Running head

The transcription factor MtSERF1 and somatic embryogenesis

Corresponding author

Dr. Ray J Rose
ARC Centre of Excellence for Integrative Legume Research
School of Environmental and Life Sciences
The University of Newcastle
University Drive
Callaghan,
NSW 2308, Australia

Phone: +61-2-49215711
Email: ray.rose@newcastle.edu.au

Journal research area

Development and Hormone Action
The transcription factor MtSERF1 of the ERF subfamily identified by transcriptional profiling is required for somatic embryogenesis induced by auxin plus cytokinin in *Medicago truncatula*.

Feky R. Mantiri, Sergey Kurdyukov, Dasharath P. Lohar, Natalya Sharopova, Nasir A. Saeed, Xin-Ding Wang, Kathryn A. VandenBosch and Ray J. Rose

Australian Research Council Centre of Excellence for Integrative Legume Research (F.R.M., S.K., N.A.S., X-D.W., R.J.R.), School of Environmental and Life Sciences, The University of Newcastle, University Drive, Callaghan, NSW 2308, Australia;
Department of Plant Biology (D.P.L., N.S. and K.A.V.), University of Minnesota, Saint Paul, Minnesota, 55108, USA.
This research was supported in part by an Australian Research Council Centre of Excellence Grant (CEO348212) to The University of Newcastle Node of the Centre of Excellence for Integrative Legume Research. Support for microarray analysis was provided by the NSF Plant Genome project (award # 0110206) and the University of Minnesota.

These two authors contributed equally

* Corresponding author; e-mail ray.rose@newcastle.edu.au
ABSTRACT

Transcriptional profiling of embryogenic callus produced from *Medicago truncatula* mesophyll protoplasts indicated up-regulation of ethylene biosynthesis and ethylene response genes. Using inhibitors of ethylene biosynthesis and perception it was shown that ethylene was necessary for somatic embryogenesis in this model legume. We chose several genes involved in ethylene biosynthesis and response for subsequent molecular analyses. One of these genes is a gene encoding a transcription factor that belongs to the AP2/ERF superfamily and ERF subfamily of transcription factors. We demonstrate that this gene designated *Mt SOMATIC EMBRYO RELATED FACTOR 1* (*MtSERF1*) is induced by ethylene and is expressed in embryogenic calli. *MtSERF1* is strongly expressed in the globular somatic embryo and there is high expression in a small group of cells in the developing shoot meristem of the heart-stage embryo. RNAi knock-down of this gene causes strong inhibition of somatic embryogenesis. We also provide evidence that *MtSERF1* is expressed in zygotic embryos. *MtSERF1* appears to be essential for somatic embryogenesis and may enable a connection between stress and development.
INTRODUCTION

There have been numerous studies concerning the hormonal induction of somatic embryogenesis (SE) in a wide range of species. In almost all cases auxin has a critical role and cytokinins are frequently involved (Fehér et al., 2003; Rose 2004). Stress is also a factor that has been increasingly recognised as having an important role in the induction of somatic embryogenesis (Touraev et al., 1997; Fehér et al., 2003, Nolan et al., 2006). Hormones and stress collectively induce dedifferentiation of differentiated cells and the initiation of an embryogenic program (Fehér et al. 2003; Ikeda-Iwai et al., 2003; Rose and Nolan, 2006).

The molecular mechanisms involved in the induction of SE from cultured tissue are not well understood. There has, however, been progress in identifying the involvement of the SOMATIC EMBRYO RECEPTOR KINASE (SERK) and a number of transcription factors. Arabidopsis transformed with the AtSERK1 gene under the control of the cauliflower mosaic virus 35S promoter showed a marked increase in SE compared to wild-type cultures (Hecht et al., 2001). Ectopic expression of the transcription factors LEAFY COTYLEDON1 (Lotan et al., 1998), LEAFY COTYLEDON 2 (Stone et al., 2001), BABY BOOM (Boutilier et al., 2002), and WUSCHEL (Zuo et al., 2002) in Arabidopsis cause spontaneous formation of somatic embryos on intact plants or explants. AGL15 is another transcription factor that promotes SE in Arabidopsis (Harding et al., 2003). In addition, many other genes are specifically expressed in somatic embryogenesis (Imin et al., 2005).

In *Medicago truncatula* high rates of somatic embryo formation can be induced in the Jemalong genotype 2HA (Rose et al., 1999) by application of the hormones auxin and cytokinin (Nolan et al., 2003). The 2HA genotype is a “super embryogenic” mutant that is 500-fold more embryogenic than wild-type Jemalong (Nolan et al., 1989; Rose et al., 1999, Rose and Nolan 2006). *M. truncatula* is a model legume (Cook, 1999) with the sequencing of the gene-rich euchromatin nearing completion (Young and Shoemaker, 2006). Mutant resources (Tadege et al., 2005), numerous expressed sequence tags, microarray chips, proteomic tools, physical and genetic maps are available for *M. truncatula* (VandenBosch and Stacey, 2003). The 2HA genotype coupled with the genomic and molecular genetics tools makes *M. truncatula* an attractive system to investigate the molecular genetics of somatic embryogenesis (Nolan et al., 2003; Imin et al., 2005; Rose and Nolan, 2006).
In addition to the application of hormones to induce somatic embryogenesis there is the stress component, induced by the excision and culture of the explant, to consider (Nolan et al., 2006). In *M. truncatula* there are many stress-related proteins associated with SE (Imin et al., 2004). A number of these proteins are differentially expressed between 2HA and Jemalong (Imin et al., 2005). The synthesis of the growth regulator ethylene can be rapidly evoked in response to a variety of biotic and abiotic stresses including wounding (Kende and Zeevart, 1997; Wang et al., 2002). Here, microarray studies on the induction of SE in *M. truncatula* identified genes predicted to encode ethylene biosynthesis and ethylene response proteins that are differentially expressed in SE. More detailed analysis of the role of ethylene in SE showed that a transcription factor of the AP2/ERF superfamily and ERF gene subfamily designated SOMATIC EMBRYO RELATED FACTOR 1 (MtSERF1), that is dependent on ethylene biosynthesis and perception for its expression, is required for SE in *M. truncatula*. MtSERF1 may enable a connection between stress and development.

**RESULTS**

**Microarray Analysis**

The use of mesophyll protoplasts was valuable for the microarray analysis as cultures are derived from one cell type and should identify critical gene expression changes more clearly than leaf explants. Leaf explants in addition to mesophyll cells contain cells of the vasculature, stomates and epidermis. Trends in gene expression from 40 to 80 day old 2HA cultures were profiled using a 16 K oligonucleotide array and Cy3 and Cy5 fluorescent labels. At 40 days the cultures are at the cell proliferation stage, at 60 days globular embryos are forming and at 80 days heart and later stage embryos are forming (Fig.1 and Supplementary Fig.1). We made direct comparisons between 40 and 60 day old cultures, 60 and 80 day old cultures and 40 and 80 day cultures. The determination of up and down-regulated genes was determined statistically using the strategies described in the methods. The statistical test is very important as the developing embryos are diluted amongst the proliferating cells and the fold change may be relatively small. Further, while there is a degree of synchrony in the production of embryos from protoplasts, embryo development is not perfectly synchronized. At 80 d of culture embryo development in many cases has reached the heart stage but synchronicity starts to be lost. Vascular tissue has also started to form in the callus at 80 d.
We have grouped genes into functional classes to assist in the interpretation. These are the first transcriptional profiling data obtained from differentiating single protoplasts using large scale microarrays.

In Fig. 2 we show the distribution of the number of genes associated with different functional classes that are up- or down-regulated for 60 d compared to 40 d and 80 d compared to 60 d. By including all genes that show statistically significant changes in expression, transcriptional changes occurring in only small numbers of cells will be included (see Supplementary Table 1). Our main interest is the time point where the cell culture (Suplementary Fig.1) switches to SE formation (60 d) from proliferation (40 d). Statistically significant changes in expression were found for more than 1500 genes at 60 d compared to 40 d: 883 and 823 genes were up- or down-regulated respectively. Comparison of 80 d and 60 d of culture revealed about 2000 genes differentially expressed from which 889 were up-regulated and 1089 down-regulated.

**Development-Related Genes**

Figure 2 shows the number of genes whose expression were up- or down-regulated within 27 functional groups. There is down-regulation of cell proliferation and protein synthesis genes (histones, DNA replication factors, ribosomal and a number of other translation associated proteins) as cells switch into SE. Two cyclin dependent kinases, cdc2Ms1 and cdcMsF, which are actively expressed during the G2-to-M phase in alfalfa cells (Magyar et al., 1997) are down-regulated at 60 days. These data are consistent with that of Thibaud-Nissen et al. (2003) in soybean where the most rapid cell division occurs in early callus formation. Our data also demonstrate changes in expression of a number of cell wall modifying enzymes as well as cell wall proteins. It is known that cells undergoing SE as well as zygotic embryogenesis show changes in cell wall polysaccharides and proteoglycans (Majewska-Sawka and Nothnagel, 2000). There is up-regulation of embryo specific genes as the somatic embryos within the callus develop. There is also increased expression of chloroplast- and photosynthesis-related genes, reflecting plastid changes associated with the development of the embryogenic callus in low light. The developmental changes are consistent with the morphological development of the embryogenic callus and support the reliability of the arrays.

**Stress-Related Genes**
There is up-regulation of genes involved in biosynthesis of flavonoids, redox, P450 and other stress related genes that could be related to general stress that is a part of cell culture and an important component in the induction of SE (Nishiwaki et al., 2000; Ikeda-Iwai et al., 2003; Belmonte and Yeung 2004; Stassola et al., 2004). Most of the enzymes from the isoflavonoid biosynthetic pathway are upregulated at 60 days (chalcone reductase and chalcone synthase, chalcone reductase, isoflavone 2'-hydroxylase, isoflavone reductase) and their product can be involved in defense or nodulation processes.

**Hormone- and Regulatory-Related Genes**

We categorised a number of functional groups that were likely to have regulatory roles and provide a useful overview of the potential contributors to the regulatory networks involved in somatic embryo induction and development. Transcriptional regulators, signal transduction and hormone biosynthesis and hormone response genes are represented. Auxin and cytokinin are the hormones supplied so it might be expected that there would be changes in gene expression for many genes directly related to these hormones. This was the case for the auxin response genes, but less so for the cytokinin response genes. What was of particular interest was the up-regulation of ethylene biosynthesis genes at both time points and ethylene response genes in the SE transition period.

To obtain a view of the major transcriptional changes involved in the induction of somatic embryogenesis we focused on a selection of genes from Supplementary Table 1 showing a two-fold or greater change (e.g. see Hass et al., 2004) for 60d compared to 40d. The data in Fig.2 are derived from all genes that are statistically up- or down-regulated and that are found in Supplementary Table 1. These data reinforce the view that stress and hormone responses are well represented. There are genes responsive to ethylene, ABA and GA, which are hormones not present in the culture medium, in addition to the auxin and cytokinin response genes.

We were interested in the contribution of stress responses to successful somatic embryogenesis. Therefore we focused on the ethylene biosynthesis genes and an ethylene response transcription factor, the AP2/EREBP homolog TC102138. The AP2/EREBP homolog was of more interest than other ethylene response genes because of its pattern of expression in qRT-PCR studies (detailed below), it showed a near 2 (1.94) fold increase and was a transcription factor. In a separate protoplast experiment the increase in expression in
AP2/EREBP occurred in the highly embryogenic 2HA at 60d but not in the near non-embryogenic Jemalong (Fig. 3). We designated the ethylene responsive AP2/EREBP homolog SOMATIC EMBRYO RELATED FACTOR 1 (MtSERF1).

**Gene Expression Analysis Using qRT-PCR**

Measurements of gene expression using qRT-PCR were carried out for both the ethylene biosynthesis genes and the ethylene response gene on leaf explants to see if these genes were similarly up-regulated as they were using mesophyll protoplasts. Leaf explants are experimentally simpler than using isolated single protoplasts and are commonly used to produce embryogenic callus for legume transformation experiments (Wang et al., 1996, Chabaud et al., 2003). Experiments can be turned over more quickly using leaf explants as they produce embryos about 40 days earlier than protoplasts These experiments were carried out with both the highly embryogenic 2HA and the near non-embryogenic wild-type Jemalong.

The ethylene biosynthesis genes are expressed quite early and expression continues throughout the culture period in the embryogenic 2HA. The expression pattern of the ethylene biosynthesis genes and MtSERF1, the ethylene response gene, is shown in Fig. 4. The peak of expression in 2HA for ACC SYNTHASE (ACS) transcription is day 1 of culture and day 1-2 in the case of ACC OXIDASE (ACO) transcription. When the expression of MtSERF1 was measured it first showed an increase in expression between day 7 and day 14 and peaked at day 21 when embryos are starting to form in a partially synchronous fashion, the transition period between day 40 and 60 in the protoplast experiments. Expression then declines but continues as more embryos are formed, the amount of expression after day 21 varies according to the amount of embryogenesis. In all four biological repeats the same inductive pattern was evident. Gene expression was also measured with the non-embryogenic line Jemalong which showed high expression of ACS but little expression of ACO and MtSERF1. The data overall indicate that inhibitors of ethylene perception (Ag+') and biosynthesis (AVG) inhibit the expression of all three genes in embryogenic tissue. In the case of ACO expression the peak of expression is delayed and clearly reduced in the AVG treatment.
As MtSERF1 is a member of the AP2/ERF family of transcription factors, the promoter region was examined for an ethylene response element (ERE). A 1,758 bp region upstream from the transcription start site was isolated, cloned and sequenced. In addition to the TATA and CAAT boxes, *in silico* analysis indicated that the promoter region contained a number of potential regulatory elements (Fig. 5). Two ERE elements were present, as well as two WUSCHEL binding sites, three Arabidopsis Response Regulator 1 (ARR1) elements that are associated with cytokinin signalling, an Auxin Response Factor (ARF) element and a tobacco EIN3-like (TEIL) element.

**Somatic Embryo Induction**

In order to further investigate the role of ethylene in somatic embryogenesis, experiments were carried out with leaf explants using stimulators of ethylene biosynthesis and inhibitors of ethylene biosynthesis and perception. These data are shown in Fig. 6 and results clearly indicate a marked influence of ethylene on SE. The addition of the ethylene precursor ACC (1-aminocyclopropane-1-carboxylic acid), the substrate for ACO (Pierik et al., 2006) caused a marked increase of SE at 10 µM. Similarly MGBG, which increases the availability of the ethylene precursor S-adenosyl-methionine (SAM), by inhibiting polyamine synthesis which utilises the same precursor, stimulates ethylene synthesis (Lee et al., 1997). MGBG at 100 µM stimulated SE to the same extent as 10 µM ACC. Conversely the ethylene biosynthesis inhibitor AVG, which inhibits the conversion of SAM to ACC (Yu and Yang, 1979) and Ag⁺, an inhibitor of ethylene perception (Bleecker, 1999), strongly inhibited SE at 1 µM and 10 µM respectively and there were no embryos at 10 µM and 100 µM respectively. Representative photographs of some treatments shown in Fig. 7 also illustrate the influence of ethylene on callus development from leaf explants. Stimulators of ethylene biosynthesis, using MGBG as the example, have small increases in callus development and inhibitors of ethylene action, such as Ag⁺, cause small decreases but still allow callus development to occur. This is clearly shown at 100 µM Ag⁺ where there is complete inhibition of embryo formation but callusing of the leaf explant has still occurred.

Given that embryogenic cultures are a mixture of embryos and callus cells, in order to establish a stronger connection with ethylene biosynthesis and embryo formation, we directly compared non-embryogenic callus with embryogenic callus, somatic embryos and ovules.
The ACS and ACO genes are consistently expressed at higher levels in embryogenic tissue, somatic embryos and ovules with globular stage embryos compared to non-embryogenic callus (Fig. 8).

**Localisation of MtSERF1 Expression and Requirement for Somatic Embryogenesis**

To localise MtSERF1 expression we carried out in situ hybridisation. The MtSERF1 specific probe was a 376 bp fragment from the 3’ region. As shown in Fig 9, MtSERF1 is strongly expressed in embryos. In the globular embryo, MtSERF1 is expressed throughout the embryo. This is very clearly shown in thick sections of fresh tissue where the callus cells show little if any hybridisation signal. Later in the heart-stage embryo, using thinner sections of paraffin-embedded tissue, we were able to show that hybridisation predominates in a small group of cells in the developing shoot meristem.

Given the MtSERF1 expression in somatic embryos, we investigated whether similar expression was present in zygotic embryos. Ovules at different times after pollination were collected and MTSERF1 expression measured using qRT-PCR. Expression increased during ovule development and then declined (Supplementary Fig.2), similar to the pattern in embryogenic callus (Fig. 4C). The peak of ovule expression corresponded to the globular stage and had clearly declined at the torpedo stage (7 dap). In-situ hybridisation studies showed expression was present in the embryo but not in the ovule wall (unpublished data).

In order to examine whether MtSERF1 expression is required for SE we used an RNAi approach. As shown in Fig 10, transgenic MtSERF1 knockdown callus produced no somatic embryos when compared to their empty vector counterparts. For the empty vector control, 90% of 28 transformed calli produced embryos (with an average of 13.6 embryos/calli). To confirm the effects of RNA knockdown, we performed qRT-PCR on the calli. Results showed that the level of the transcripts in knockout calli was only 15% of that of empty vector calli. We also obtained transformed plants using an inducible vector containing RNAi and produced calli in the presence and absence of dexamethasone. The induction of RNAi by dexamethasone reduced the number of calli producing embryos by 90%. The empty vector control showed no change in the presence or absence of dexamethasone.
Sequence and Phylogenetic Analyses of the Transcription Factor MtSERF1

MtSERF1 is a protein of molecular mass 23 kDa and contains 201 amino acids. The amino acid sequence of MtSERF1 contains a single AP2/ERF domain as shown by position specific iterated and pattern hit initiated BLAST. As indicated by Nakano et al. (2006) this domain is characteristic of the AP2/ERF superfamily and the ERF subfamily contains a single domain. An alignment of this domain with other proteins containing a single AP2/ERF domain shows high similarity (Fig 11A).

In order to further investigate this uncharacterised ethylene-induced transcription factor, phylogenetic analyses of AP2 domain sequences of all 65 transcription factors of A. thaliana identified as members of the ERF subfamily (Sakuma et al., 2002; Nakano et al., 2006) and other well-characterised ERFs from other species were conducted. Phylogenetic analyses using the neighbour-joining method (Saitou and Nei, 1987) on Clustal X 1.8 software showed that MtSERF1 belongs to GROUP IX of the classification of Nakano et al. (2006) or GROUP B-3 according to the classification of Sakuma et al. (2002) as shown in an unrooted cladogram (Fig. 11B). To find orthologs of MtSERF1 in other species, phylogenetic analyses of the entire amino acid sequences of ERF proteins included in GROUP IX were performed. As shown in Fig 11C, we found that the MtSERF1 is clustered together with two uncharacterized genes of A. thaliana, with At5g61590 being the closest ortholog. This gene shares 41% identity with MtSERF1 (data not shown). Both genes also share similar motifs outside the AP2 domain when analyzed using the motif discovery software MEME (http://meme.nbcr.net). These findings suggest that MtSERF1 is distinct from all AP2 domain-containing transcription factors of known function.

DISCUSSION

Transcriptional profiling of the development of somatic embryos from single isolated mesophyll protoplasts of the highly embryogenic M. truncatula genotype 2HA revealed changes in the expression of many transcripts. These data showed increased transcription of ethylene biosynthesis genes and ethylene response genes, which were of interest because of their common involvement in stress and development responses. Subsequent experiments on
ethylene response genes identified an ethylene responsive transcription factor MtSERF1 which was essential for somatic embryogenesis.

Some of the major changes in the microarray data relate to stress, reflected in a range of genes connected to abiotic, biotic and oxidative stresses. This may have been predicted given that protoplast isolation (Pasternak et al. 2002) and tissue excision (Nolan et al., 2006) associated with the induction of SE is a very stressful, wound-related procedure. Transcriptional profiling in response to mechanical wounding has been carried out in Arabidopsis (Cheong et al., 2002; Delessert et al., 2004) and a diverse groups of genes previously related more specifically to wounding, pathogen attack, abiotic stress and plant hormones are up-regulated. In this study *M. truncatula* flavonoid biosynthesis genes were also up-regulated and have also been related to stress protection (Winkel-Shirly, 2002). In *M. truncatula* embryogenic cultures, there are many stress-related proteins associated with SE (Imin et al., 2004; Imin et al., 2005) as there are in *Medicago sativa* (Domoki et al., 2006). In soybean, somatic embryogenesis is induced by 2,4-D in cotyledons and is associated with up-regulation of oxidative stress and defense genes (Thibaud-Nissen et al., 2003). Studies by Che et al. (2006) involving microarray analysis of shoot, root and callus development in Arabidopsis tissue culture also noted an increased expression of specific stress-related genes.

Amongst the most highly induced genes in our study was an ethylene biosynthesis gene (Table 1). Up-regulation of transcripts of ethylene biosynthesis genes has also been seen in wounding (Cheong et al., 2002; Delessert et al., 2004) and somatic embryogenesis in soybean cotyledons (Thibaud-Nissen et al., 2003). ACC synthase was up-regulated on an auxin-rich callus induction medium (Che et al., 2006) in Arabidopsis. We also noted an up-regulation of ethylene-response genes and this contributed to ethylene becoming a focus of our studies. In addition to the suite of up-regulated genes related to ethylene, it was of interest to note that it might be expected that as auxin and cytokinin were present in the medium auxin and cytokinin response genes would be the only prominently featured hormone-related genes. However, this was not the case. Genes related to ABA, gibberellin and brassinosteroids were also featured. We have recently discussed the possible roles of these hormones in somatic embryogenesis (Rose and Nolan, 2006).

While there is value in focusing on mesophyll protoplasts as a uniform source of starting cells, experimentally leaf explants are commonly used and are experimentally much more
straightforward. Leaf explants also produce embryos more quickly, about 40 days earlier than protoplasts. We were able to show that ethylene biosynthesis and ethylene response genes in leaf explants were also up-regulated. The first question that arises is whether the ethylene biosynthesis genes really reflect a requirement for ethylene for somatic embryogenesis in *M. truncatula*. Results with an inhibitor of ethylene biosynthesis (AVG) and ethylene perception (Ag⁺) strongly support the idea that ethylene is essential for SE in *M. truncatula*. Consistent with this, the stimulation of ethylene biosynthesis by ACC and MGBG increased SE. This contrasts with the effect of ethylene on auxin-induced *in vitro* root formation in *M. truncatula*, where the ethylene-insensitive mutant *sickle* had enhanced root formation in comparison to wild type (Rose et al., 2006). Reported effects of ethylene on SE are variable and this is not surprising as ethylene concentration and signalling interactions with other hormones (Pierik et al., 2006) are likely to be species, developmentally and environmentally dependent. However in a defined experimental system of a developmental process, as with other hormones, there are most likely specific roles to play in the genetic networks (Nemhauser et al., 2006).

In order to examine the role of ethylene we examined the expression of ethylene response genes that were up-regulated as cells entered into SE. We specifically focused on TC102138 which based on our investigations we designated *MtSERF1*. This gene is a member of the ERF subfamily based on the classification of Nakano et al. (2006). Further phylogenetic analysis placed MtSERF1 in the group nine of Nakano et al. (2006) which includes the AtERF5 gene induced by wounding in Arabidopsis (Cheong et al., 2002). We have shown that *MtSERF1* expression is inhibited by AVG and Ag⁺ indicating its ethylene dependence. The connection of *MtSERF1* to SE is shown by the minimal expression of *MtSERF1* in rarely embryogenic Jemalong as opposed to the highly embryogenic 2HA, the localization of *MtSERF1* expression to the early embryo and later to a specific shoot pole region of the heart-stage embryo, and the inhibition by of SE by RNAi directed against *MtSERF1*. It is noteworthy that a number of genes implicated in somatic embryogenesis induction are expressed in developing zygotic embryos: *SERK1* (Hecht et al., 2001), *LEC1* (Lotan et al., 1998), *LEC2* (Stone et al., 2001) and *WUSCHEL* (Zuo et al., 2002). The pattern of *MtSERF1* expression in developing ovules of *M. truncatula* is consistent with expression in the zygotic embryo.
The lack of *MtSERF1* expression in Jemalong, rarely embryogenic and near isogenic with respect to 2HA, provides a focus for further analysis. There is a small inhibition of *ACC SYNTHASE* expression and a more strongly reduced *ACC OXIDASE* expression in Jemalong. This could ultimately lead to reduced signaling and reduced *MtSERF1* expression. We also know that Jemalong and 2HA respond to auxin by producing roots but when cytokinin is added to the auxin only 2HA forms embryos (Nolan et al., 2003) and Jemalong usually forms callus only. The significance of the localisation of *MtSERF1* expression to the early embryo and to a localized region of the shoot pole of the heart-stage embryo also requires further investigation. We also note that the *MtSERF1* promoter contains WUSCHEL binding sites and *WUSCHEL* is implicated in the induction of somatic embryogenesis as well as stem cell maintenance in apical meristems (Zuo et al., 2002, Rose and Nolan, 2006).

The finding of a relationship between an ERF subfamily gene and the formation of somatic embryos *in vitro* is consistent with an emerging picture of the involvement of ERF transcription factors in developmental processes studied *in vitro*. The *ENHANCER OF SHOOT REGENERATION* 1 and 2 (Banno et al., 2001; Ikeda et al., 2006) and *RAP 2.6L* (Che et al., 2006) have a role in shoot regeneration in Arabidopsis. These transcription factors are all members of the AP2/ERF superfamily, as is *BABY BOOM* which induces somatic embryogenesis when overexpressed in *Arabidopsis* and *Brassica napus* (Boutilier et al., 2002). Heterologous expression of *BABY BOOM* in *Nicotiana tabacum* enhances regeneration capacity (Srinivasan et al., 2007). Baby Boom is a member of the AP2 family as it has two repeated AP2/ERF domains (Boutilier et al., 2002; Nakano et al., 2006). Recently the ERF transcription factor ERN, required for nodulation, has been identified in *Medicago truncatula* (Middleton et al., 2007). *MtSERF1* is in group 9 of the ERF subfamily while ERN is in group 5 (Fig. 11B). The AP2/ERF superfamily has a mix of transcription factors which relate to growth and development, abiotic and biotic stressors, and ethylene response (Alonso et al., 2003; Nakano et al., 2006;). This may relate to the need to link growth and stress in the evolution of sessile plants.

**MATERIALS AND METHODS**

**Protoplast Isolation and Culture**
Protoplasts were isolated from leaves of the highly embryogenic 2HA genotype of *Medicago truncatula* cv. Jemalong. A wild-type Jemalong plant frequently produces no embryos. The highest embryo producing plant we have ever recorded was one embryo per 6 explants (Nolan et al., 1989). The 2HA genotype was derived from a rare regenerated plant obtained by a single cycle of tissue culture from wild type Jemalong. This regenerated Jemalong showed enhanced somatic embryogenesis and the seed progeny segregated into types with and without the capacity to produce somatic embryos. Seed from the regenerated Jemalong was used to continue to select for high embryogenicity over four seed generations (Rose et al. 1999). The 2HA genotype can be considered to be a near isogenic, highly embryogenic mutant of Jemalong. Plants were grown in controlled environment rooms at low light intensity, as described by Tian and Rose (1999). The flotation procedure utilised for protoplast isolation yields almost exclusively mesophyll protoplasts. Isolated protoplasts were grown in 1% low melting point agarose droplets and then transferred to agar plates on P4 10:4 (10 µM NAA and 4 µM BAP) medium for culture as described by Rose and Nolan (1995). For microarray analysis calli derived from these isolated single protoplasts were taken at the following stages of development (Fig.1 and supplementary Fig.1) - the cell proliferation stage (40 days of culture), the early globular stage (60 days of culture), and the heart and later stages (80 days of culture).

**Cultured Leaf Explants**

Cultured *Medicago truncatula* leaf explants were obtained from glasshouse grown 2HA or wild type Jemalong. Seeds of wild type Jemalong were originally obtained from the National *Medicago* Collection, South Australian Research and Development Institute (SARDI), Adelaide. The standard leaf culture procedure was as described by Nolan et al. (2003). Explants were cultured on P4 10:4 for three weeks before transfer to P4 10:4:1 (10 µM NAA, 4 µM BAP and 1µM ABA).

**The 16K Oligo Microarray Slides**

The *Medicago* 16 K microarray was utilised and has a probe set mapped to the *Medicago* Gene Index Release 8.0 (http://www.tigr.org/docs/tigr/scripts/medicago/ARRAYS/array.TC mapping). The 70-mer oligos were synthesised by Qiagen-Operon and the slides printed at The University of Arizona in the laboratory of Dr. David Galbraith. After printing, the slides were baked for 80 min at 80°C. The oligonucleotide array elements were immobilised by UV
cross-linking at 300 mJ then washed twice with gentle rocking, for 2 min each wash, in 2X SSC + 0.2% SDS. The slides were then immersed in boiling hot water for 2 min, blotted briefly and transferred to ice cold ethanol for 2-5 min. Slides were then dried by centrifugation at 1500 rpm for 2-5 min and finally stored in a light proof box under cool dry conditions.

**RNA Preparation, cDNA Synthesis, and Hybridization of Microarrays**

Calli grown from individual protoplasts in an isolation that produced thousands of embryogenic microcalli, consistent with high protoplast quality, were collected at 40, 60 and 80 days after initiation of culture. The calli were frozen in liquid nitrogen and stored at -80°C until RNA was isolated. RNA was isolated as described by Lohar et al. (2006) and stored at -80°C. Total RNA (22 µg per sample) was pooled from three biological replicates giving 66 µg of RNA and 33 µg of RNA was aliquoted into two separate tubes. The Eppendorf tubes containing 33 µg of RNA were thawed on ice, spun dried in a speed vac and immediately returned to -80°C. The RNA was shipped from Newcastle, Australia to St. Paul, USA on dry ice and transferred to -80°C until required. This maintained the quality of the RNA. The 33 µg of RNA was resuspended in 8 µL of nuclease free double distilled water and used for cDNA synthesis with an RT primer for labelling with either Cy3 or Cy5 dyes using a 3DNA Array50 Kit (Genisphere) as previously described (Lohar et al., 2006).

Experiments were conducted using a regular dye-swap design as described earlier in Lohar et al (2006). Microarrays for 40 d, 60 d, for 60 d, 80 d and 40 d, 80 d comparisons were hybridised with cDNAs from the two different time points labelled with different dyes. Each hybridisation was repeated a total of six times to sample the technical variability, with three repeats of each dye combination to control for dye effects (Lohar et al., 2006).

**Microarray Analysis**

Methods for array analysis were as described for a 6K microarray (Lohar et al., 2006). Briefly, microarray slides were scanned using an Axon two-laser scanner and image analysis was performed using GenePix (Axon) software. Background-subtracted mean intensities for both tissues were log transformed and normalized before further analysis. Normalization of microarray data was performed using a statistical module developed as a part of Lab Information System, which includes several scripts and modules written in PERL and R languages.
Normalization steps included i) within-slide normalization using local linear regression (LOWESS function) (Yang et al., 2000); ii) between-slide normalization using four-way ANOVA with replications for multi-slide dye-swap experiments (Kerr et al., 2000). More detailed description of pre-processing steps, such as log2 transformation of background-subtracted Cy5 and Cy3 intensities are described in Lohar et al. (2006). Identification of differentially expressed genes was done using SAM software (Stanford), which allows flexible monitoring of the False Discovery Rate (Tusher et al., 2001). We applied a False Discovery Rate of < 0.1 % and the highest q value was <0.06%.

All genes of statistical significance with predicted or known function or that showed significant homology to characterised genes (annotated in the TIGR database at http://compbio.dfci.harvard.edu/tgi/) have been manually divided into 27 classes. Genes that did not fit readily into one of these classes have been classified as “other genes with defined function” and “genes with unknown function”. Supplementary Table 1 lists all the genes incorporated into these classes. To obtain a subset of genes that passed a statistical significance test we have also imposed a fixed ratio threshold of 2.

Real Time PCR

Total RNA was isolated from calli at different time points and from intact leaves (as a calibrator) using the RNAqueous®-4PCR Kit (Ambion, Austin, USA) according to the manufacturer’s instructions. cDNA synthesis was performed using the Superscript II™ First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, USA) starting with 2 µg of total RNA with oligo (dT)15 primers. Real-time PCR was performed using SYBR® GreenER™ qPCR SuperMix Universal Kit (Invitrogen, Carlsbad, USA) and analyzed in the DNA Engine Opticon® 2 Continuous Fluorescence Detection System (BioRad, formerly MJ Research). Primers 5’- TCATACGCCATCATCTCTTAGGT-3’ (forward) and 5’-AGGGTTTGGTTCTTTGAAGAT-3’ (reverse) were designed to quantify the MtSERF1 expression levels, which were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), primers 5’-TGGTCATCAAACCCTCAACA-3’ (forward) and 5’-CCTCGTTCTTTCCGCTATCA-3’ (reverse), in each sample. To quantify the expression levels of ACS the primers were 5’-CCCACACAAATTGCGCTCTT-3’ (forward) and 5’-TCACCATGTCCATCACCAGT-3’(reverse); while for ACO the primers were 5’-
GGGATTCTTTGAGCTGGTGA-3’ (forward) and 5’-GACGAACATGGAAGGTGCTT-3’ (reverse). PCR cycling conditions included a 94 °C heating step for 1 min at the beginning of every run. The tubes were then cycled at 94 °C for 30 s, annealed at 60 °C for 60 s, and extended at 72 °C for 60 s. A melting curve was generated at the end of every run to ensure product uniformity. PCR reactions were performed in triplicate in at least two biological repeats. Transcript abundance was estimated using a modification of the comparative threshold cycle (Ct) method and was calculated as $E^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{target} - Ct_{GAPDH})_{Time x} - (Ct_{target} - Ct_{GAPDH})_{Calibrator}$ and E is the estimated amplification efficiency, which was calculated employing the linear regression method on the log(fluorescence) per cycle number data for each amplicon using the LinRegPCR software (Ramakers et al. 2003).

**In Situ Hybridisation**

To generate the RNA probes, a 376 bp fragment specific to *MtSERF1* was first amplified by PCR with the primers 5’-CTGTGAAATTGATGCTGCAAA-3’ (forward) and 5’-TGACATAATTGAGCTCActCC-3’ (reverse). Then, the promoter sequences of T7 and SP6 RNA polymerase were introduced to this fragment by a two-step PCR. The first primers used were 5’-GAGGCCGCGTCTGTGAAATTGATGCTGCAAA-3’ (forward) and 5’-ACCCGGGGCTTGACATAATTGAGCTCActCC-3’ (reverse). The second set of primers used were 5’-TTATGTGAAATTGAGCTCActATAGGGAGGCGGCGT-3’ (forward) and 5’-CCAAATTAGGTGACACTATAGGATAAGTACCCGGGGCT-3’ (reverse). This PCR product was subsequently used as a template for *in vitro* transcription employing T7 and SP6 RNA polymerase to synthesize digoxigenin-labelled sense and anti-sense single-stranded RNA probes respectively using a DIG RNA Labeling Kit (Roche Diagnostics GmbH, Penzberg, Germany; Cat. 11 093 274 910). Two different cytological procedures were used; paraffin embedding and fresh tissue sectioned with a vibratome. For the paraffin procedure four to five week old 2HA calli from leaf explants were fixed in 4% formaldehyde in 0.025 M phosphate buffer at pH 7.2, dehydrated through an ethanol and ethanol: histolene (Fronine lab supplies) series, embedded in paraffin, sectioned (8 µM), and hybridized with the digoxigenin-labelled sense and anti-sense probes according to the manufacturer’s instructions. For the fresh tissue procedure, the 2HA embryogenic tissue from leaf explants tissue was embedded in agar and 40 µM sections cut with a vibratome. In both cases hybridisation was detected using a Fluorescent Antibody Enhancer Set for DIG detection (Boehringer Cat. 176756) and was visualized as a red/purple color after the NBT/BCIP color reaction (Roche Diagnostics). In all cases, no signal over background was observed using control sense-strand probes.
Construction of Constitutive and Inducible RNAi Plasmids

For *MtSERF1* RNAi construction, specific sequences in the 3’ end of *MtSERF1* mRNA were selected for construction of RNAi fragments. A cDNA fragment of *MtSERF1* was amplified by PCR with the primers, 5’-CTGTGAAATTGATGCTGCAAA-3’ (forward) and 5’-TGACATAATTGGTGAGCTCCTCT-3’ (reverse). The *MtSERF1* specific PCR products were cloned into the vector pCR®8/GW/TOPO® (Invitrogen, Carlsbad, USA). After linearization of the plasmids, the Gateway LR recombination reaction (Invitrogen, Carlsbad, USA) was conducted according to the manufacturer’s protocol to incorporate the *MtSERF1* specific fragment into the binary T-DNA destination vector pH7GW1WG2(II) (Karimi et al., 2002) and pOpOff2(hyg) (Wielopolska et al., 2005) for constitutive and inducible RNAi constructs, respectively. The resulting constructs were introduced into *Agrobacterium tumefaciens* strain AGL1 by electroporation.

**Transformation of *Medicago truncatula***

Transformation of *M. truncatula* 2HA leaf explants was carried out as described by Wang et al. (1996) with some modifications. In brief, leaf pieces were prepared and sterilized according to the method described by Nolan et al. (2003) and were dipped into bacterial solution, followed by co-cultivation for 2-5 days. After co-cultivation, the explants were decontaminated by dipping in a solution containing 750 mg/L Augmentin (5 parts amoxicillin/L part clavulanic acid; Beecham Laboratories, France) before plating onto solid media as described previously in the section on cultured leaf explants. Transformed calli were screened for Hygromycin resistance by including Hygromycin at 15 mg/L in the media. 500 mg/L Augmentin was also added in the media to eliminate the *Agrobacterium*. The explants were subcultured on fresh media every 4 weeks. RNAi constructs were induced by 2.5 uM dexamethasone.

**Sequence Analysis and Construction of Phylogenetic Trees**

Multiple alignment analyses were performed with ClustalW using a Clustal X 1.8 software package. Phylogenetic trees were constructed using the neighbour-joining method (Saitou and Nei, 1987) included in the Clustal X 1.8 software. Phylogenetic trees were drawn using TreeView (Win32) 1.6.0 software (Page, 1996).

**Promoter sequence isolation and in silico analysis**
Isolation of the MtSERF1 promoter was carried out according to the GenomeWalker Kit (Clontech) with minor modifications. In brief, for the first round of amplification biotinylated gene-specific primer and the adaptor primer AP1 were used. Immobilisation of the PCR product to streptavidin-coated particles and washing steps were conducted according to the Dynal kilobase BINDER Kit (Invitrogen). A one tenth part of these beads was used for nested PCR as described in the Genome Walker kit and the fragment obtained sequenced. The proximal region of the promoter was analyzed using a eukaryotic transcription start site prediction software NNPP version 2.2 (Reese, 2000, 2001) (www.fruitfly.org/seq_tools/promoter.html). Search for core and responsive element motifs was performed in silico by means of Web Signal Scan Program (Higo et al., 1999; Prestridge, 1991) (http://www.dna.affrc.go.jp/Sigscan/signal.html) and MatInspector (Cartharius, 2005) (http://www.genomatix.de/products/MatInspector/).

ACKNOWLEDGEMENTS

We wish to thank Yoko Nitanai for assistance with “gridding” of the microarray signals and Dr. Kim Nolan for assistance with tissue culture and Dr. Kim Nolan and Dr. Michael Sheahan for helpful discussion.

LITERATURE CITED

Allen MD, Yamasaki K, Ohme-Takagi M, Tateno M, Suzuki M (1998) A novel mode of DNA recognition by a beta-sheet revealed by the solution structure of the GCC-box binding domain in complex with DNA. EMBO J 17: 5484–5496

Alonso, JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk, R, Gadrinab C, Heller C, Jeske A, Koesema E., Meyers CC, Parker H, Prednis L, Ansari Y, Choy N, Deen H, Geralt M, Hazari N, Hom E, Karnes M, Mulholland C, Nubakku R, Schmidt I, Guzman P, Aguilar-Henonin L, Schmid M, Weigel D, Carter DE, Marchand T, Risseewu E, Brogden D, Zeko A, Crosby WL, Berry CC, Ecker JR (2003) Genome-wide insertional mutagenesis of Arabidopsis thaliana. Science 301: 653-657

Banno H, Ikeda Y, Niu QW, Chua NH (2001) Overexpression of Arabidopsis ESR1 induces initiation of shoot regeneration. Plant Cell 13: 2609-2618

Belmonte MF, Yeung EC (2004) The effects of reduced and oxidized glutathione on white spruce somatic embryogenesis. In Vitro Cell & Dev Biol - Plant 40: 61-66

Bleecker AB (1999) Ethylene perception and signalling: an evolutionary perspective. Trends Plant Sci 4: 269-274
Boutilier K, Offringa R, Sharma VK, Kieft H, Ouellet T, Zhang LM, Hattori J, Liu CM, van Lammeren AAM, Miki BLA, Custers JBM, Campagne MMV (2002) Ectopic expression of BABY BOOM triggers a conversion from vegetative to embryonic growth. Plant Cell 14: 1737-1749

Cartharius K, Frech K, Grote K, Klocke B, Haltmeier M, Klingenhoff A, Frisch M, Bayerlein M, Werner T (2005) MatInspector and beyond: promoter analysis based on transcription factor binding sites. Bioinformatics 21: 2933-2942

Chabaud M, de Carvalho-Niebel F and Barker DG (2003) Efficient transformation of Medicago truncatula cv. Jemalong using the hypervirulent Agrobacterium tumefaciens strain AGL1. Plant Cell Rep 22: 46-51

Che P, Lall S, Nettleton D, Howell SH (2006) Gene expression programs during shoot, root, and callus development in Arabidopsis tissue culture. Plant Physiol 141: 620-637

Cheong YH, Chang HS, Gupta R, Wang X, Zhu T, Luan S (2002) Transcriptional profiling reveals novel interactions between wounding, pathogen, abiotic stress, and hormonal responses in Arabidopsis. Plant Physiol 129: 661-677

Cook DR (1999) Medicago truncatula - a model in the making! Curr Opin Plant Biol 2: 301-304

Delessert C, Wilson IW, Van der Straaten D, Dennis ES, Dolferus R (2004) Spatial and temporal analysis of the local response to wounding in Arabidopsis leaves. Plant Mol Biol 55: 165-181

Domoki M, Györgyey J, Biró J, Pasternak TP, Zvara A, Bottka S, Puskás LG, Dudits D, Fehér A (2006) Identification and characterization of genes associated with the induction of embryogenic competence in leaf-protoplast-derived alfalfa cells. Biochimica et Biophysica Acta 1759: 543-551

Fehér A, Pasternak TP, Dudits D (2003) Transition of somatic plant cells to an embryogenic state. Plant Cell Tissue and Org Cult 74: 201-228

Harding EW, Tang WN, Nichols KW, Fernandez DE, Perry SE (2003) Expression and maintenance of embryogenic potential is enhanced through constitutive expression of AGAMOUS-Like 15. Plant Physiol 133: 653-663

Hass C, Lohrmann, J, Albrecht V, Sweere U, Hummel F, Yoo S D, Hwang I, Zhu T, Schäfer E, Kudla J, Harter K (2004) The response regulator 2 mediates ethylene signalling and hormone signal integration in Arabidopsis. Embo J 23: 3290-3302

Hecht V, Vielle-Calzada JP, Hartog MV, Schmidt EDL, Boutilier K, Grossniklaus U, de Vries SC (2001) The Arabidopsis SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1 gene is expressed in developing ovules and embryos and enhances embryogenic competence in culture. Plant Physiol 127: 803-816

Higo K, Ugawa Y, Iwamoto M, Korenaga T (1999) Plant cis-acting regulatory DNA elements (PLACE) database:1999. Nucleic Acids Res 27: 297-30

Ikeda Y, Banno H, Niu Q-W, Howell SH, Chua N-H (2006) The ENHANCER OF SHOOT REGENERATION 2 gene in Arabidopsis Regulates CUP-SHAPED COTYLEDON 1 at the Transcriptional Level and Controls Cotyledon Development. Plant Cell Physiol. 47: 1443-1456

Ikeda-Iwai M, Umehara M, Satoh S, Kamada H (2003) Stress-induced somatic embryogenesis in vegetative tissues of Arabidopsis thaliana. Plant J 34: 107-114

Imin N, De Jong F, Mathiesius U, van Noorden G, Saeed NA, Wang XD, Rose RJ, Rolfe BG (2004) Proteome reference maps of Medicago truncatula embryogenic cell cultures generated from single protoplasts. Proteomics 4: 1883-1896

Imin N, Nizamidin M, Daniher D, Nolan KE, Rose RJ, Rolfe BG (2005) Proteomic analysis of somatic embryogenesis in Medicago truncatula. Explant cultures grown...
under 6-benzylaminopurine and 1-naphthaleneacetic acid treatments. Plant Physiol 137: 1250-1260

Karimi M, Inze D, Depicker A (2002) GATEWAY(TM) vectors for Agrobacterium-mediated plant transformation. Trends Plant Sci 7: 193-195

Kende H, Zeevaart JAD (1997) The five "classical" plant hormones. Plant Cell 9: 1197-1210

Kerr MK, Martin M, Churchill GA (2000) Analysis of variance for gene expression microarray data. J Computational Biol 7: 819-837

Lee M M, Lee SH and Park KY (1997) Effects of spermine on ethylene biosynthesis in cut carnation (Dianthus caryophyllus L.) flowers during senescence. J. Plant Physiol. 151: 68-73

Lohar DP, Sharopova N, Endre G, Penuela S, Samac D, Town C, Silverstein KAT, VandenBosch KA (2006) Transcript analysis of early nodulation events in Medicago truncatula. Plant Physiol 140: 221-234

Lotan T, Ohto M, Yee KM, West MAL, Lo R, Kwong RW, Yamagishi K, Fischer RL, Goldberg RB, Harada JJ (1998) Arabidopsis LEAFY COTYLEDON1 is sufficient to induce embryo development in vegetative cells. Cell 93: 1195-1205

Magyar Z, Meszaros T, Miskolczi P, Deak M, Feher A, Brown S, Kondorosi E, Athanasiadis A, Pongor S, Bilgin M, Bako L, Koncz C, Dudits D (1997) Cell cycle phase specificity of putative cyclin-dependent kinase variants in synchronized alfalfa cells. Plant Cell 9: 223-235

Majewska-Sawka A, Nothnagel EA (2000) The multiple roles of arabinogalactan proteins in plant development. Plant Physiol 122: 3-9

Middleton PH, Jakab J, Penmetsa RV, Starker CG, Doll J, Kaló P, Prabhu R, Marsh JF, Mitra RM, Kereszt A, Dudas B, VandenBosch K, Long SR, Cook DR, Kiss GB, Oldroyd GED (2007) An ERF transcription factor in Medicago truncatula that is essential for nod factor signal transduction. Plant Cell 19: 1221-1234

Nakano T, Suzuki K, Fujimura T, Shins H (2006) Genome-wide analysis of the ERF gene family in Arabidopsis and rice. Plant Physiol 140: 411-432

Nemhauser JL, Hong F and Chory J (2006) Different plant hormones regulate similar processes through largely nonoverlapping transcriptional responses. Cell 126: 467-475

Nishiwaki M, Fujiwara K, Koda Y, Masuda K, Kikuta Y (2000) Somatic embryogenesis induced by the simple application of abscisic acid to carrot (Daucus carota L.) seedlings in culture. Planta 211: 756-759

Nolan KE, Irwanto RR, Rose RJ (2003) Auxin up-regulates MtSERK1 expression in both Medicago truncatula root-forming and embryogenic cultures. Plant Physiol 133: 218-230

Nolan, K. E.; Rose, R. J., and Gorst, J. E. (1989) Regeneration of Medicago truncatula from tissue culture: increased somatic embryogenesis from regenerated plants. Plant Cell Rep. 8:278-281.

Nolan KE, Saeed NA, Rose RJ (2006) The stress kinase gene MtSK1 in Medicago truncatula with particular reference to somatic embryogenesis. Plant Cell Rep 25: 711-722

Page RDM (1996) TreeView: An application to display phylogenetic trees on personal computers. Bioinformatics 12: 357-358

Pasternak TP, Prinsen E, Ayaydin F, Miskolczi P, Potters G, Asard H, Van Onckelen HA, Dudits D, Feher A (2002) The role of auxin, pH, and stress in the activation of embryogenic cell division in leaf protoplast-derived cells of alfalfa. Plant Physiol 129: 1807-1819
Pierik R, Tholen D, Poorter H, Visser EJW, Voesenek L (2006) The Janus face of ethylene: growth inhibition and stimulation. Trends Plant Sci 11: 176-183

Prestridge DS (1991) SIGNAL SCAN: A computer program that scans DNA sequences for eukaryotic transcriptional elements. CABIOS 7: 203-206

Ramakers C, Ruijter JM, Lekanne Deprez RH, Moorman AFM (2003) Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neuroscience Lett 339: 62-66

Reese MG (2000) Computational prediction of gene structure and regulation in the genome of Drosophila melanogaster, PhD Thesis, UC Berkeley/University of Hohenheim.

Reese MG (2001) Application of a time-delay neural network to promoter annotation in the Drosophila melanogaster genome. Comput Chem 26:51-56

Rose RJ (2004) Somatic embryogenesis in plants. In RM Goodman, eds, Encyclopedia of plant and crop science, Marcel Dekker Inc, New York, pp. 1165–1168

Rose RJ, Nolan KE (1995) Regeneration of Medicago truncatula from protoplasts isolated from kanamycin-sensitive and kanamycin-resistant plants. Plant Cell Rep 14: 349-354

Rose RJ, Nolan KE (2006) Genetic regulation of somatic embryogenesis with particular reference to Arabidopsis thaliana and Medicago truncatula. In Vitro Cell & Dev Biol - Plant 42: 473–481

Rose RJ, Nolan KE, Bicego L (1999) The development of the highly regenerable seed line Jemalong 2HA for transformation of Medicago truncatula - Implications for regenerability via somatic embryogenesis. J Plant Physiol 155: 788-791

Rose RJ, Wang X-D, Nolan KE, Rolfe BG. (2006) Root meristems in Medicago truncatula tissue culture arise from vascular-derived procambial-like cells in a process regulated by ethylene. J Exp Bot 57: 2227-2235

Saitou N, Nei M (1987) The neighbour-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406-425

Sakuma Y, Liu Q, Dubouzet JG, Abe H, Shinozaki K, Yamaguchi-Shinozaki K (2002) DNA-binding specificity of the ERF/AP2 domain of Arabidopsis DREBS, transcription factors involved in dehydration- and cold-inducible gene expression. Biochem Biophys Res Commun 290: 998-1009

Srinivasan C, Liu ZR, Heidmann I, Supena EDJ, Fukuoka H, Joosen R, Lambalk J, Angenent G, Scorza R, Custers JBM, Boutilier K (2007) Heterologous expression of the BABY BOOM AP2/ERF transcription factor enhances the regeneration capacity of tobacco (Nicotiana tabacum L.). Planta 225: 341-351

Stasolla C, Belmonte MF, van Zy1 L, Craig DL, Liu WB, Yeung EC, Sederoff RR (2004) The effect of reduced glutathione on morphology and gene expression of white spruce (Picea glauca) somatic embryos. J Exp Bot 55: 695-709

Stone SL, Kwong LW, Yee KM, Pelletier J, Lepiniec L, Fischer RL, Goldberg RB, Harada, J. J. (2001) LEAFY COTYLEDON 2 encodes a B3 domain transcription factor that induces embryo development. Proc Natl Acad Sci USA 98:11806-11811;

Tadge M, Ratet P, Mysore KS (2005) Insertional mutagenesis: a Swiss army knife for functional genomics of Medicago truncatula. Trends Plant Sci 10: 229-235

Thibaud-Nissen FO, Shealy RT, Khanna A, Vodkin LO (2003) Clustering of microarray data reveals transcript patterns associated with somatic embryogenesis in soybean. Plant Physiol 132: 118-136

Tian D, Rose RJ (1999) Asymmetric somatic hybridisation between the annual legumes Medicago truncatula and Medicago scutellata. Plant Cell Rep 18: 989-996.

Touraev A, Vicente O, HeberleBors E (1997) Initiation of microspore embryogenesis by stress. Trends Plant Sci 2: 297-302
Tusher VG, Tibshirani R, Chu G (2001) Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci USA 98: 5116-5121

VandenBosch KA, Stacey G (2003) Summaries of legume genomics projects from around the globe. Community resources for crops and models. Plant Physiol 131: 840-865

Wang JH, Rose RJ, Donaldson BI (1996) Agrobacterium-mediated transformation and expression of foreign genes in Medicago truncatula. Aust J Plant Physiol 23: 265-270

Wang KL, Li H, Ecker JR (2002) Ethylene biosynthesis and signaling networks. Plant Cell 14: S131-S151

Wielopolska A, Townley H, Moore I, Waterhouse P, Helliwell C (2005) A high-throughput inducible RNAi vector for plants. Plant Biotech J 3: 583-590

Winkel-Shirley B (2002) Biosynthesis of flavonoids and effects of stress. Curr Opin Plant Biol 5: 218-223

Yang YH, Buckley MJ, Dudoit S, Speed TP (2000) Comparison of methods for image analysis on cDNA microarray data. Technical Report 2000. Statistics Department, University of California, Berkley.

Young ND, Shoemaker RC (2006) Genome studies and molecular genetics. Part 1: Model legumes. Exploring the structure, function and evolution of legume genomes. Curr Opin Plant Biol 9: 95-98

Yu Y-B, Yang SF (1979) Auxin-induced ethylene production and its inhibition by aminoethoxyvinylglycine and cobalt ion. Plant Physiol 64: 1074-1077

Zuo JR, Niu QW, Frugis G, Chua NH (2002) The WUSCHEL gene promotes vegetative-to-embryonic transition in Arabidopsis. Plant J 30: 349-359
FIGURE LEGENDS

**Fig. 1.** Main stages of embryogenic callus development starting from single protoplasts. Microcalli (A), proliferating stage, 40 days of culture (B), appearance of embryos, globular stage, 60 days of culture (C), callus with embryos at heart and later stages of development, 80 days of culture (D). Arrows indicate embryos. Bars = 5 mm

**Fig. 2.** Distribution of number of genes of different functional classes that are up- or down-regulated, for 60 days versus 40 days and 80 days versus 60 days of culture from single protoplasts. Derived from Supplementary Table 1 which contains the significantly up- and down-regulated genes.

**Fig. 3.** *MtSERF1* (TC102138) expression in embryogenic 2HA and non-embryogenic Jemalong after 40 and 60 days of protoplast culture using qRT-PCR. SEM indicated.

**Fig. 4.** Relative Expression of *ACC SYNTHASE* (TC95406) [A], *ACC OXIDASE* (TC106655) [B], and *MtSERF1* (TC102138) [C] during the development of embryogenic cultures from leaf explants of 2HA or non-embryogenic Jemalong using qRT-PCR. The 2HA cultures were also treated with the ethylene biosynthesis inhibitor AVG at 10 µM or the ethylene perception inhibitor Ag⁺ at 10 µM. All data for each time point are derived from the same cDNA. The fold change is normalised to the starting leaf tissue. SEM indicated.

**Fig. 5.** Core and responsive element motifs in a 1,758 bp region upstream from the transcription start site of *MtSERF1*. In addition to the TATA and CAAT boxes, *in silico* analysis indicated that the promoter region contained a number of potential regulatory elements indicated in the Figure.
**Fig. 6.** The effects of ethylene biosynthesis stimulators (ACC and MGBG) and inhibitors of biosynthesis and perception (AVG and Ag⁺) on embryo number in 2HA. To modify the levels of ethylene production the following compounds were added to the media: (1) the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) which stimulates ethylene biosynthesis, (2) the stimulator of ethylene biosynthesis methylglyoxal bis-(guanylhydrazone) (MGBG), (3) the inhibitor of ethylene perception silver nitrate (AgNO₃), and (4) the inhibitor of ethylene biosynthesis aminoethoxyvinylglycine (AVG). Three different concentrations, i.e. 1, 10, and 100 µM, were employed for ACC, MGBG, and AgNO₃; whereas AVG was employed at 0.1, 1, and 10 µM. Embryo numbers were counted after 11 weeks of culture. SEM indicated.

**Fig. 7.** The effect of the ethylene biosynthesis stimulator (MGBG) and inhibitor of perception (Ag⁺) on the development of embryogenic callus from 2HA leaf explants. Embryos have developed in the dark for 11 weeks. Note large numbers of embryos at 100 µM MGBG and callus development without embryos in the case of 100 µM Ag⁺. Arrows point to embryos. Bars = 8 mm. In parallel experiments with Jemalong only 2 embryos were produced across all treatments. This confirms that wild type Jemalong rarely produces embryos.

**Fig. 8.** Relative Expression of *ACC SYNTHASE* (ACS, TC95406) and *ACC OXIDASE* (ACO, TC106655) in non-embryogenic callus (Jem) compared to embryogenic callus (2HA) obtained from leaf explants, somatic embryos (S embryos) and ovules with globular stage embryos four days after pollination. (ZE 4DAP). The SE data are shown only for the embryogenic 2HA and ZE (zygotic embryogenesis) is shown only for Jem.

**Fig. 9.** In situ hybridization of *MtSERF1* RNA probe. The globular stage embryo shows expression over the whole embryo (left) while in the heart stage embryo (right) expression is localized to a small group of cells (arrow) in the developing shoot meristem just below the apical notch (arrow). S = suspensor like structure C = callus cells Bars = 40 μm.

**Fig. 10.** The effect of *MtSERF1* knockdown using RNAi on embryo development. Empty vector control (left) and *MtSERF1* knockdown (right). Bars = 5 mm. The histogram indicates the reduction in *MtSERF1* expression due to RNAi.
**Fig. 11.**  A) Alignment of the AP2 domain of *Medicago truncatula* *MtSERF1* with AP2 domains from other plant species. Sequences were aligned with CLUSTAL W implemented in Clustal X (1.8) using default parameters. Black and grey shading of the residues indicate identical and similar amino acid residues, respectively. Gaps required for optimal alignment are indicated by dashes. The black bar and arrows represent predicted α-helix and β-sheet regions, respectively, within the AP2/ERF domain (Allen et al., 1998). B) An unrooted cladogram of *MtSERF1* and all known members of the ERF subfamily of *A. thaliana* and other well-characterized genes from other species. The name of the genes were given when they are well-characterized, otherwise they are presented as TIGR ID. The tree was generated using the neighbour-joining method (Saitou and Nei, 1987) on Clustal X 1.8 software. Groups were named according to Nakano et al (2006). Classification by Sakuma et al. (2002) is indicated in parentheses. The analysis is based on the amino acid sequences of the AP2 domain. C) An unrooted phylogenetic tree of *MtSERF1* and ERFs clustered in Group IX, using the entire amino acid sequence. Branch lengths are drawn to scale. Bar is estimated amino acid substitutions per site.

**Supplementary Fig. 1.** Histological examination of 40 d and 60 d callus used in microarray experiments. 40 d tissue did not contain embryos and rapid cell division appears to occur in nodular groups of cells. 60 d tissue showed that some globular embryos were present (arrow) and were only visible in sectioned material. Histology preparation according to Rose et al. (2006).

**Supplementary Fig. 2.** The bar graph represents the level of *MtSERF1* expression normalised to the *GAPDH* gene. RNAs were isolated from ovules at different stages of embryogenesis (from open flowers and from pods at two to seven days after pollination (DAP)) that spans embryogenesis till its late torpedo stage. “Green seeds” represented material collected at 25 DAP. Bars show SEM.
TABLES

Table 1
Genes upregulated ≥ 2 times (and *MtSERF1) at 60 days of protoplast culture versus 40 days culture. List derived from Supplementary Table 1. This selection of genes is from the 883 genes statistically up-regulated.

Table 2
Genes downregulated ≥ 2 times at 60 days of protoplast culture versus 40 days culture. List derived from Supplementary Table 1. This selection of genes is from the 823 genes statistically down-regulated.

Supplementary Table 1
All statistically up-regulated and down-regulated genes at 60 versus 40 days, 80 versus 60 days and 80 versus 40 days of protoplast culture. A False Discovery Rate of <0.1% was applied and the highest q value was <0.06%.
Table 1. Genes upregulated ≥ 2.00 times at 60 days of protoplast culture versus 40 days culture

| Fold change | IDs | Similarity to known proteins |
|-------------|-----|------------------------------|
| **chalcone and flavonoid metabolism** | | |
| 2.8-3.5 | TC100398, TC100400, TC100398 | Chalcone reductase |
| 2.0-2.3 | TC106536, TC106544 | Chalcone synthase |
| 3.4 | TC107720 | UDP-glucose:flavonoid glycosyltransferase |
| 3.2-3.4 | TC94281, TC96312 | Isoflavone reductase |
| 2.1 | TC100787 | Isoflavone 2'-hydroxylase |
| **transcription factors** | | |
| 2.5-3.6 | TC107549, TC94651, TC1015293 | MYB |
| 2.3-2.8 | TC97324, TC101761 | WRKY |
| 2.1 | TC100528, TC96130 | No apical meristem (NAM) |
| **nodulins** | | |
| 2.5-3.2 | TC111031, AL383966 | NOD-like membrane protein |
| 2.5-3.6 | TC100836, TC94419, TC107353 | Nodule specific |
| 2.2 | TC103767 | ENOD18 |
| 2.2 | TC95616 | N21-like protein |
| **cytokinin response** | | |
| 2.2 | TC94601 | Serine/threonine protein kinase |
| **auxin response** | | |
| 2.6 | TC95234 | Auxin-induced protein homolog F8F16.140 (Arabidopsis thaliana) |
| 2.6 | TC94351 | MAIZE IN2-2 protein |
| **ethylene biosynthesis** | | |
| 2.8-6.9 | TC106654, TC106655 | 1-aminocyclopropanecarboxylic acid oxidase |
| 2.3 | TC95406 | 1-aminocyclopropanecarboxylic acid synthase |
| **ethylene response** | | |
| 2.8-3.3 | TC43436, TC105017 | Ethylene-induced esterase/lipase (Citrus sinensis) |
| 1.94* | TC102138 | MtSERF1 |
| **ABA response** | | |
| 2.2-3.9 | TC95327, TC106638 | Abscisic acid and environmental stress inducible protein |
| **gibberellin response** | | |
| 5 | TC100404 | LTCOR11, |
| 3.8 | TC94215 | GAST-like gene product (Fragaria x ananassa) |
| 2.2 | TC95411 | Snakin-1 (Solanum tuberosum) |
| **gibberellin metabolism** | | |
| 2.9 | TC103730 | Gibberellin 2-oxidase (Pisum sativum) |
| **jasmonic acid biosynthesis** | | |
| 2.2 | TC107322 | Allene-oxide cyclase |
| **stress and defense** | | |
| 2.3 | TC106640 | Ferritin |
| 2.1-4.7 | TC106639, TC106641, TC100143 | Cold and drought-regulated protein |
| 2.2 | TC108259 | Dehydration stress-induced protein (Brassica napus) |
| 2.8 | TC101709 | Putative esterase |
| 2.1-7 | TC101688, TC95383, TC94626, TC94626 | Pathogenesis-related protein |
| 2.4 | TC94759, TC100686 | Putative disease resistance protein |
| 2.2-5 | TC108315, TC107261, TC106851, TC95164, TC100746 | Peroxidase |
| 4.7 | TC100966 | Environmental stress-induced protein |
|   | TC102781 | Temperature stress-induced lipocalin, partial (87%) |
|---|----------|-------------------------------------------------|
| 2 | TC97485  | Beta-glucan elicitor receptor - soybean         |
| signal transduction          |                      |
| 2.7 | TC100498 | Protein kinase MMK4, cold- and drought-induced - alfalfa, complete |
| 2.2 | TC109312 | Putative LRR receptor-like kinase               |
| 2 - 2.5 | TC102218, TC94601, TC94008 | Serine/threonine-specific protein kinase |
| 2.5 | TC103069 | Similarity to calmodulin, partial (46%)         |
| Ca2+ related                  |                      |
| 2.3 | TC108816 | Ca2+/H+-exchanging protein                        |
| chloroplasts/photosynthesis   |                      |
| 2 - 2.7 | TC106570, TC93920, TC94106 | RubisCO small subunit                           |
| 2 - 2.2 | TC100390, TC100390,TC106432 | Chlorophyll a/b-binding protein                  |
| cytochrome P450                |                      |
| 2.1- 2.3 | TC100502, TC100504, BE941365 | Cytochrome P450                                 |
| lipid transport               |                      |
| 2.1- 4.5 | TC94445, TC95002, TC94143, TC94138, TC93922 | Nonspecific lipid-transfer protein precursor (LTP) |
| protein integrity             |                      |
| 3.5 | TC106781 | Kunitz proteinase inhibitor                      |
| redox-related                 |                      |
| 2 | TC104047 | Thioredoxin 3                                    |
| cell wall structure and biosynthesis |                      |
| 2.3- 3.1 | TC106576, TC106582 | Extensin-like protein                           |
| 2.5- 2.7 | TC94063, TC94968 | Cyanogenic Beta-Glucosidase                     |
| 2.7 | TC100486 | Xyloglucan endotransglycosylase, Brassinosteroid-regulated protein BRU1 [Soybean] |
| 2.1- 2.4 | TC101143, TC94366 | Pectinesterase-like protein                     |
| 2.2 | TC100597 | Haloacid dehalogenase-like hydrolase, putative ripening-related protein {Vitis vinifera} |
| 2.1 | TC100580 | Proline-rich cell wall protein                   |
| 2 - 2.5 | TC94670, TC93935 | Glucan endo-1 3-beta-d-glucosidase             |
Table 2. Genes downregulated $\geq$ 2 times at 60 days of protoplast culture versus 40 days culture

| Fold change | N of genes | Similarity to known proteins |
|-------------|------------|-----------------------------|
| **Cell proliferation** | | |
| 2.4 | TC99694 | DNA topoisomerase II |
| 2 | TC109376 | Histone H3 |
| 2 – 2.1 | TC95372, TC100976 | Histone H2A |
| **Transcription regulators** | | |
| 2 | TC103975 | TGACG-motif binding protein - (fava bean) |
| **Auxin response** | | |
| 5 | TC106520 | Proline rich protein auxin-induced - alfalfa |
| 2.7 | TC102735 | Auxin-induced protein AUX28 |
| 2.2 | TC110388 | Auxin-induced protein 22E (Indole-3-acetic acid induced protein ARG14) |
| **Abscisic acid response** | | |
| 6.7 | TC106508 | Abscisic stress ripening protein homolog \( (Prunus armeniaca) \) |
| **Ethylene biosynthesis** | | |
| 2.4 | TC104552 | 1-Aminocyclopropane-1-carboxylate synthase |
| **Nodulins** | | |
| 4.8 | TC106522 | Early nodulin 12 \( (Vicia sativa) \) |
| 2.7 | TC109877 | Nodulin protein \( (Arabidopsis thaliana) \) |
| **Stress and defense** | | |
| 2 - 2.3 | TC106484, TC95489 | Peroxidase precursor |
| 2.3 | TC94562 | Pathogenesis-related protein PR-1 precursor \( (Medicago truncatula) \) |
| 2 | TC107866 | Defensin AMP1 - Dahlia merckii |
| **Aquaporins** | | |
| 2.3 | TC100851 | Multifunctional aquaporin \( (Medicago truncatula) \) |
| **Signal transduction** | | |
| 2.2 - 2.4 | TC108015, TC103969 | Cdc2-like protein kinase - alfalfa |
| 2.3 | TC109033 | Serine/threonine-specific protein kinase homolog - Arabidopsis thaliana |
| 2.3 | TC95992 | DNA-binding protein phosphatase 2C \( (Nicotiana tabacum) \) |
| **Cytoskeleton** | | |
| 2.1 | TC100408 | Tubulin alpha-1 chain \( (Pisum sativum) \) |
| **Protein integrity** | | |
| 2.1 | TC102787 | Ubiquitin-conjugating enzyme \( (Arabidopsis thaliana) \) |
| 2 | TC95356 | Serine proteinase - Arabidopsis thaliana |
| **Redox-related** | | |
| 2 | TC95190 | L-Ascorbate oxidase |
| **Cell wall structure and biosynthesis** | | |
| 2.3 - 4.8 | TC100994, TC94484, TC100993, TC94613 | Alpha-expansin |
| 2.2 - 3.3 | TC112360, TC107321 | Arabinogalactan-protein |
| 2 - 2.5 | TC94066, TC96847, TC96066 | Beta-1 3-glucanase |
| 3 | TC98773 | Cellulase precursor - garden pea |
| 3.2 | TC104377 | Cellulose synthase \( (Arabidopsis thaliana) \) |
| 2.4 | TC94609 | Endo-beta-1 4-glucanase \( (Fragaria x ananassa) \) |
| 2.1 - 3.1 | TC108758, TC110405 | Extensin-like protein |
| 3.2 | TC101945 | Mannan endo-1 4-beta-mannosidase \( (Arabidopsis thaliana) \) |
| 2.3 - 3.5 | TC100685, TC109691 | Pectate lyase |
The images contain bar charts that compare gene expression levels across different conditions. The x-axis represents various biological processes or categories, and the y-axis shows the percentage or fold change in expression.

The charts are color-coded to indicate different conditions:
- Pink: up in 60 vs. 40
- Cyan: down in 60 vs. 40
- Red: up in 80 vs. 60
- Blue: down in 80 vs. 60

Categories include:
- Transcription regulators
- Cell proliferation
- Chromatin structure
- Protein synthesis
- Protein integrity
- Signal transduction
- Redox-related
- Stress and defense
- Chalcone and flavonoid metabolism
- Embryo/seed specific
- Cell wall structure and biosynthesis
- Cytoskeleton
- Chloroplast/photosynthesis
- Nodulins
- Cytokinin response
- Cytokinin metabolism
- Auxin response
- Ethylene response
- ABP3 response
- Gibberellin response
- Gibberellin metabolism
- brassinosteroid response
- brassinosteroid metabolism
- Jasmonic acid synthesis
- brassinosteroid synthesis
- E2 related
- Cytochrome P450
- Lipid/vesicle transport

The charts show a comparison of gene expression changes between two sets of conditions (60 vs. 40, 80 vs. 60) across these categories.
