Isolation of a somatic embryogenesis receptor kinase gene from wheat and assessment of its role in transformation

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INTRODUCTION

Wheat transformation via particle bombardment of immature embryos is a well established and relatively efficient method[1]. The protocol works better with some genotypes than others but has been used successfully on a range of elite genotypes. However, the method is labour intensive and not always practical for all laboratories due to the need to constantly generate high quality immature embryos. The generation of transgenic plants on a consistent basis requires access to controlled environment growth facilities year round. The use of *Agrobacterium* for wheat transformation is becoming routine but all reports to date have used either immature embryos or embryogenic callus derived from immature embryos and transformation efficiencies are generally lower than those achievable with particle bombardment[1]. In addition, transformation efficiency is higher with highly regenerable model genotypes such as Bobwhite than with commercial cultivars[1]. The use of explants such as mature embryos has been explored but these explant sources suffer from higher genotype dependency and lower regeneration rates as compared to immature embryos[2]. The main barrier to the development of high-throughput wheat transformation protocols is the ability to regenerate whole plants from explant tissues[3]. The development of methods which improve regeneration efficiency would allow the use of easier to obtain explants, reduce genotype dependency and increase the overall efficiency of wheat transformation.

The regeneration efficiency (number of shoots produced per explant cultured) is dependent on the ability of cells in a callus to form somatic embryos in response to a set of environmental stimuli. The isolation of genes involved in regulating this process may provide insights and technology to increase regeneration rate in a variety of explants. Schmidt et al.[4] reported the isolation from *Daucus carota* cells of a receptor-like kinase gene where expression started in single cells competent for embryogenesis and continued until the globular stage. Due to this association with somatic embryogenesis the gene was named somatic embryogenesis receptor-like kinase (*DcSERK*). Receptor-like kinases are characterised by an extracellular domain, a transmembrane domain and an intracellular kinase domain[5] and therefore play a role in the transduction of extracellular signals. The SERK protein may act as a transmembrane receptor of signals present in the culture medium and trigger somatic embryogenesis.

Based on homology to *DcSERK,* related genes have been isolated from other species including *Arabidopsis*, maize (*Zea mays*) and rice (*Oryza sativa*). In *Arabidopsis* 5 SERK-like genes were found indicating the presence of a gene family[6]. The family member with the highest homology to *DcSERK* was designated *AtSERK1* and the embryogenic competence of callus derived from seedlings overexpressing this gene was 3-4 times higher than with wild-type callus. This suggests that the use of SERK genes could be used to improve regeneration frequency. This concept was further developed by Hu et al.[7] as they demonstrated that overexpression of a rice SERK (*OsSERK1*) could enhance the regeneration rate from cultured cells while inhibition of *OsSERK* expression by RNA interference (RNAi) reduced the regeneration rate. In maize two *SERK* genes were identified and designated *ZmSERK1* and *ZmSERK2*[8]. *ZmSERK1* had an expression pattern limited to reproductive tissues *in vivo* but was also expressed in both embryogenic and non-embryogenic calli. In contrast, *ZmSERK2* was expressed constitutively in all tissues examined including vegetative tissues. The effect of these two genes on regeneration frequency from in vitro cultures has not been reported.

With the aim of increasing shoot regeneration frequency (and ultimately transformation efficiency) from wheat callus cultures we identified and cloned a wheat *SERK* gene (designated *TaSERK2*) and tested the ability of this gene to enhance transformation efficiency and shoot regeneration.

METHODS

As part of the International Triticeae EST Consortium (ITEC) approximately 5,000 ESTs were sequenced from each of three cDNA libraries derived from developing seed at 5, 15 and 25 days after anthesis[9]. The sequences were annotated by BLAST analysis to the GenBank database of non-redundant genes and the top 5 BLAST hits were recorded in a searchable database. This annotated database was used to identify EST sequences with homology to *SERK* genes by searching the database with the keyword 'SERK'. The two clones identified were obtained and the inserts fully sequenced. The sequences were analyzed for open reading frames. The 628 amino acid open reading frame derived from clone TaE05032D01 was used for sequence analysis. Multiple sequence alignment was performed using CLUSTALW

to generate a dendogram. The sequences to be aligned were of known SERK proteins (from GenBank) and the predicted protein sequences of TaSERK2 (this study) and TC252811 (from the Dana Farber Cancer Institute Wheat Gene Index).

The putative *SERK* gene from TaE05032D01 was cloned in forward orientation between the intron and NOS terminator of pAHC17[10] to create pAAFC61-1. TaE05032D01 has 2 internal BamHI sites which generate a 486bp fragment after digestion with BamHI. This fragment was cloned into BamHI cut pAHC17 in the forward orientation to create pAAFC-33-9. This plasmid was in turn cut with BglII which cuts in the exon fragment located after the ubiquitin promoter but before the intron in pAHC17 (and pAAFC-33-9). The 484bp *SERK* fragment was ligated into BglII cut pAAFC-33-9 in the reverse orientation to create the RNAi vector pAAFC34-12.

A plasmid suspension at a concentration of 1 µg/µL was used for transformation of wheat cultivar 'Fielder' by particle bombardment as essentially as described in Jordan[11] with minor modifications. A 2-day explant pre-culture was used prior to bombardment using 0.6 µm gold, 650 psi rupture disks and a 3cm target distance. The DNA used for bombardment was a 1:1 mixture of pAAFC34-12 (or pAAFC61-1) and pAAFC15-1 which carries the hygromycin phosphotransferase gene controlled by the maize ubiquitin promoter[10]. Selection medium contained 25 mgL^{-1} of hygromycin. In each of three independent bombardment experiments the embryos were equally divided among both of the vector combinations (pAAFC34-12/pAAFC15-1 and pAAFC61-1/pAAFC15-1).

All T1 plants were checked for the presence of the complete *TaSERK2* transgene by Southern blot analysis. Southern positive plants were grown to maturity and 15 T2 seeds from each plant were sown. Lines in which all 15 plants were positive for the transgene (by PCR with the same primers used to make probes for hybridization) were assumed to be homozygous and two of these lines (708-2b10 and 706-1b6) were used for the retransformation experiment to test the ability of *TaSERK2* to increase transformation efficiency. Immature embryos from the transgenic lines 708-2b10 and 706-1b6, along with 'Fielder' controls, were transformed as above except using the plasmid pAct1bar[12] and selection on 5 mgL^{-1} glufosinate. The experiment was repeated 3 times with all three genotypes used in each bombardment experiment.

The same two transgenic lines (plus Fielder controls) were also tested for regeneration efficiency by culturing immature embryos on callus induction medium[11] for two weeks in the dark followed by transfer to regeneration medium in the light. After 4 weeks the number of shoots formed on each callus was recorded. This experiment was repeated 3 times.

RESULTS AND DISCUSSION

An initial search of annotations (derived from BLAST hits) from the in house EST collection using 'SERK' as a keyword identified three EST sequences corresponding to two clones. The clones were fully sequenced and clone TaE05032D01 was found to contain an open reading frame of 628 amino acids with high homology to known full length SERK genes. The predicted protein sequence was analysed by multiple sequence alignment followed by phylogenetic analysis with SERK sequences identified from other species. These included *Daucus carota* SERK (AAB61708), *Arabidopsis* SERK1 (NP_177328) and SERK2 (AAK68073), *Zea mays* SERK1 (CAC37638) and SERK2 (CAC37639) and *Oryza sativa* SERK1 (AY652735). The resulting cladogram (Fig. 1) shows that the wheat sequence is most closely related to *Zea mays* SERK2 (ZmSERK2) and has therefore been designated TaSERK2. The SERK sequences from eudicotyledonous species cluster as a group whereas the monocotyledonous SERKs break into SERK1 and SERK2 clusters.

In *Zea mays*, *ZmSERK1* gene expression exhibited broad expression in reproductive tissues while *ZmSERK2* showed constitutive expression in all tissues examined by Baudino et al.[8] The expression pattern of *TaSERK2* was examined by northern analysis (data not presented) and expression was seen in a range of reproductive tissues, developing seeds, embryogenic and regenerating callus as well as roots and leaves. The pattern more closely resembles that of *ZmSERK2* as opposed to *ZmSERK1* however in this analysis the probe used (derived from the coding sequence) would very likely cross-hybridize to transcripts from a putative *TaSERK1* gene if they are as closely related as *ZmSERK1* and *ZmSERK2*.

It is not known if the two maize SERK proteins perform different biological functions in and of themselves or whether they have similar functions in different tissues. In *Arabidopsis*[6] and rice[7] the *SERK1* gene was capable of enhancing somatic embryogenesis and shoot regeneration in each species respectively and are therefore functional analogues. In order to determine if TaSERK2 was also a functional analogue of SERK1 proteins (which would mean SERK1 and SERK2 proteins are both capable of similar functions) *TaSERK2* was used to generate transgenic wheat plants overexpressing this gene in a constitutive manner. The use of *TaSERK2* did not result in higher transformation efficiency (data not shown) and retransformation of independent homozygous lines containing the *TaSERK2* transgene (Table 1) did not show any positive effect on transformation efficiency over controls. In addition, immature embryos collected from the transgenic lines and placed on callus induction medium followed by transfer to regeneration medium after two weeks (as per our standard regeneration procedure) did not show an increase in shoot regeneration frequency for the transgenic lines (0.93 ± 0.2 shoots per cultured embryo over three replicated experiments) over the control lines

(1.21± 0.25 shoots per cultured embryo) (data not shown). These results could indicate that TaSERK2 is not capable of acting in a similar manner to type 1 SERK proteins or that SERK genes are not important in wheat regeneration and transformation using our protocols.

To further explore the role of TaSERK2 in transformation a transformation vector designed to reduce the expression of *TaSERK2* by RNA interference (RNAi) was constructed. In transformation experiments comparing the *TaSERK2* overexpression vector with the RNAi vector there was a marked reduction in the number of transgenic plants produced using the RNAi vector. In addition, fewer of the transgenic plants produced using the RNAi vector contained the *SERK* derived elements in addition to the marker gene (Table 2). This suggests SERK proteins are indeed necessary for efficient production of transgenic wheat (at least using this protocol). The transgenic T0 plants containing complete copies of the hairpin portion of the RNAi vector were grown to maturity but all of the resulting T1 progeny analysed by Southern analysis were lacking the diagnostic fragments indicating presence of the hairpin construct (data not shown). This suggests that SERK proteins may be necessary for some aspect of reproduction *in vivo*.

If SERK proteins are indeed necessary for efficient shoot regeneration and transformation of wheat, but TaSERK2 is not involved, it is reasonable to assume that a functional homologue to ZmSERK1 and OsSERK1 exists. There currently exists over 850,000 EST sequences for *Triticum aestivum*. The Wheat Gene Index curated by the Dana Farber Cancer Institute (DFCI) contains represents a database of wheat EST sequences assembled into contigs along with annotations. Searching the database by nucleotide BLAST using the *OsSERK1* sequence as the query resulted in the identification of TC252811 as having high homology (2.0e-247) to *OsSERK1*. In contrast, the three EST sequences identified in this study all assemble into TC253788. The consensus sequence corresponding to TC252811 was translated in all reading frames and the amino acid sequence of the largest open reading frame was aligned with the other SERK sequences. In the cladogram (Fig. 1) it can be seen that TC252811 is more closely related to OsSERK1 and ZmSERK1 than TaSERK2. The consensus sequence of TC252811 does not reveal a full length coding sequence.

For increases in wheat transformation frequency it is possible that wheat SERK genes could be used to increase shoot regeneration as has been shown in rice[7] and *Arabidopsis*[6]. The involvement of SERK proteins in wheat transformation has been shown by a reduction in transformation efficiency when the RNAi vector for *TaSERK2* was used. However, the lack of effect of *TaSERK2* overexpression in improving transformation or shoot regeneration efficiency is likely due to this form of SERK not being the critical form for involvement in embryogenesis. It is more probable that a wheat SERK protein with greater homology to the SERK1 type (as opposed to SERK2) would have a positive impact on regeneration and transformation. That a TaSERK1 exists is implicated in the EST database and cloning of the full length version of the gene corresponding to TC252811 has recently been reported[13].

Fig. 1

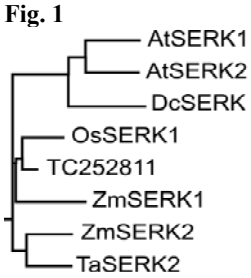

Table 1. Comparison of transformation efficiency (%) between 'Fielder' and two 'Fielder'-derived transgenic wheat lines carrying full length copies of *TaSERK2*.

Fielder	Plant 708-2b10	Plant 706-1b6
0.4	0.2	0.05

Table 2. Comparison of wheat transformation efficiencies between the *SERK* expression vector pAAFC61-1 and the *SERK* RNAi vector pAAFC34-12 following co-bombardment with the selectable marker plasmid pAAFC15-1

Plasmid	Transformation Efficiency (%)	Co-Transformation Efficiency (%)
pAAFC61-1 pAAFC15-1	2.14	94
pAAFC34-12 pAAFC15-1	0.54	50

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