. S2), and contained approximately 10⁶ colonies, with average insert size of 1.0 kb, and 82% of full-length cDNA clones as determined by random sequencing of 5' ends. Amino acid sequences of predicted *L. crassifolium* proteins had on average 84% identity with the most similar Arabidopsis proteins. *L. crassifolium* proteins were most similar to *A. thaliana* (41%) and *A. lyrata* (38%) proteins, suggesting that *L. crassifolium* is closely related to species in the Arabidopsis genus.

Screening for stress tolerance

To identify *L. crassifolium* genes, which could enhance stress tolerance of Arabidopsis, a multi-step screening and validation procedure was designed (Fig. 1). 40 000 transgenic Arabidopsis lines were generated which corresponded to approximately 60 000 randomly inserted T-DNAs (Szabados *et al.* 2002), each carrying different *L. crassifolium* cDNAs. Pooled seeds of 25 lines were tested for germination efficiency (Fig. S3) and used for screening to osmotic, salt and oxidative stress tolerance. 1040 seedlings were identified which were larger than average in one of the stress conditions (Fig. 2A).

The screening programme included a novel non-destructive method, based on chlorophyll fluorescence of plantlets subjected to osmotic stress. Imaging of chlorophyll fluorescence with Image PAM allows the visualization of photosynthetic performance and estimation of rapid changes in stress conditions (Oxborough 2004). Osmotic stress was applied for 2 days before imaging, to avoid gross differences in growth influencing the results. Screening the osmotically stressed seedlings with Image PAM lead to the identification of 42 seedlings, with enhanced or reduced maximum quantum yield of PSII (Fv/Fm)

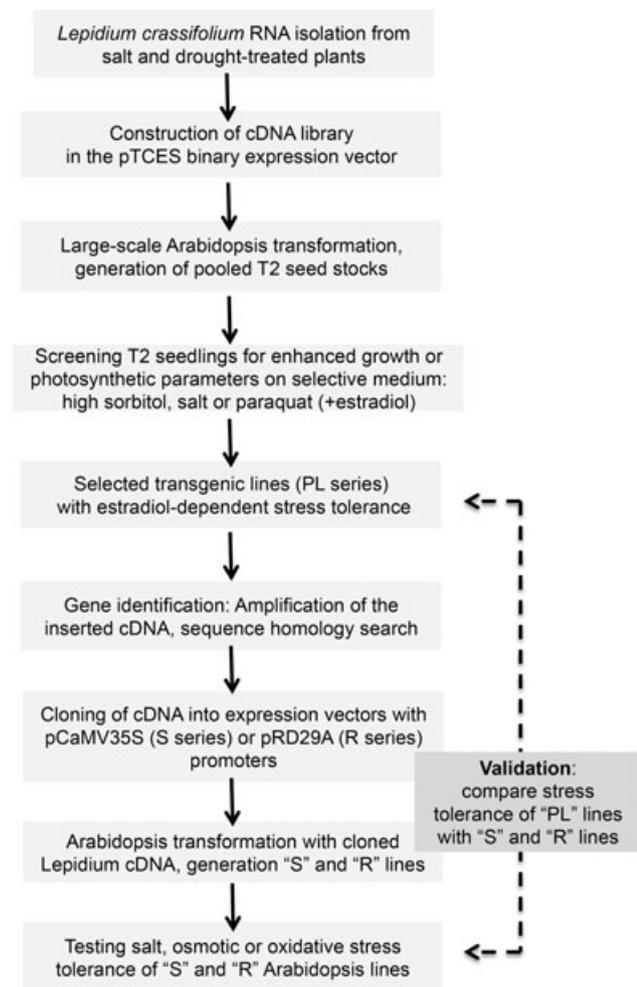


Figure 1. Outline of the Conditional cDNA Overexpression System adapted to interspecific gene transfer. cDNA library was prepared from *L. crassifolium*, transferred and overexpressed in *A. thaliana*. Consecutive steps of the cDNA library preparation, transfer to Arabidopsis, screening, gene identification, cloning and validation procedures are shown. pTCES.

or effective PSII quantum yield (Φ_{PSII}) (Fig. 2B–D). Most of the rescued plants survived and produced seeds.

Progenies of several of the selected plants showed some degree of stress tolerance. Therefore, extensive validation was performed to confirm the tolerance characteristics associated with the identified genes. The estradiol-inducible expression system has the advantage of producing conditional phenotypes, which is a reliable indication that the inserted cDNA is indeed responsible for the altered stress tolerance (Papdi *et al.* 2008). Estradiol-dependent stress tolerance of the progenies of selected plants was observed in 12 lines, while six lines from the PAM screen had higher and one had lower chlorophyll fluorescence when compared with wild type (Table 1). Tolerance of the other tested lines to the stress conditions was either estradiol independent or did not respond clearly to the inducer.

Gene identification and cloning

PCR amplification and sequencing of the inserted *L. crassifolium* cDNA allowed the identification of the encoded

proteins by sequence homology searches of public databases. Based on sequence homology, 82% of the inserted cDNAs were full-length with complete open reading frame, while 18% of them were truncated at their 5' termini. As the cDNA library was generated in a Gateway expression vector, PCR amplification of the inserted cDNA conserved the Gateway recombination sites, facilitating their cloning in the entry vector pDONR222 and subsequent transfer into plant destination expression vectors (Rigo *et al.* 2012). Stress tolerance phenotypes were validated by generating new transgenic plants, in which the inserted cDNA was controlled by constitutive (pCaMV35S) or stress-induced (pRD29A) promoters (S and R lines, respectively, see Fig. 1). Three examples are presented to illustrate the gene identification programme.

The PL542Na1 line is tolerant to salt stress

The PL542Na1 line was derived from a plant, which grew better on high salt medium (Fig. 2A). PL542Na1 plants were more tolerant to salt stress than Col-0 in the presence of estradiol, but were similar to wild type in the absence of the inducer (Fig. 3A–C). Estradiol alone had no influence on growth of PL542Na1 plants. The 1179 bp open reading frame of the 1.6 kb cDNA insert encoded a predicted protein of 392 amino acid residues (Fig. S4A,B). The predicted amino acid sequence showed highest similarity to the GDSL-like lipase/acylhydrolase family protein MVP1/GOLD36/ERMO3, encoded by *AT1G54030* in Arabidopsis and were named LcMVP1 (Fig. S4C). To verify that LcMVP1 is responsible for the salt tolerance, the full-length cDNA was cloned, introduced and overexpressed in wild type Arabidopsis plants and under the control of constitutive (S12 series) or stress-induced promoters (R12 series) (Fig. S5). Fresh weight accumulation, survival and chlorophyll content of S12 and R12 plants was superior to wild type on high salt medium (Fig. 3D–F Fig. S6). PS II maximum quantum yield (Fv/Fm) of soil-grown S12 plants was less affected than wild type, when irrigated with saline solution (Fig. 3G). These results confirmed that LcMVP1 overexpression could confer salt tolerance to Arabidopsis.

The paraquat-tolerant PL372Pq1 line

The PL372Pq1 plants grew better than wild type on paraquat-containing medium only in the presence of estradiol (Fig. 4A,B). PL372Pq1 and Col-0 plants were indistinguishable on standard culture medium (Fig. 4A). The inserted cDNA was 0.6 kb, which encoded a predicted protein of 69 amino acids (Fig. S7A,B). The most similar protein in Arabidopsis was the unknown gene product of *AT3G52105* (92% identity), with predicted signal peptide, but no other conserved domain (Fig. S7C). Sequence homology search revealed that similar proteins exist in all plants. The amplified insert was introduced and overexpressed in transgenic Arabidopsis plants (S10 and R10 lines, Fig. S8). Enhanced paraquat tolerance of S10 lines was confirmed in growth tests (Fig. 4C,D) as well as in greenhouse, where plants were sprayed with 20 μ M paraquat (Fig. 4E). Paraquat spray drastically reduced Fv/Fm values in

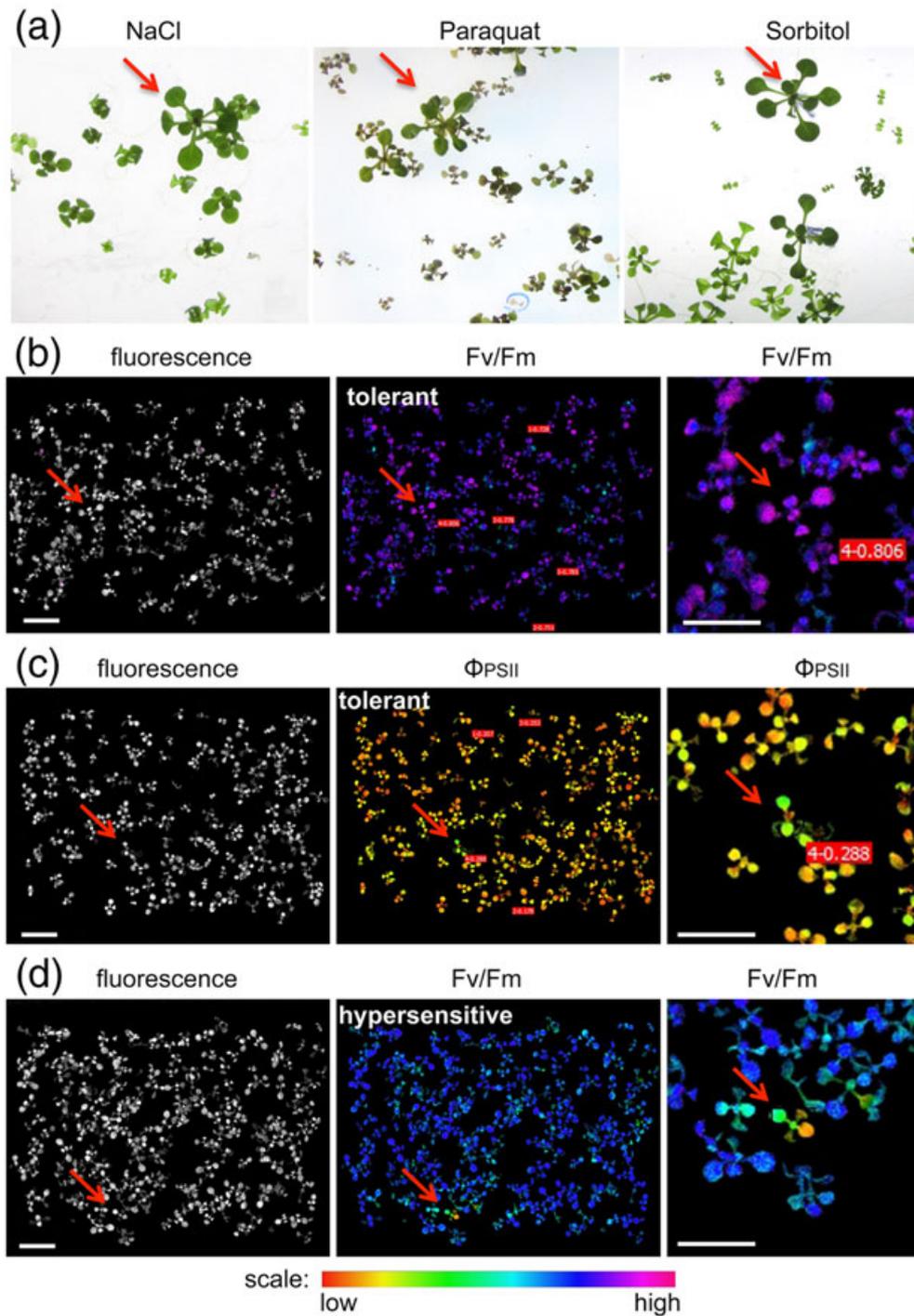


Figure 2. Screening for salt, oxidative and osmotic stress tolerance of Conditional cDNA Overexpression System-transformed Arabidopsis seedlings. (a) Three weeks-old plantlets growing on $\frac{1}{2}$ MS medium supplemented by $5 \mu\text{M}$ estradiol and one of the following additives: 150 mM NaCl or $0.2 \mu\text{M}$ paraquat or 200 mM sorbitol. Arrow indicates plantlets with enhanced growth. (b–d) PAM imaging of Arabidopsis plantlets, subjected to osmotic stress (600 mM sorbitol, 2 days). Arrow indicates plantlets with higher maximal photosystem II (PS II) quantum yield [variable fluorescence/maximum fluorescence (Fv/Fm)] (b), higher effective PS II quantum yield (Φ_{PSII}) (c), or reduced Fv/Fm (d) values. Scale bar corresponds to 1 cm.

Col-0 plants, but was only slightly affected in S10 (Fig. 4F). These results confirmed that overexpression of the *L. crassifolium* cDNA from PL372Pq1 can indeed enhance paraquat tolerance.

The PL127P4 line is tolerant to osmotic stress

Progenies of PAM-selected plants were re-tested for chlorophyll fluorescence, and estradiol-dependent changes were

Table 1. Selected transgenic lines with altered stress tolerance

Line	Screen	Protein encoded by the inserted cDNA	Validation
PL1210Na2	growth, NaCl	—	
PL542Na1	growth, NaCl	GDSL-like Lipase/Acylhydrolase protein	S12, R12
PL720Na1	growth, NaCl	—	
PL749Na4	growth, NaCl	Ribosomal protein S19e family protein	
PL900Na1	growth, NaCl	gamma-glutamyl hydrolase 2, GGH2	
PL051Pq2	growth, PQ	—	
PL1012Pq2	growth, PQ	unknown protein	
PL1265Pq1	growth, PQ	—	
PL1400Pq1	growth, PQ	—	
PL372Pq1	growth, PQ	unknown protein	S10, R10
PL1225So1	growth, Sorb.	—	
PL226So1	growth, Sorb.	hypothetical protein (DUF814 domain)	
PL037P4	PAM, Sorb.	unknown protein (DUF581 domain)	
PL039P4	PAM, Sorb.	Ribosomal protein L2, embryo defective 2296	
PL120P4	PAM, Sorb.	Ribosomal protein L10 family protein	
PL127P4	PAM, Sorb.	Acyl CoA binding protein 6, ACBP6	S16, R16
PL142P6	PAM, Sorb.	glutathione S-transferase phi 8, GST6, GSTF8	
PL142P6	PAM, Sorb.	RNA polymerase II mediator complex subunit	
PL151P6	PAM, Sorb.	translationally controlled tumour protein, TCTP	
PL181P3	PAM, Sorb. (HS)	—	

Explanations: Line: identification code of the Arabidopsis line, Screen: type of screen the line was identified. Protein: annotation of closest sequence based on the predicted amino acid sequence. Validation: identification code of the lines used for validation of the stress tolerance trait. cDNA were overexpressed under the control of pCaMV35S (S series) or pRD29A (R series) promoters. PQ, Paraquat.

confirmed in seven lines. Six of them showed enhanced Fv/Fm and/or Φ_{PSII} values, while in one line chlorophyll fluorescence was reduced upon estradiol treatment (Fig. 5A,B). In standard culture medium ($\frac{1}{2}$ MS) Fv/Fm and Φ_{PSII} values of these lines were not significantly different from wild type and were not influenced by the inducer (Fig. 5A,B).

PL127P4 had higher Fv/Fm value than other seedlings on high osmotic medium (Fig. 2B), which was reproduced in the progenies (Fig. 5A). The 0.8kb recovered cDNA had a 270 bp open reading frame encoding a protein with high sequence similarity to the Arabidopsis Acyl CoA binding protein (ACBP)6 (*ATIG31812*, Fig. S9A,B) and was therefore named LcACBP. The protein had a conserved ACBP domain and had 90% identity to ACBP6 of *A. thaliana* and *A. lyrata* (Fig. S9C). Overexpression of LcACBP cDNA in Arabidopsis by the stress-induced RD29A promoter (Fig. S10) improved growth on high osmotic medium (Fig. 5C). Growth and maximal PS II quantum yield (Fv/Fm) was less affected in osmotically stressed R16 plants than wild type in greenhouse conditions (Fig. 5D,E). These results confirmed, that overexpression of LcACBP can enhance tolerance to osmotic stress.

DISCUSSION

Halophytes have been recognized as valuable gene sources for stress tolerance (Nevo & Chen 2010). Introgression of multigene-controlled tolerance traits to crops however needs detailed Quantitative trait locus maps and molecular markers linked to tolerance loci (Arrauadi *et al.* 2012; Chankaew *et al.* 2014; Pandit *et al.* 2010). Moreover, incompatibility is a serious barrier, which prevents gene transfer between species.

To overcome such problems, we have adapted the COS system (Papdi *et al.* 2008; Rigo *et al.* 2012) for interspecific gene transfer and showed that it can facilitate the identification and transfer of tolerance genes from an extremophile plant to a sensitive one. The Arabidopsis relative, *L. crassifolium* was employed as gene source, whose tolerance to salinity and drought was demonstrated.

The Lepidium COS collection contains pooled seed stocks of 40 000 transgenic Arabidopsis lines, suitable for screening purposes. In contrast to the *T. salsuginea* cDNA library with constitutive expression (Du *et al.* 2008), the COS system permits controlled transcription of the inserted cDNA, regulated by a chemical inducer. Thus the COS system generates conditional dominant phenotypes which facilitates unambiguous assignment of gene-phenotype linkage as well as the recovery of such genes whose overexpression can cause lethality or affect fertility (Joseph *et al.* 2014). Further advantage of the COS system is, that gene identification and cloning is a simple and straightforward task because of facile PCR amplification of the inserts and re-cloning with the flanking Gateway recombination sites (Rigo *et al.* 2012). We could amplify cDNA most inserts from the selected lines and determine their identity by sequence homology searches.

Systematic screening the Lepidium COS collection for tolerance to salt, osmotic and oxidative stress, lead to the identification of 19 lines, which showed superior growth in controlled conditions or altered chlorophyll fluorescence under stress. The non-invasive PAM imaging technology (Oxborough 2004) was optimized for high throughput screening to detect alterations in maximum quantum yield (Fv/Fm) or photosynthetic yield (Φ_{PSII}) in osmotically stressed plantlets. While

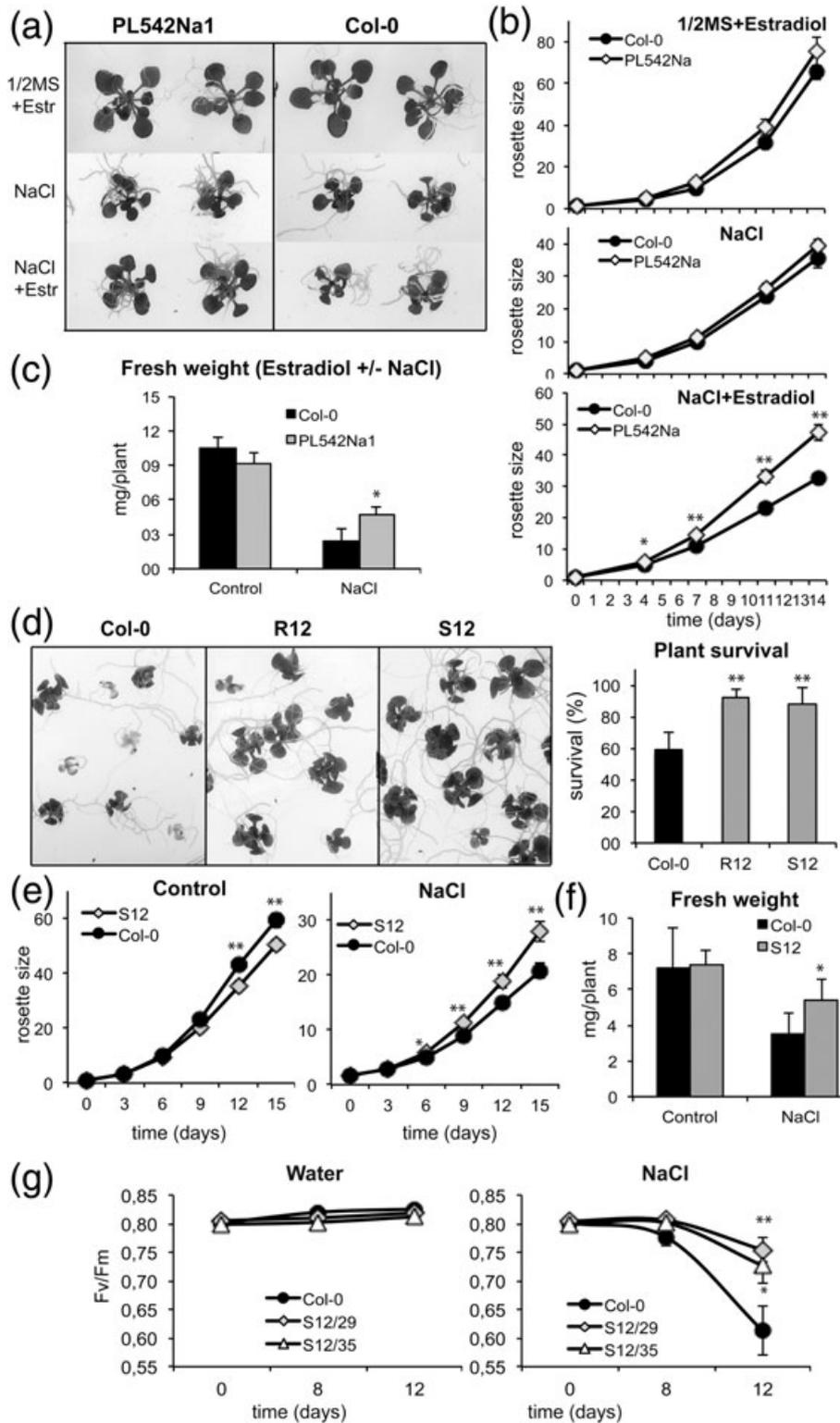


Figure 3. Salt tolerance of PL542Na1, S12 and R12 plants. S12 and R12 lines overexpress the PL542Na1-derived cDNA under the control of constitutive pCaMV35S or stress-induced pRD29A promoters, respectively. (a) 5 days-old seedlings were transferred to $\frac{1}{2}$ MS medium supplemented by 5 μ M estradiol, estradiol and 125 mM NaCl or NaCl alone. Typical rosettes of PL542Na1 and Col-0 plants are shown. (b) Relative rosette sizes of Col-0 and PL542Na1 plantlets on $\frac{1}{2}$ MS and high salt medium. Rosette sizes were normalized to wild type (Col-0) at the start of the experiment (day 0). (c) FW of Col-0 and PL542Na1 plantlets, measured on 12th day after transfer to estradiol-containing $\frac{1}{2}$ MS or high salt medium. (d) Survival of wild type, S12 and R12 plantlets, transferred to saline medium (150 mM NaCl) for 15 days. Diagram shows % of surviving, green plants. (e,f) Rosette growth (e) and fresh weight (f) of Col-0 and S12 plants grown on control ($\frac{1}{2}$ MS) and high salt medium (150 mM NaCl). (g) Maximal photosystem II quantum yield [variable fluorescence/maximum fluorescence (Fv/Fm)] of S12 and Col-0 plants grown in greenhouse and irrigated with water or 200 mM NaCl at day 0, 4, 8. Bars on diagrams indicate standard deviation, * and ** show significant differences to Col-0 wild type at $p < 0.05$ and $p < 0.005$, respectively (Student *t*-test).

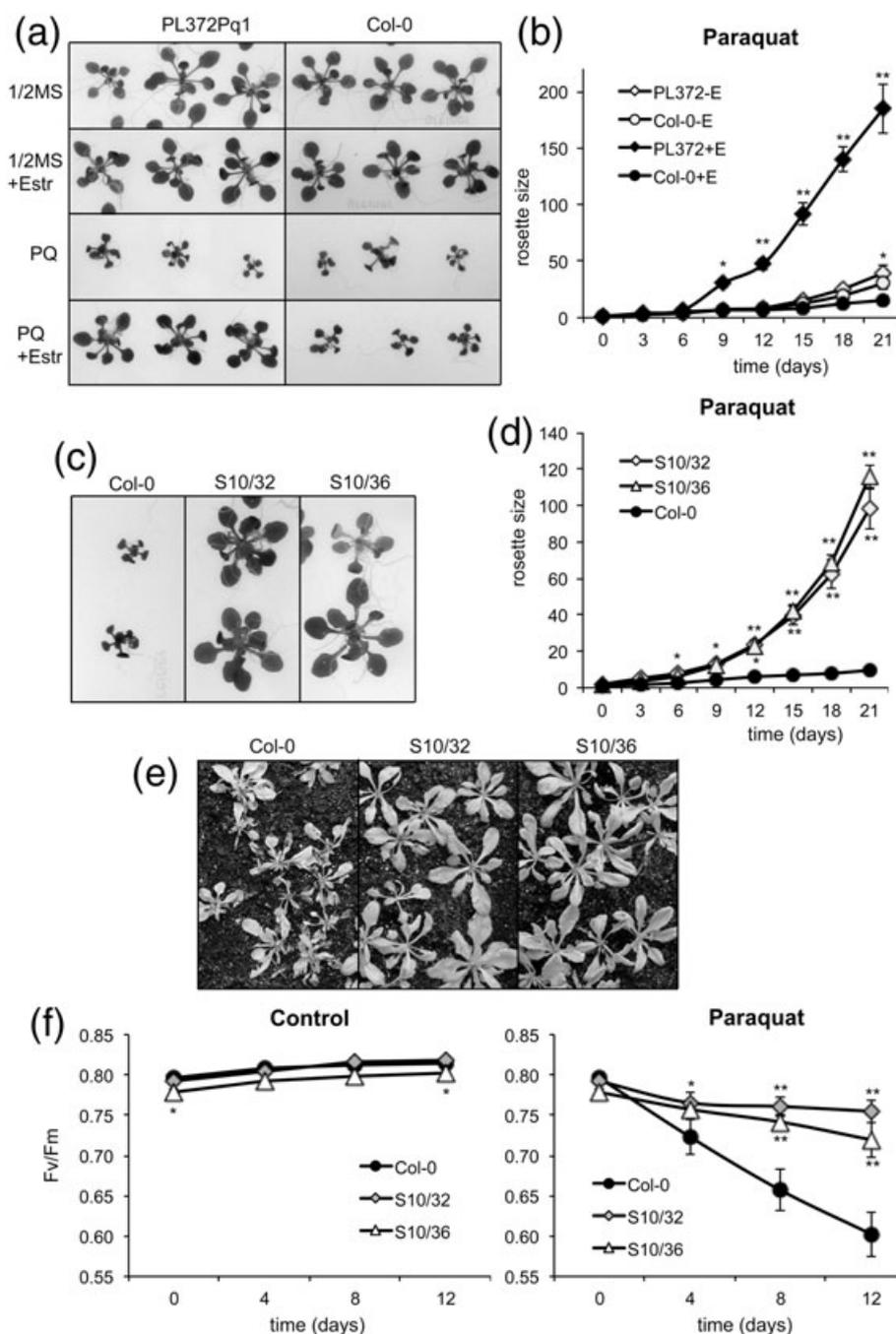


Figure 4. Paraquat (PQ) tolerance of PL372Pq1 and S10 plants, overexpressing the PL372Pq1-derived cDNA under the control of pCaMV35S promoter. (a) PL372Pq1 and wild type plants grown on standard 1/2MS media supplemented by 0,2 μ M paraquat (PQ) and/or 5 μ M estradiol (Estr). (b) Rosette growth on paraquat-containing medium with (+E) or without (–E) estradiol. (c,d) Rosette sizes of Col-0 and S10 plants, grown on paraquat-containing (0,2 μ M) medium. (e) Wild type and S10 plants in soil, 8 days after spraying with paraquat (20 μ M). (f) Chlorophyll fluorescence [variable fluorescence/maximum fluorescence (Fv/Fm)] of soil-grown plants, sprayed with 20 μ M paraquat. Rosette sizes were measured and statistics were calculated as described for Figure 2.

chlorophyll fluorescence was previously used as marker to identify mutants or genotypes with altered photosynthetic activity associated to drought or cold tolerance (Mishra *et al.* 2014; Niyogi *et al.* 1998; Woo *et al.* 2008), combining the COS system with PAM imaging offers new possibilities for gene identification.

To illustrate the potential of the gene identification system, three lines were characterized and their tolerance traits were

subsequently verified in independent transgenic plants. A GDSL-lipase/esterase family protein was responsible for salt tolerance of PL542Na1. It was closely related to MVP1/GOLD36/ERMO3 of Arabidopsis, which is implicated in maintenance of endoplasmatic reticulum integrity, protein trafficking and endoplasmatic reticulum-related defenses (Jancowski *et al.* 2014; Marti *et al.* 2010; Nakano *et al.* 2012).

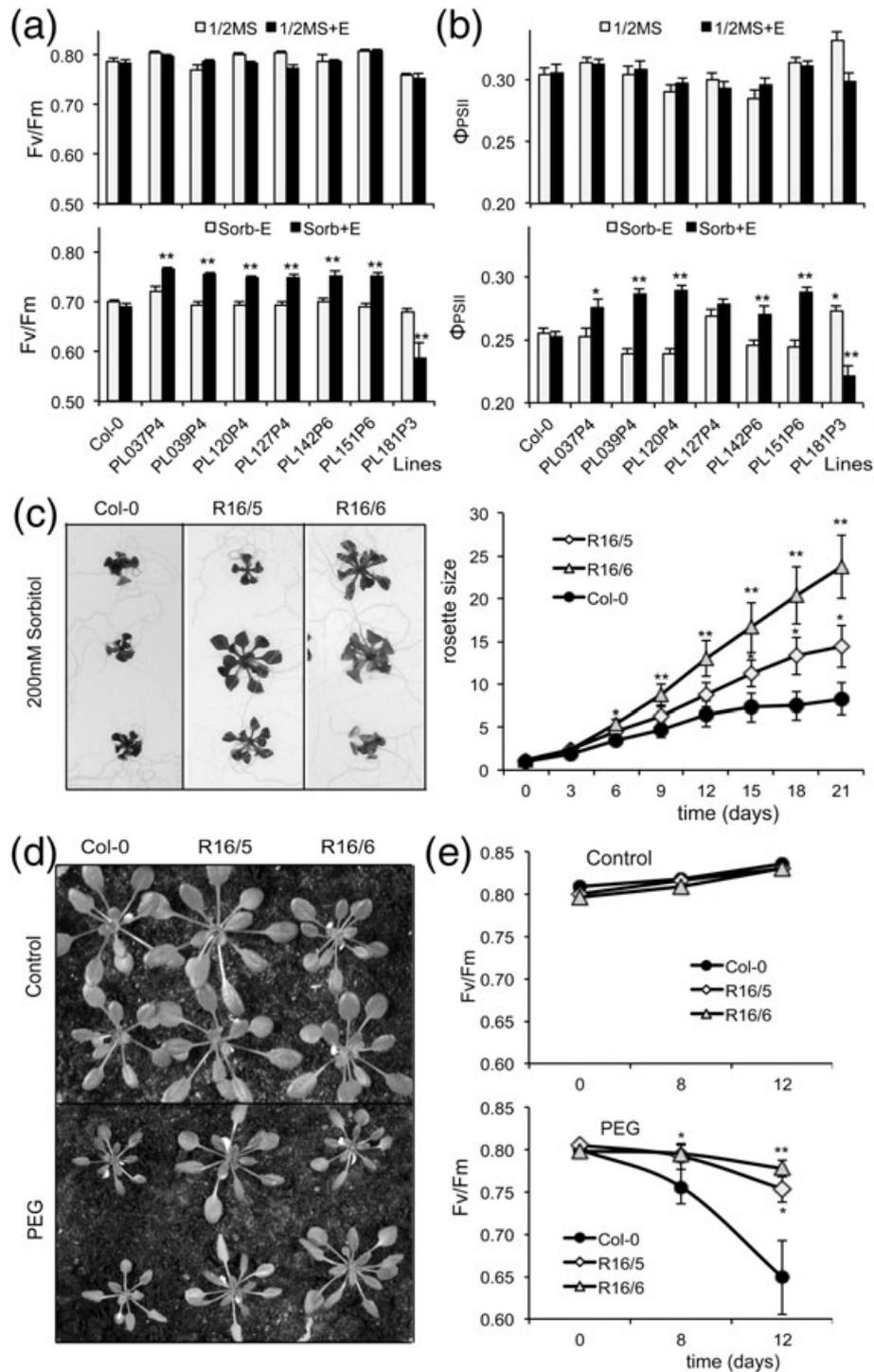


Figure 5. Osmotic stress tolerance and chlorophyll fluorescence of transgenic *Arabidopsis* plants identified by PAM imaging. (A,B) Chlorophyll fluorescence [A: variable fluorescence/maximum fluorescence (Fv/Fm), B: Φ_{PSII} values] of selected lines on standard ($\frac{1}{2}$ MS) and high osmotic medium (600 mM Sorbitol), both of the containing $5 \mu\text{M}$ Estradiol (+E). (C) Growth of Col-0 wild type and two independent LcACBP overexpressing plants (R16) on high osmotic medium (200 mM sorbitol). Diagram shows relative rosette sizes. (D) Soil-grown wild type and R16 plants, irrigated with water or 10% polyethylene glycol (PEG) 6000. (E) Maximal photosystem II (PS II) quantum yield (Fv/Fm) of Col-0 and R16 plants stressed by PEG treatment. Rosette sizes were measured and statistics were calculated as described for Figure 2.

ATIG54030 is upregulated by drought, osmotic stress, UV-B, wounding and certain pathogens (eFP Browser, <http://bbc.botany.utoronto.ca>), suggesting that the *AtMVP1* gene is involved in defenses against various stresses.

The cDNA insert in PL372Pq1 rendered remarkable tolerance to paraquat and encoded a small protein, with closest similarity to the predicted gene product of *AT3G52105*, a gene with unknown function. Genes which can influence paraquat

resistance have been reported to encode amino acid or polyamine transporters (Fujita *et al.* 2012; Li *et al.* 2013), enzymes which regulate reactive oxygen species levels, antioxidant capacity, modulate nitric oxide levels and regulate cell death (An *et al.* 2014; Chen *et al.* 2009; Fujibe *et al.* 2004). The short protein identified in PL372Pq1 is apparently not related to these proteins, suggesting that it is implicated in a novel mechanism of tolerance, further demonstrating the potential of our screening approach for gene discovery.

PL127P4 had superior chlorophyll fluorescence in osmotically stressed plants. The full-length cDNA encoded a small Acyl CoA binding protein (LcACBP) with highest similarity to Arabidopsis ACBP6. Small ACBPs are highly conserved in all eukaryotes, bind long-chain acyl-CoA esters, and are implicated in plant lipid metabolism, transport and signalling, some of them modulate plant development and stress responses (Li-Beisson *et al.* 2013; Xiao & Chye 2011). AtACBP6 was shown to regulate phosphatidylcholine and phosphatidic acid levels and improve freezing tolerance (Chen *et al.* 2008; Liao *et al.* 2014). ACBP6, together with ACBP4, ACBP5 was shown to control phospholipase D, modulate ABA sensitivity in seed development and germination (Hsiao *et al.* 2014). Whether small Acyl CoA binding protein enhances tolerance to osmotic stress through modulation of phosphatidylcholine and phosphatidic acid metabolism remains to be elucidated by further studies.

Here, we demonstrated that the properly designed COS system is suitable to explore natural variability of wild species, facilitate interspecific gene transfer and contribute to our efforts to understand molecular bases of drought and salt tolerance. Further studies are required and are in progress to elucidate the precise molecular and biological function of the identified genes **Q17** and their relevance in stress responses. Identified genes can further be utilized as molecular tools to improve stress tolerance of crops.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Salt and drought tolerance of *A. thaliana*, *T. salsuginea* and *L. crassifolium*.

Figure S2. Structure of the pTCES vector.

Figure S3. Germination of Col-0 wild type and COS-transformed *Arabidopsis* seeds.

Figure S4. Sequence analysis of the insert in PL542Na1.

Figure S5. Expression of LcMVP1 in transgenic S12 and R12 *Arabidopsis* plants.

Figure S6. Chlorophyll and carotene content of Col-0 wild type and S12 plants.

Figure S7. Sequence analysis of the insert in PL372Pq1.

Figure S8. Expression of PL372Pq1 cDNA in transgenic S10 and R10 plants.

Figure S9. Sequence analysis of the insert in PL127P4.

Figure S10. Expression of LcACBP in transgenic R16 and S16 *Arabidopsis* plants.

Table S1. DNA oligoes and primers used in this study.

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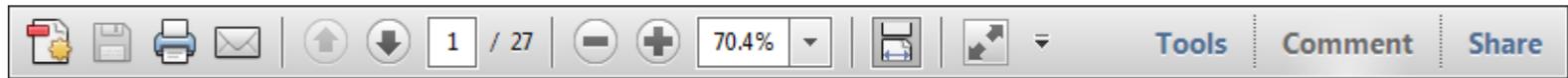
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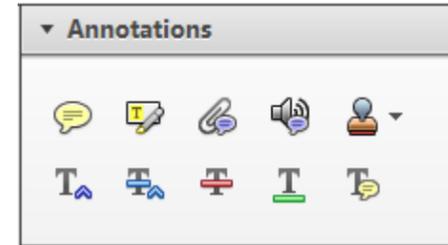
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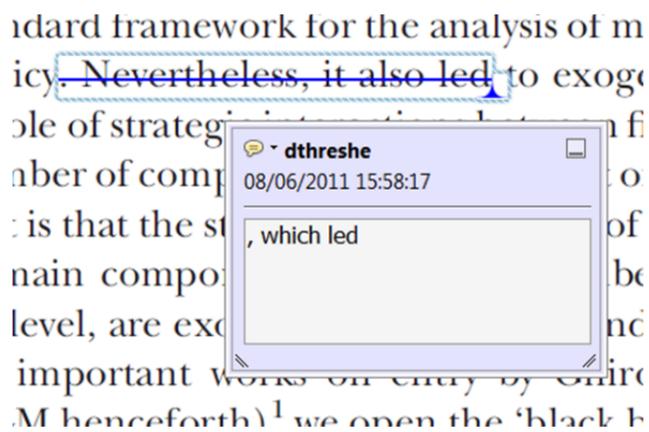
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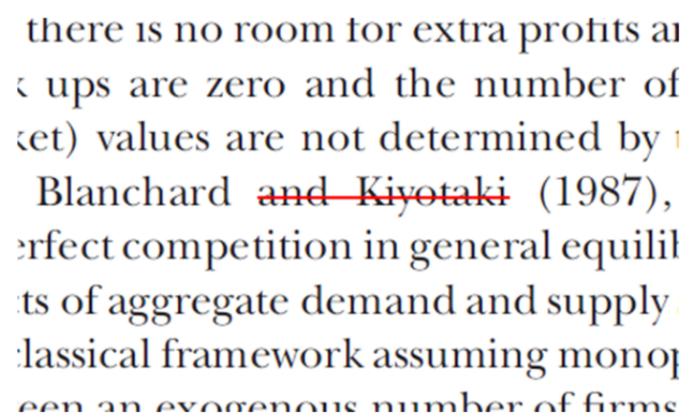
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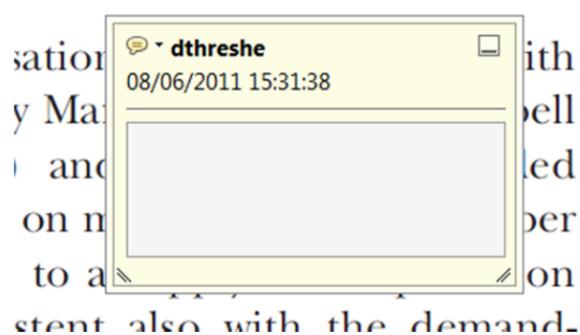
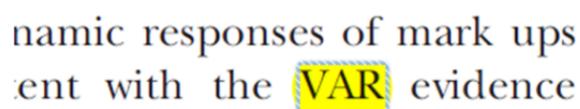
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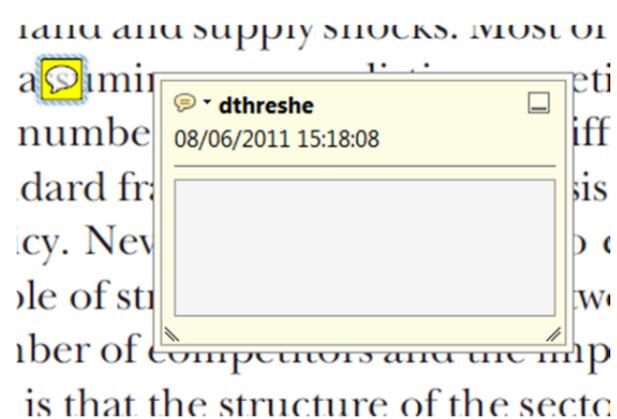
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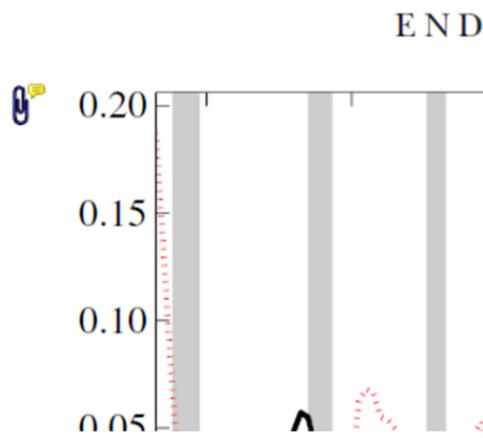
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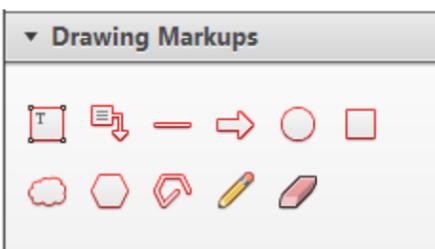


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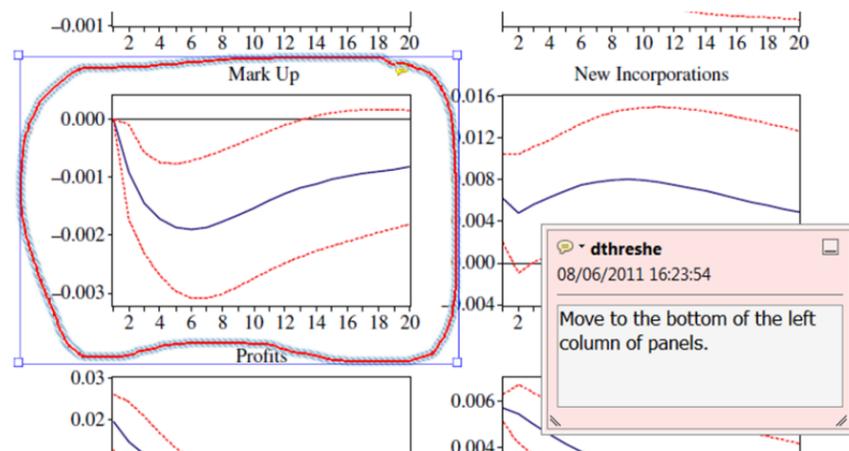


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