Towards unravelling the genetic determination of the acquisition of salt and osmotic stress tolerance through *in vitro* selection in *M. truncatula*

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i. Summary

Changes in global climate and the non-stop increase in demographic pressure have provoked a stronger demand for agronomic resources at a time where land suitable for agriculture is becoming a rare commodity. They have also generated a number of abiotic stresses which exacerbate effects of diseases and pests and result in physiological and metabolic disorders that ultimately impact on yield when and where it is most needed. Therefore, a major scientific and agronomic challenge today is that of understanding and countering the impact of stress on yield. In this respect, *in vitro* biotechnology would be an efficient and feasible breeding alternative, particularly now that the genetic and genomic tools needed to unravel the mechanisms underlying the acquisition of tolerance to stress have become available. Legumes in general play a central role in a sustainable agriculture due to their capacity to symbiotically fix the atmospheric nitrogen, thereby reducing the need for fertilizers. They also produce grains that are rich in protein and thus are important as food and feed. However, they also suffer from abiotic stresses in general and osmotic stress and salinity in particular. This chapter provides a detailed overview of the methods employed for *in vitro* selection in the model legume *Medicago truncatula* for the generation of novel germplasm capable of resisting NaCl and PEG-induced osmotic stress. We also address the understanding of the genetic determination in the acquisition of stress resistance, which differs between NaCl and PEG. Thus, the expression of genes linked to growth (*WEE1*), *in vitro* embryogenesis (*SERK*), salt tolerance (*SOS1*) proline synthesis (*P5CS*) and ploidy level and cell cycle (*CCS52* and *WEE1*) was up-regulated under NaCl-stress, while under PEG treatment the expression of *MtWEE1* and *MtCCS52* was significantly increased but no significant differences were observed in the expression of genes *MtSERK1* and *MtP5CS*, and *MtSOS*1 was down-regulated. A number of morphological and physiological traits relevant to the acquisition of stress resistance were also assessed and methods used to do so are also detailed.

ii. Key Words

Callus, cell cycle checkpoint, *in vitro* selection, *Medicago truncatula*, NaCl, PEG6000, proline, stress resistance, endoreduplication

1. Introduction

Given its agroecological relevance (1), *Medicago truncatula* has been the object of many biotechnology studies (2-7), including on the generation of novel germplasm capable of tolerating salinity following *in vitro* selection with a step-up recurrent strategy (8), and of osmotic stress tolerance after selection on PEG 6000 (9).

M. truncatula grows in a wide range of habitats with varying environmental stress notably soil salinity and drought (10, 11), to which it is more tolerant than other legume crops such as pea, bean and soybean (12-15). Moreover, it includes a large diversity of ecotypes useful for exploring the molecular bases of environmental adaptation mechanisms. In addition, genetic tools are available to identify genes that could improve abiotic stress tolerance offering a range of approaches for exploiting such variability (16). In spite of this, previous studies particularly on water stress resistance in *M. truncatula* mostly concerned gene transfer (5, 7, 17), while assessment of the physiological responses (18) and their genetic mechanisms (10) is more limited.

2. When salinity is the problem

Salinity is typically caused by sodium chloride accumulated in the soil top-layers (19, 20) where the electrical conductivity (EC) is 1.30 mM/cm^{-1} or more at 40 mM NaCl, and this is a salt level that dramatically decreases productivity of many crops (21-24).

Salt tolerant ecotypes exist in different species that have developed mechanisms to control salt accumulation (e.g. exclusion, vacuolar sequestration or control of sodium transport) by selecting other ions normally present in the soil at lower levels, such as potassium (K^+) and nitrate (NO_3^-), but compatible with plant growth (25). Such mechanisms are not easily switched on by the plant, and high NaCl levels (≥ 40mM) may generate an imbalance of cellular ions provoking toxicity and/or osmotic stress. This results in increased reactive oxygen species

coupled with reduced photosynthesis (26), which can in time result in whole plant death (27, 28, 29).

A typical feature of the acquisition of physiological tolerance to salt and osmotic stress is an increased endogenous content of osmolytes including soluble sugars, proline and glycinebetaine (30, 31), which play an adaptive role in mediating osmotic adjustment to protect subcellular structures via the exclusion or vacuolar sequestration of Na^+ ions (20). In many plants, a positive correlation has been observed between the accumulation of glycinebetaine and proline, and stress tolerance (20, 32, 33). Interestingly, using *M. truncatula* nodules, Aydi et al. (34) also found that salt stress tolerance was correlated to nodule osmotic adjustment due to Na^+ sequestration and an accumulation of soluble sugars and amino acids, and coupled with an adequate nitrogen metabolism due to a high glutamine synthetase activity. Plant cells accumulate osmolytes including water soluble carbohydrates and proline to combat water loss in response to salt stress (41). The increase in proline is driven by induction of the P5CS gene that encodes Δ1-pyrroline -5-carboxylate synthetase, involved in proline biosynthesis (35). P5CS is highly expressed in salt-and drought-tolerant plant species (36, 37), and also after *in vitro* selection of tissues on media with high NaCl contents (8, 38).

Phenotypic changes that can enable salt tolerance also include a modification of plasma membrane permeability through a differential lipid content whereby NaCl uptake is controlled, proteins stabilised and cell antioxidant capacity enhanced (39, 40, 41). In this context, plant ecotypes able to tolerate 100 mM NaCl or more can accumulate NaCl in vacuoles or exclude Na^+ (20, 42). The *SALT OVERLY SENSITIVE* (*SOS*) gene family is important in maintaining ion homeostasis (43, 44) and conferring salt tolerance as recently reported in *Arabidopsis thaliana*, where *sos1*, *sos2* and *sos3* mutants were hypersensitive to 60 or 120 mM NaCl with root growth strongly suppressed compared to the wild type (45). This was mainly due to the fact that *SOS1* encodes a membrane bound antiporter that is regulated by the *SOS2*/*3* kinase

complex. The effect of over-expression of *P5CS* and *SOS1* genes on inducing tolerance seems however to be species-specific and, within the plant, related to a particular stage of development.

A recent transcriptional profiling of roots of *M. truncatula* under 180 mM NaCl using microarrays identified transcription factor *MtCBF4*, a member of the *AP2-EREBP* transcription factor family, as playing an important role between primary and secondary metabolism pathways in response to salt stress and may thus be a good candidate gene to obtain salt tolerant plants (46).

3. When the problem is water shortage or osmotic stress

Osmotic stress, frequently equated to water deficit or drought, is defined as an absence of the adequate moisture needed for a plant to grow and complete its life cycle normally (47). The three main mechanisms that enable plants to withstand osmotic stress are escape, avoidance and tolerance (48-50), but water stress may also alter plant physiology, whereby plants acclimate to withstand drier conditions by mitigating osmotic stress through the production of osmolytes such as proline, and soluble sugars to protect cells against osmotic perturbation (8, 36, 51-53). On the other hand, water potential induces morphological variation in stressed tissues at the cellular level, and particularly in terms of cell shape and size (54, 55) as recently shown in osmotic stress resistant cells of *M. truncatula* (9).

Plants respond to abiotic stress by altering the expression of scores of genes resulting in many cellular and physiological modifications (5, 56, 57), but information about the effect on cell cycle genes for plants exposed to high NaCl or to osmotic stress remains more limited (8, 9, 58-61).

In Arabidopsis, *WEE1* which is a negative regulator of mitosis (62), is strongly expressed in response to abiotic stress (58), but not in the context of salinity or drought, while we recently

showed that in *M. truncatula* the expression of *WEE1* is dramatically increased both by NaCl (8) and osmotic stress (9). West et al. (63) had shown that the effects of salt stress on growth are mediated by effects on cell division and, more recently, Skirycz et al. (64) showed that endoreduplication is also associated with osmotic stress in Arabidopsis. Taken together, there is increasing evidence for a central role of the *WEE1* gene in association with endoreduplication (65- 67), as well as its strong expression when the DNA replication and DNA damage checkpoints are invoked (58), and in response to abiotic stresses such as salinity and osmolarity (8, 9). Likewise, another key marker for endoreduplication is *CCS52* (cell cycle switch) (68); whose expression in *Medicago sativa* was enhanced in differentiating cells undergoing endoreduplication (69). In this context, the studies of Elmaghrabi et al. on *in vitro* selection for salt (8) and osmotic stress tolerance (9) showed that the acquisition of tolerance was coupled with the onset of endoreduplication and a concomitant increased expression level of *CCS52* too.

4. Contribution of *in vitro* biotechnology tools to selection for NaCl and osmotic stress tolerance

In vitro culture is a very powerful tool for the study of plant development. Given the potential regeneration of plants from cells, tissues, or organs cultured under suitable media, it can therefore be used also for *in vitro* selection to improve abiotic stress tolerance. From a fundamental perspective, *in vitro* selection can also be used to unravel the mechanism(s) underlying the acquisition of stress tolerance coupled to other tools as described above. The first *in vitro* plant regeneration strategy for *M. truncatula* was based on indirect somatic embryogenesis from callus tissues (70), and was thereafter followed by many protocols inducing indirect somatic embryogenesis from a range of explants including leaves, hypocotyls, cotyledons, petioles and flower parts (71-74). Direct somatic embryogenesis in *M. truncatula*

has been achieved in both liquid and solid media (75-79).

It is well known today that during *in vitro* culture some tissues may exhibit somaclonal variation (a random, non-directed, non-controlled variation of regenerating cells and/or callus sectors; 80), even when the objective may have been the production of true-to-type regenerated plants. This apparent defect can be turned into an advantage by exploiting it in terms of *in vitro* selection by culturing tissues on media including abiotic stress-inducing factors. This can in turn be used for fundamental studies to probe mechanisms induced by abiotic stresses or for applied research in providing new salt and drought tolerant germplasm, provided the regeneration capacity is maintained. Somaclonal variation is characterised by changes in ploidy level and DNA sequence, coupled with movement of transposable elements to other chromosomal sites and also including epigenetic changes, such as DNA methylation and histone modification (81). Abiotic factors (drought, salinity, extreme temperatures) can induce both somaclonal and epigenetic changes when applied over long culture periods as used for *in vitro* selection protocols (82-84).

Tissues and plants resistant to salinity have been obtained after *in vitro* culture on selection media for many years in species as wide apart as cherry (85, 86), potato (87) or *Zoysia matrella* L. (88) among many others but the fundamental mechanisms underlying the acquisition of salinity tolerance often remained partially unexplained. This was likely due to the fact that the extent and range of DNA instability *in vitro* depends on many abiotic factors, among which the age of cell cultures, the genotype studied, the type of explants, the hormonal composition of the medium, and the type of exogenous abiotic factor added to induce (physical and/or chemical) stress. However, literature on *in vitro* selection for salt resistance has addressed the cellular mechanisms involved in salt tolerance (19, 85, 89, 90) and also the genetic determinism of acquisition of resistance to salinity (8, 37, 44, 62, 63) by using selected NaCl-tolerant cell lines as study systems. Several reports also examined alternative ways to exploit stress *in vitro* to

characterize the biology and genetic diversity of the early stages of plantlet growth (33, 87, 91-97), and the effects of salinity stress on plant morphology have also been extensively studied (26, 31, 33, 98-99). However, for example, cellular studies mainly focused on the effects of abiotic stresses on cell size (100) until recent studies on the effects on cell morphometry of NaCl and osmotic stress in resistant cells of *M. truncatula* (8, 9). In this context and as a direct consequence of this lack of detailed analysis, some of the apparently resistant tissues obtained were only acclimated (101) followed by physiological adaptation to the stress induced, but not truly resistant. Thus the trait would not be heritable unless a recurrent selection strategy, encompassing back and forth passages from a stress to a non-stress condition, was applied (102). Such a strategy results in a progressive enrichment of the selected cell population in stress resistant cells facilitated by a re-programming of gene expression pattern responses to the stress, e.g. receptor kinases (103, 104), transcription factors (105), enzymes and structural proteins with a central physiological and metabolic role under stress (80). Such recurrent selection strategies can be direct (where the maximum resistance level aimed for is added to the culture medium from the start (102), or indirect where tissues are successively transferred to increasing concentrations of the stress-inducing factor until the level of resistance sought is reached (8). As mentioned above, exposure to increasing NaCl levels through acclimation may result in long-term tolerance of cells/tissues to salinity (88, 106, 107) and water stress (108). Increases in both embryogenic and organogenic callus have also been achieved by acclimation (87, 89, 109, 110), even if best conditions for *in vitro* embryogenesis are highly species specific in terms of both medium and explant (87,111). Therefore, a useful marker for embryogenic callus is the *SERK* (*SOMATIC EMBRYOGENESIS RECEPTOR KINASE*) gene (103, 112) a key gene required for somatic embryogenesis and whose expression in rice, was up-regulated in embryogenic calli (113). *SERK* was also recently found to be up-regulated following selection for NaCl tolerance *in vitro* in *M. truncatula* (8).

Likewise, water stress tolerant cell lines have been selected *in vitro* and resistant plants were recovered in crops like alfalfa, tomato, soybean, and wheat (114-116). Most frequently, the stress-agent used was PEG 6000 (85, 115, 117, 118) whose adsorbant features imposes on cells and tissues the same or comparable effects to those obtained by drying soil at the same φw but generally without any other associated detrimental effects (119). Note however that PEG 6000 was also reported to induce DNA damage on *in vitro* plantlets (120) and seeds (121) of *M. truncatula*. Thus, PEG 6000 imitates soil water shortage (122) and also increases total soluble sugars (9, 123), while stimulating somatic embryogenesis *in vitro* (124, 125), including in *M. truncatula* (9).

5. Protocols for *in vitro* plant regeneration in M. truncatula.

In *M. truncatula*, the most frequently used and most successful medium components for the induction and maintenance of somatic embryogenesis are various combinations of auxins such as 2,4-D (2,4-D, dichlorophenoxyacetic acid), NAA, (α-naphthalenacetic acid) or Picloram with either BAP (6-benzylaminopurine) or kinetin as cytokinins (70, 73, 126, 127) as summarized in Table 1.

The synthetic auxin, 2,4-D is known to generate callus for indirect embryogenesis in legumes and particularly in Medicago species (73, 128, 129), with a clear dose-response mechanism in action whereby the optimum concentration for somatic embryo induction is highly species- and genotype-specific (2, 130). As far as NAA is concerned, its importance is mostly for the induction of callus formation rather than for the differentiation of somatic embryos, although it also proved successful in that respect with both *M. truncatula* (70) and *M. polymorpha* (131). Production of callus tissue followed by embryo formation has been achieved in *M. truncatula* on solid medium supplemented with the cytokinin, BAP (6-benzylaminopurine) (73). However, induction of somatic embryogenesis by a single cytokinin

is relatively rare with legumes, and sequences of different cytokinins (e.g. BAP, Kinetin, Thidiazuron) are often required (111, 132). The molecular processes activated during the induction and development of embryogenesis are still poorly understood. However, recently, the *SERK* gene from *M. truncatula* (*MtSERK1*) has been cloned and its expression evaluated on defined culture media. (74, 104). Its expression is strongly linked to somatic embryogenesis.

Lowering or a complete removal of auxin is a key for embryo development and maturation beyond the globular stage in *M. truncatula* (73, 75-79). In addition, moderate reductions in cytokinin levels are sometimes also useful for a normal progress of embryogenesis *in vitro* (71, 76). Moreover, a total removal of phytohormones from the culture medium is often the only way to enable somatic embryos to convert into a robust plantlet (2, 79), and the stage of embryo maturation at which auxins are removed is also critical for its subsequent development (71, 79). Last but not least, *in vitro* cultures in general, and those of recalcitrant species like legumes in particular, tend to lose regeneration competence as they age (2, 88, 111), and this may have dramatic effects on the efficiency of long-term *in vitro* selection studies on callus and cell suspension cultures, as the subsequent regeneration of plants from the tolerant tissues might be impaired.

6. Protocols for *in vitro* selection for abiotic stress in *M. truncatula*.

6.1. Callus and cell suspension culture initiation and maintenance

In two recent articles, Elmaghrabi et al. reported protocols useful for the *in vitro* selection of NaCl-induced salinity (8) and PEG 6000-induced osmotic stress (9) with *M. truncatula* and the procedures employed are described and discussed below.

For both protocols, a recurrent selection strategy was adopted, where *M. truncatula* cv. Jemalong line (A17) (2n=2x=16, 1C value = 0.48 pg) was used as the source of material.

As in any *in vitro* selection study, the first step was the optimization of the composition of culture conditions required for the induction of callus tissues and also for the initiation and

maintenance of cell suspension cultures from such callus. This entailed a number of preliminary assessments to identify the best original explant source, basal salts mixture and hormonal composition of the culture medium and light and temperature regimes. During such tests, hypocotyls, cotyledons and green leaves from aseptically germinated seedlings were compared as explant sources, all being wounded with a scalpel in order to ensure contact of the explant tissue with the culture medium, and they were cultured on four different media all based on Murashige & Skoog (133) salts formula, with supplements as detailed in Table 2.

For culture, multi-well 5 X 5 dishes with 2 mL of medium per well were used, providing 25 replicates for each treatment (medium and explant), and all experiments were repeated twice. Dishes were kept at 24/22 C°, in the dark or under a 16/8 h (light/dark) photoperiodic regime of 90 µmol m^{-2} s^{-1} from warm white fluorescent tubes for initiation of callus proliferation. After four weeks, explants were sub-cultured on the four different media and the frequency of callus initiation assessed as reported elsewhere (127). The optimum medium was MANA and the most responsive explants were leaves, where mature callus cultures were established within 5 to 6 months with monthly subculturing.

6.1. Full experimental protocol for *in vitro* acclimation, recurrent selection, growth assessments and characterization of stress-resistant material produced

During establishment and once no remaining material from the initial explants was left within the culture tissues, the callus was observed by light microscopy for signs of embryogenesis and organogenesis. Then, calli were transferred to Petri dishes (10 cm diameter x 2 cm) with 25 mL MANA medium supplemented with or without 50 mM NaCl. One month into the 50 mM treatment, embryogenic callus only was stepped up to 100 mM NaCl for one month, when half of such calli were kept at 100 mM NaCl and the other half was stepped up to 150 mM NaCl. Likewise, one month into this treatment, calli were split into two halves where one was subcultured onto fresh medium with 150 mM NaCl and the other half stepped up onto

either 250 or 350 mM NaCl for 3 months (Fig. 2). Thus, by five months from the start of the acclimation strategy calli were available that had remained continuously on 50 mM NaCl (6 months), 100 mM NaCl (5 months), 150 mM NaCl (4 months) and 250 or 350 mM NaCl (3 months), in addition to the controls on 0 mM NaCl (10 months). This first phase of acclimation was termed Phase I. At this point in time, the first Phase of indirect recurrent selection (Phase II) was initiated by transferring all calli onto MANA medium minus NaCl for two months and then back to the same Phase I NaCl concentration they came from. Phase III (second cycle of recurrent selection) was a repeat of Phase II, and by the end of Phase III calli were 24 months old.

In parallel to these treatments, five month old control calli were also tested for their growth potential on medium containing PEG 6000 (1%, w/v) and they were submitted to the same strategy only that there were no step-up stages, thereby constituting a direct recurrent selection methodology when it came to the induction of tolerance to osmotic stress.

For the quantification of growth, data were first examined for linearity following log transformation of y axis coordinates, and exponentially growing calli were then measured in terms of absolute (g month^{-1}) and relative growth rates (RGR g g $^{-1}$ day^{-1}), as follows:

For the relative growth rate calculation the formula used was

$$\mathrm{RGR} = \frac{lnB - \ln A}{t}$$

where A and B are the sampling times within the exponential phase of growth for each treatment and t is the time interval between successive samplings. Thereafter, when results conformed to linear growth, they were analyzed by regression and growth (g month^{-1}) determined according to the gradient of the straight line (y = mx+c), and finally statistically significant regressions were then used to calculate growth (g m^{-1}).

When growth was neither exponential nor linear, absolute callus growth per month was estimated as

$$\mathrm{GR} = \frac{FW2 - FW1}{t}$$

where FW1 and FW2 are the fresh weight of callus at the beginning and end of treatments, respectively and t spans this time interval

6.2 Assessment morphogenetic potential

Once Phase III above was completed, stress-tolerant callus tissues were sub-cultured onto an embryo development medium (EDM), derived from that reported by Iantcheva et al. (134) and supplemented with the same NaCl concentration as used during Phase III, for 4-6 weeks. Then, such calli were transferred for a further two months onto an embryo conversion and rooting medium (ECR) (134) supplemented again with the same NaCl concentration as in the previous passage (Fig. 2). At this stage, callus tissues were examined under a binocular loupe and the extent of embryo and/or organ formation at each concentration of NaCl was recorded.

6.3. Endogenous K+ and Na+ measurements

For the determination of the sodium accumulation in the callus, 100 mg fresh weight pieces were collected from three independent replicates following Phases I, II and III, i.e. with calli that were 11, 17, and 23 months-old, respectively. The dry weight of each replicated fresh callus piece was determined by incubation in a glass beaker for 24 h in an oven at 80 ºC. Then, 10 mL of 15.8 M HNO_3 (Fisher-MOS) was added to each beaker and beakers were placed on a sand bath at 240 ºC in a fume cupboard for 2-3 h until the digests started to clear (more acid should be added as necessary to prevent the digest from drying up), and once samples had cooled (15 min) at room temperature, they were filtered and made up to 25 mL with ultra-pure water. Na^+ accumulation for each treatment was measured using an atomic absorption spectrophotometer (Varian- Spectr AA-100, version 2.00 software) and [Na^+] calculated as:

$$\frac{\mu g \text{ ion}}{g \text{ sample material}} = \frac{ppm\ value\ \left(\frac{\mu g}{cm3}\right) x\ volume\ x\ dilution\ factor}{dry\ weight}$$

The same methodology was used to measure K^+ accumulation in 23 months-old calli.

6.4. Proline measurement after selection for NaCl and osmotic stress tolerance

For all experiments on *in vitro* selection for salt and osmotic stress tolerance with *M. truncatula* we evaluated the proline content (8, 9) following methods reported by Troll and Linsley (135) and Boukel and Houassine (136). Briefly, 100 mg of callus tissue per sample per treatment was examined over the concentration range 0, 50, 100, 150, 250 and 350 mM NaCl, repeated three times. Samples were collected in 30 mL tubes and 2 mL of 40% ethanol was added per tube prior to heating in a water- bath at 85° C for 60 min. After cooling samples (2 h, room temperature), 1 mL extract per sample was transferred to a tube with 1 mL acetic acid and 1 mL ninhydrin reagent (120 mL distilled water + 30 mL acetic acid + 80 mL orthophosphoric acid (density 1.7) + 25 mg ninhydrin). This mixture (sample and reagent) was then boiled (30 min) and after cooling at room temperature, 5 mL benzene was added. At this time, an upper layer yellow- coloured band indicated the presence of proline (136), whereby determination of the sample optical densities was performed using a spectrophotometer (LNICAM: uv-vis spectrometry) at a wavelength of 528 nm. A standard proline solution was prepared from a stock solution (125 mg proline in 100 mL 0.3 M HCl) that was diluted with 0.3 M HCl to obtain concentrations of 0.1, 0.2 and 0.4 mg mL^{-1} for the calibration used to determine the proline concentration of different samples.

6.5. Water soluble carbohydrate measurements

Water soluble sugars were measured with the anthrone method of Plummer (137) by extraction from three 100 mg callus samples from each NaCl treatment (0, 50, 100, 150, 250 and 350 mM

NaCl), that were immersed for 48 h in 3 mL of 80% ethanol and then were heated in a water bath at 70 °C (30 min). Then, 2 mL were taken from the extract solution from each sample and were diluted 10 times with 80% ethanol plus 4 mL of anthrone (0.2 g dissolved in 100 mL of 18M H_2SO_4, prepared at least 4 h in advance). Tubes were shaken, placed in a water bath at 92 °C for 8 min, and finally cooled (30 min on ice in the dark). The water soluble sugar content was read spectrophotometrically (LNICAM: uv-vis spectrometry) at a 585 nm wavelength, and the data were converted to mg l^{-1} using pre-established calibrations.

6.6. Osmolarity measurements.

All measurements of osmolarity were carried out using a Wescor (model VAPRO 5520, USA) vapour pressure micro-osmometer on a minimum of three 10-µl samples for each treatment and the intracellular osmolarity and that of MANA medium were also evaluated at the end of step-up acclimation, i.e. Phase I.

For assessments at the intracellular level, 2 g fresh weight of callus were collected from all salt stress treatments of 0, 50, 100, 150, 250, and 350 mM NaCl after callus terminated Phase I, and also for callus tissues selected for resistance to PEG-induced osmotic stress. They were crushed in an Eppendorf tube with a pestle, then centrifuged (1000g, 10 min, 4ºC), and finally, the supernatant was employed for measurements of osmolarity after calibrating the micro-osmometer. For calibration, three standard solutions are used: 100 ± 0.3 mM/Kg (equivalent to osmosed deionized water osmolarity), 290 ± 0.5 mM/Kg (equivalent to milliQ water osmolarity) and 1000 ± 1.4 mM/Kg (equivalent to sea water osmolarity) (54).

Calibration is a delicate and time-consuming operation, as a minimum of five measurements of each standard solution are performed in order to determine precisely the range of technical error to be found in the subsequent measurements. Thus, the variations from the standard solution data evoked above are those corresponding to the actual measurements performed at the time

of experiments, but they are not necessarily identical to figures that may be obtained every time the experiments are repeated. In general, an admissible margin of error would be of ≤ 1.5 mM/Kg against the 100 mM standard, ≤ 3.5 mM/Kg against the 290 mM standard and up to 6 mM/Kg against the 1000 mM standard.

Results, are the mean ± S.E of measurements performed at least three times and are expressed in terms of mM/kg and, for all parameters assessed, should be analysed statistically ($P \geq 0.05$).

6.7. Determination of the water content of callus tissues

For determinations of the water (according to 138), about 0.3g fresh weight of 11 month old calli, at the end of Phase I, was gathered in three replicates from each NaCl treatment: 0, 50, 100, 150, 250 and 350 mM. The fresh weight (FW) was determined and they were oven-dried (70 °C, for 72 h) to determine the dry weight (DW). Then, the water content (WC) was simply calculated according to the formula: WC (%) = FW- DW/ FW X 100 (139).

6.8. Determination of mitotic index, cell viability, and cell morphology

C-value of calli was determined following *in vitro* selection for several months as compared to leaf tissues from the original plants in order to confirm their cytogenetic stability. Thus, nuclei were isolated from abiotic stress resistant calli and also from leaves of greenhouse grown *M. truncatula* A17 plants, by chopping tissues in 400 mL of nuclear extraction buffer to which 1.6 mL of staining buffer containing 4, 6 diamidino-2-phenylindole (DAPI; Sysmex), an A-T binding specific fluorochrome, were added (140). The resulting nucleus suspension was filtered through a 20 mm nylon mesh and DNA contents of the isolated nuclei was analyzed using a Partec PAS-II flow cytometer equipped with an HBO-100 W mercury lamp and a dichroic mirror (TK420). Ten replicated calli for each treatment were analyzed, with a minimum of 3000 to 10000 nuclei per run. The mitotic index was calculated according to the formula: MI D 4 x

4C/6 2C C 4C, where 2C and 4C correspond to the mean integrated value of nuclei in G1 phase and G2, respectively (140).

Cell viability was estimated by dual staining with fluorescein diacetate (FDA; Sigma, Poole, United Kingdom) and propidium iodide (PI; Sigma, Poole, United Kingdom). Cell suspensions (75 mL) from each treatment were mixed with 75 mL of dual staining solution containing FDA (200 mg mL^{-1}; 141) and propidium iodide (PI at 120 mg mL^{-1}) on ice and incubated for 20 min. FDA is cleaved by esterases in the cytoplasm into acetate and fluorescein which is hydrophilic and accumulates in the cytoplasm of metabolically active cells. Thus, after excitation under the UV light live cells fluoresce yellow–green, while dead cells appear red using a fluorescent microscope. A minimum of 300 cells are counted and results are expressed as the percentage of fluorescing cells referred to the total number of cells in the field.

Following application of a long-term *in vitro* selection strategy, the cell morphology should also be characterized (1). For this, FDA stained slides of control and stress-treated cells are observed using a microscope under UV light. The surface area of cells and nuclei is determined after variable times of culture (generally 2, 4, and 6 months), using image acquisition programs such as ArchimedPlus and Histolab (Microvision, France) as reported by Ochatt et al. (54) or with Image J software, and it is also advisable to apply a shape coefficient (55) once selected cells are stabilized under the stress factors tested, i.e. at about 6 months of culture. Briefly, this shape coefficient (SC) is calculated based on the half length of the cell along its longest (a) and shortest (b) axes, as:

$$SC = \frac{\sqrt{a^2 - b^2}}{a}$$

For each treatment, nucleus and cell size should be measured on 10 to 20 cells, and results are expressed as the mean ± SE. The SC distinguishes round from elongated shapes, since SC values close to 1.0 correspond to elongated cells while SC values close to 0.5 correspond to rounder cell shapes (55).

For vacuolar measurement, calli having completed Phase III (23 months ± acclimation) on 0, 50, 100, 150, 250 and 350 mM NaCl were used. Vacuoles were measured by placing 50 mg callus in small vials containing a 1% (w/v) aqueous solution of Lucifer yellow–dipotassium salt (Sigma) for 2 hs. Lucifer yellow is a trace that stains the apoplast and accumulates in the pro-vacuole; it cannot penetrate membranes but it can penetrate the tonoplast (142). Size of stained vacuoles was measured by image analysis using Sigmascan–pro (objective: DPlan Apo 20 UV, 0.70, 160/0.17).

6.8. Real Time PCR

In the *in vitro* selection studies of Elmaghrabi et al. for salt (8) and osmotic stress resistance (9), all procedures were according to Spadafora et al. (143, 144). Thus, RNA was extracted using TRI Reagent (Sigma Aldrich, UK) with a ratio of about 200 mg of plant material to 2 mL of TRI Reagent. Keeping this ratio is important to ensure sufficient concentration of the resulting RNA without contamination from proteins and other cellular components. Leaf or callus material was ground to a fine powder using liquid nitrogen in a sterile pestle and mortar (pre-chilled to -20 ^{o}C) ensuring that the plant material remained frozen throughout. Then, 2 mL of TRI Reagent were added to the mix and ground to form a homogeneous paste and the paste was transferred to a 1.5 mL Eppendorf tube. Following a brief (15 sec) vortex the extract was left at room temperature for 5 min, before being centrifuged at 12 000 rpm for 10 minutes at 4 ^{o}C in a microcentrifuge (BECKMAN COULTERTM- AllegraTM 12R Centrifuge) to pellet the cellular debris. The resulting supernatant was added to 0.2 mL of chloroform, vortexed (10 sec) and left to stand at room temperature for 5 min. This was followed by centrifugation at 12000 rpm in a microcentrifuge (as above) for 15 min at 4 °C. The supernatant produced contained an aqueous layer of RNA which was carefully removed, avoiding the interface between the two phases and mixed with 0.5 mL of isopropanol to precipitate the nucleic acids at room temperature for 10 min. The mix was then centrifuged for 10 min at 12000 rpm in a

microcentrifuge at 4 ºC (as above). The supernatant was removed and the pellet washed in 1 mL of 75% ethanol to remove residual salts. A quick vortex followed before another round of centrifugation for 10 min at 12000 rpm in a microcentrifuge at 4 ºC (as above). The pellet was air dried thoroughly (in a flow cabinet or fume hood to increase air flow and accelerate the process) and resuspended in sterile distilled water. The RNA was kept on ice until used or stored at -80 ºC. Concentration and purity were tested using a Nanodrop spectrophotometer (Nano Drop® ND-1000 spectrophotometer) and by agarose gel electrophoresis (Fig. 2) to visualize the rRNA bands. This is important to assess integrity of the RNA. To ensure that the RNA was not degraded during electrophoresis, the tank, comb and tray were soaked in 0.1 M NaOH for at least 10 min and then rinsed thoroughly with distilled water before use.

To ensure that real time PCR results are a true reflection of changes in gene expression it is very important to remove residual genomic DNA. DNase treatment was therefore used to remove the residual genomic DNA. Depending on the RNA concentration, from 2 to 16 µl of RNA (2µg) were added to a solution of 2 µl of RQ1 DNAase 10X buffer (Promega), 2 µl of RQ1 DNAse and sterile water to a 20 µl final volume and were incubated at 37 ºC for 10 min. To stop the reaction 2 µl of RQ1 DNAse stop solution were added to the mix, and incubated at 65 ºC for 10 min. To test whether the DNAse treatment was successful, the absence of contaminating genomic DNA was verified by PCR amplification with primers to 18S rRNA (PUV2F 5`-TTCCATGCTAATGTATTCAGAG-3` and PUV4R 5`-ATGGTGGTGACGGGTGAC-3`) using appropriate positive and negative controls (plant genomic DNA and sterile distilled water), and products were analysed by agarose gel electrophoresis. If residual genomic DNA was present the DNAse treatment was repeated, checking the integrity and concentration of the RNA again after the second round of DNAse treatment.

Purified RNA (2 µg of in a total volume of 19 µL) was retrotranscribed using an Ambion kit (RETROscript® Reverse transcription for RT-PCR) and cDNA was stored at -80 ^{o}C until future processing. cDNA quality was tested by PCR using 18S rRNA primers and the primers for the target genes of interest. PCR using 18S rRNA and limited cycle number was used to equalize the cDNA concentrations prior to real time PCR.

An ABsoluteTM QPCR SYBR® Green Mix (Thermo Scientific) kit was used in the real time PCR process, where each reaction consisted of a total volume of 25 µl: 5 µl cDNA (diluted 1:20), 12.5 µl ABsolute^{TM} QPCR SYBR® Green Mix, 1.75 µl of each primer (10 µM) and 4 µl H2O. Reactions were cycled in an MJ Research OPTICONTM 2, in triplicate, at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s and finally one cycle of 72 °C for 30 s. A melting curve analysis (60 to 98 °C with an increasing heat rate of 0.5 °C s^{-1}) was performed to test primer specificity after amplification. Mt18S rRNA primers were used to normalise the results in Elmaghrabi et al. (8, 9) with *M. truncatula* samples. 18S rRNA is a reliable reference gene that has been used widely for developmental and stress-response studies with many species (145, 146). To ensure correct quantification, the real time PCR was repeated adjusting the dilution of the cDNA if cycle number variability amongst the samples was > 2 cycles. At least two technical and three biological replicates are recommended for real time PCR. The relative quantification of gene expression data was carried out with the 2-DDCT or comparative CT method (147).

7. Conclusion

Use of *in vitro* selection for the development of salt and drought tolerant germplasm is underpinned by a range of techniques described above that assess the physiological, biochemical and gene expression status of the cells as they progress through the selection process. This ensures that enhancement of the tolerance can be followed and optimized

throughout these long experiments. Using the three phase step protocol in *M. truncatula* resulted in a change in these parameters over the treatments (Fig 3) (8, 9). For example, mean callus growth, and *SOS1* gene expression changed from moderate decrease to moderate increase compared to controls during the course of the experiment. In contrast many other parameters showed a consistent but moderate increase even from the earliest time points measured at 6 months after the start of the selection. As techniques for the analysis of gene expression become cheaper and faster, future analyses may incorporate approaches such as RNAseq, proteomics, metabolomics and whole genome methylation experiments to assess the overall status of the germplasm, offering new insights into the cellular changes that are taking place and the mechanisms that underlie those changes (148, 149).

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Legends for figures:

Figure 1: Full *in vitro* culture protocol from callus initiation to selection for NaCl resistance. **(A)** Leaf explants from *in vitro* germinated plantlets are grown on various media for callus initiation, which is best on MANA medium where they are kept by either continuous culture on 0 mM NaCl (control treatment) or, after 5 months entered in the acclimation protocol; **(B)** Acclimation protocol (until month 10 of culture, Phase I) begins by subculturing calli onto 50 mM NaCl for one month and thereafter, monthly, calli are also stepped-up to 100 mM NaCl then to 150 mM NaCl and finally to 250 mM or 350 mM NaCl where they were kept for 4, 3 and 2 months, respectively. This marks completion of Phase I, which is followed by recurrent selection consisting of two back and forth passages between stress-free and stress-inducing medium, repeated twice i.e., Phases II and III (the same protocol is applied for PEG6000 only that direct recurrent selection is applied). **(B)** Callus morphology by the end of Phase I as calli are acclimated to increasing NaCl concentrations from 0 to 350 mM NaCl.

Figure 2: Quality checks for RNA quality and primer specificity **(A)** Gel electrophoresis of RNA extracted from leaf and callus from *Medicago truncatula* grown with different levels of abiotic stress. Lanes: 1= 1 kb ladder, 2= leaf (0 mM NaCl), 3= callus (0 mM NaCL), 4= callus (50 mM NaC), 5= callus (100 mM NaCL), 6= callus (150 mM NaCl), 7= callus (250 mM NaCl), 8= callus (10 v/v PEG) showing sharp bands for both major rRNAs **(B)** Melting curve of Mt18S gene as control, following Phase I of *M. truncatula* calli on NaCL stress treatments (See Fig 1 for further details) showing a sharp single peak indicating that there are no primer-dimers or other non-specific products in the real time PCR reaction.

Figure 3. Summary of physiological responses and molecular results following in vitro selection for NaCl and PEG resistance with M. truncatula cultures compared with non-stressed

control tissues. Blue color indicates increase/upregulation (moderate= light, high= dark), red color indicates decrease/downregulation (moderate= light, high= dark) and when non-significant compared to controls white is used. *, P = < 0,05; ** P = < 0,01; ND = not determined.

Table 1. Culture media used to induce somatic embryogenesis in *Medicago truncatula*

Medium Code	**Basal Medium**	**Supplements***	**References**
B5E1	B5 (150) + 3% sucrose + 7 g/L agar	1 mg/L 2,4-D + 0.2 mg/L Kin + 1 mg/L adenine + 500 mg/L CEH + 500 mg/L myo-inositol	134
EID	MS (133) + 3% sucrose + 7 g/L agar	1 mg/L 2,4-D + 0.2 mg/L BAP + 1 g/L CEH + 100 mg/L myo-inositol	134
B5,4	B5 (151) + 3% sucrose + 7 g/L agar	4 mg/L 2,4-D + 0.2 mg/L Kin + 1 mg/L adenine + 500 mg/L CEH	76
MS4	MS (133) + 3% sucrose + 7 g/L agar	4 mg/L 2,4-D + 0.2 mg/L BAP + 100 mg/L myo-inositol	77
PCI-4	SH (152) + 3% sucrose + 7 g/L agar	4 mg/L 2,4-D + 0.2 mg/L BAP	72
R2B5	B5 (151) + 3% sucrose + 7 g/L agar	1 mg/L IAA + 5 mg/L Kin+ 1 g/L CEH	91
CIM-D	MS (150) + 3% sucrose + 8 g/L agar	1 mg/L 2,4-D + 2 mg/l Z + 100 mg/L myo-inositol	154
CIM-C	UM (Uchimiya and Murashige; 153) + 3% sucrose + 8 g/L agar	1 mg/L 2,4-D + 2 mg/L Z + 100 mg/L myo-inositol + 0.2 mg/L bacto-tryptone	71
MANA	MS (150) + 3% sucrose + 7 g/L agar	2 mg/L NAA + 0.5 mg/L BAP + 100 mg/L myo-inositol	8, 9, 111
MPIC	MS (150) + 3% sucrose + 7 g/L agar	0.2 mg/L Picloram + 0.52 mg/L Kin + 100 mg/L myo-inositol	8, 9, 111

* 2,4-D, 2,4-dichlorphenoxyacetic acid; BAP, 6-benzylaminopurine; CEH, casein enzymatic hydrolysate; IAA, 3-indoleacetic acid; Kin, Kinetin (6-furfurylaminopurine); NAA, alpha-naphthalene acetic acid; Pic, Picloram (4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid)

Table 2. The four different media used to optimize the ideal conditions of *in vitro* growth of callus derived from explants of *Medicago truncatula.* See also Figure 1.

Medium Codes[a]	Growth regulators and other supplements (mg l^{-1})		
	Auxin[b]	Cytokinin[c]	Other[d]
MANA	NAA (2.0)	BAP (0.5)	Nil
EID	2,4-D (1.0)	BAP (0.2)	CEH (1,000)
MS4	2,4-D (4.0)	BAP (0.2)	Nil
MPIC	Pic (0.2)	Kin (0.5)	Nil

[a] MANA and MPIC media, as in Ochatt et al. (111), EID : Embryo Induction Development, modified from Iantcheva et al. (134). See also Table 1.
[b] NAA = alpha-naphthalene acetic acid, 2,4-D = 2,4-dichlorphenoxyacetic acid; Pic = Picloram (4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid). See also Table 1.
[c] BAP = 6-benzylaminopurine; Kin = Kinetin (6-furfurylaminopurine). See also Table 1.
[d] CEH = casein enzymatic hydrolysate. See also Table 1.

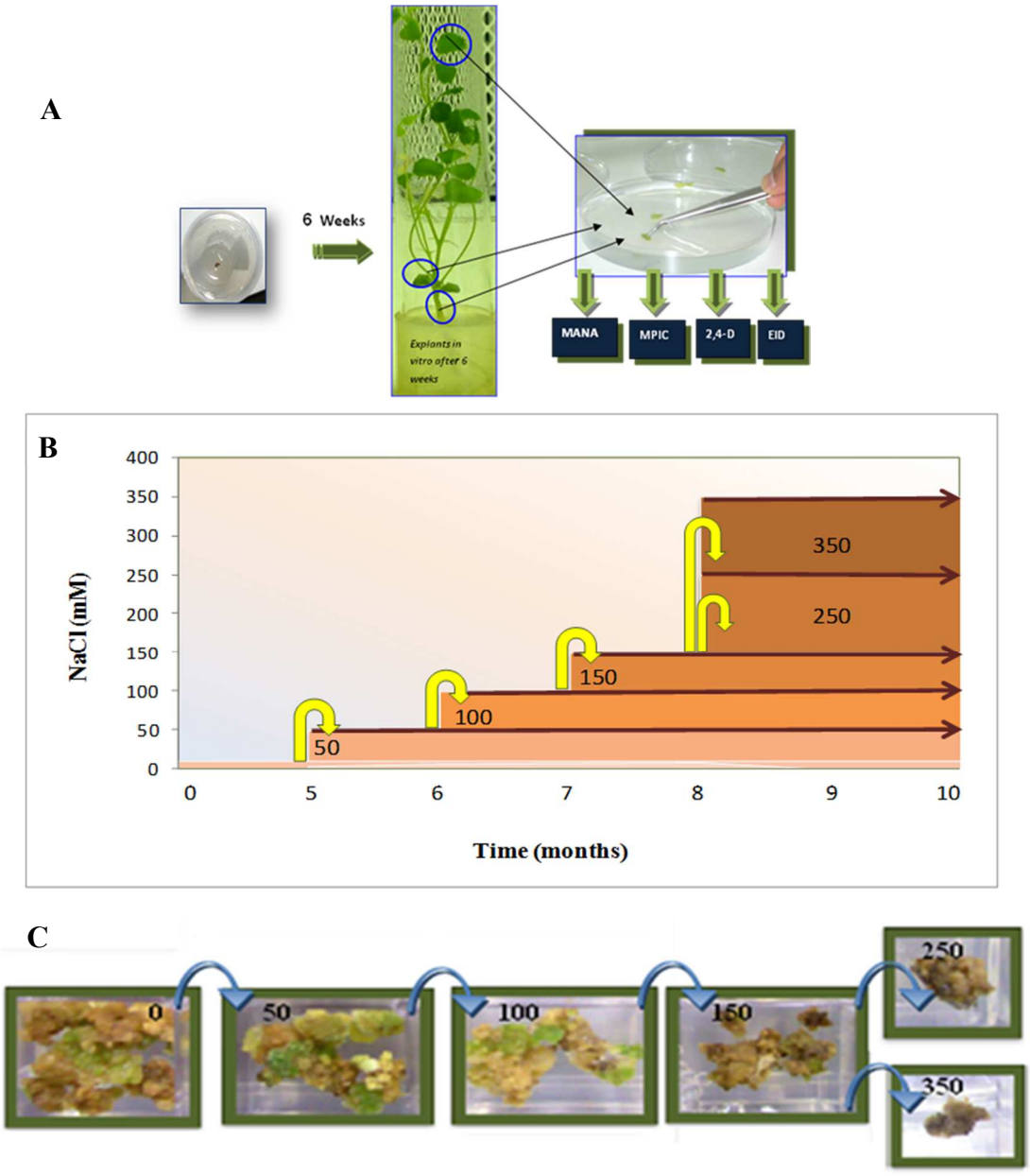
A
6 Weeks
Explants in vitro after 6 weeks
MANA
MPIC
2,4-D
EID
B
NaCl (mM)
Time (months)
0
50
100
150
250
350
C
0
50
100
150
250
350

Figure 1

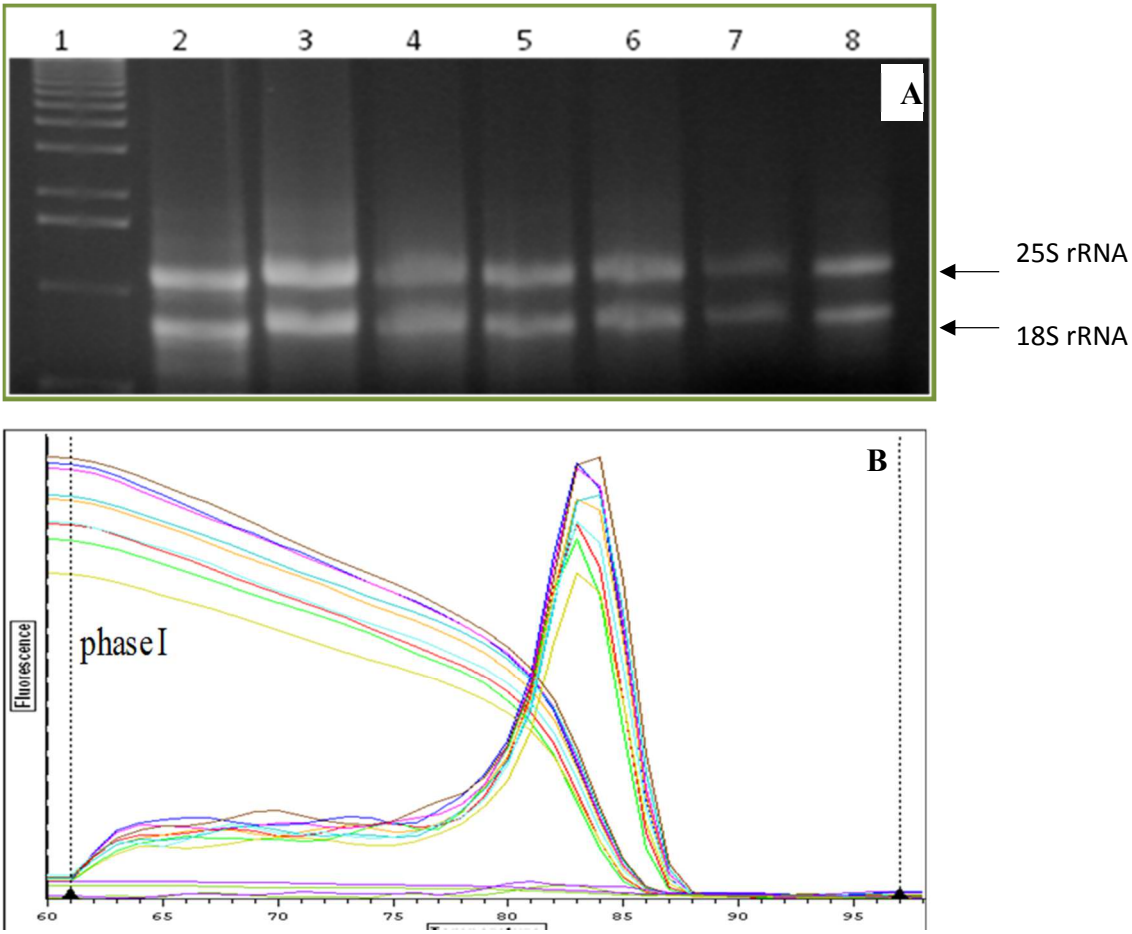
1
2
3
4
5
6
7
8
A
25S rRNA
18S rRNA
B
phase I
Fluorescence
60
65
70
75
80
85
90
95

Figure 2

	Treatments															
NaCl (mM)	50			100			150			250			350			PEG 6000 (1%, w/v)
Phases	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	
Mean Callus Growth				*		*	*		*	**	**	**	**	**	**	
Na^+ accumulation	*	*	*	*	*	*	*	*	*	**	**	**	**	**	**	
K^+ accumulation	ND	ND		ND	ND	*	ND	ND	*	ND	ND	**	ND	ND	**	
Proline	ND	*	*	ND	*	**	ND	*	**		*	*		*	*	*
Soluble sugars	ND	ND		ND	ND		ND	ND	*			*				*
Water content		ND	ND		ND	ND		ND	ND	*	ND	ND	*	ND	ND	
Osmolarity	*	ND	ND	*	ND	ND	*	ND	ND	**	ND	ND	**	ND	ND	*
Vacuolar size	ND	ND		ND	ND	*	ND	ND	*	ND	ND	*	ND	ND	*	
SOS1 expression	*	*		*	*	**	**	**	*	ND	ND	ND	ND	ND	ND	**
P5CS		**	*	*	**	**	**	**	**	ND	ND	ND	ND	ND	ND	
SERK1	*	*	*	*	**	**	**	*	*	ND	ND	ND	ND	ND	ND	
WEE1	*	*	*	*	**	**	**	*	**	ND	ND	ND	ND	ND	ND	*
CCS52	*	*	*	*	**	**	**	**	**	ND	ND	ND	ND	ND	ND	*

Figure 3