UNIVERSIDADE FEDERAL RURAL DE PERNAMBUCO

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA

SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE (SERK): APLICABILIDADE COMO MARCADOR EMBRIOGÊNICO EM ALGODOEIRO

TAIZA DA CUNHA SOARES

RECIFE - PE

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Tese apresentada ao Programa de Pós-Graduação em Biotecnologia – RENORBIO na Universidade Federal Rural do Pernambuco – UFRPE, como requisito para obtenção do título de Doutor.

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Orientador: Dr. Péricles de Albuquerque Melo Filho

Co-orientadores: Dra. Roseane Cavalcanti dos Santos e Dra. Julita Maria Frota Chagas de Carvalho

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TESE DE DOUTORADO ELABORADA POR:

TAIZA DA CUNHA SOARES

***Somatic Embryogenesis Receptor-Like Kinase* (*SERK*): aplicabilidade como marcador embriogênico em algodoeiro**

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Aos meus pais, Francisco e Marizete,

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RESUMO GERAL

Soares, T. da C. *Somatic Embryogenesis Receptor-Like Kinase (SERK)*: aplicabilidade como marcador embriogênico em algodoeiro. 2018. 87f. Tese (Doutorado) – Pós-graduação em Biotecnologia, Universidade Federal Rural de Pernambuco, Recife.

O lançamento de cultivares transgênicas de espécies recalcitrantes como o algodoeiro pode ser retardado devido à dificuldade em regenerar o tecido transformado. Os protocolos de regeneração existentes limitam-se a poucas variedades responsivas, como a Coker, normalmente utilizadas como receptoras durante a transformação genética. Um dos fatores que podem estar ligados à recalcitrância, é a ineficiência na ativação de diferentes genes durante o cultivo *in vitro* que podem estar associados à aquisição de competência embriogênica. Dentre os genes citados na literatura, destaca-se o *Somatic Embryogenesis Receptor-Like Kinase (SERK)* retratado como responsável pela transição do estado celular somático para embriogênico em diferentes espécies de plantas. A potencialidade deste gene como marcador de embriogênese somática tem sido investigada. Neste trabalho, foram analisadas a relação dos níveis de expressão de *GhSERK1* com a aquisição de competência embriogênica em genótipos de algodoeiro. Para isto, uma sequência curta (186 pb) deste gene foi selecionada a partir de região conservada entre o algodão e cinco outras dicotiledôneas (*Theobroma cacao*, *Citrus sinensis, Vitis vinifera*, *Glycine max* e *Phaseolus vulgaris)* de modo a permitir que a sonda também possa ser aplicada na seleção de outras culturas. Inicialmente, os testes de expressão gênica via RT-qPCR foram realizados em seis genótipos com capacidade embriogênica conhecida (embriogênicos – Coker 312, BRS Rubi e BRS Seridó e não embriogênicos – BRS 201, CNPA Precoce 1 e BRS Topázio), a partir de RNA extraído de tecidos zigóticos meristemáticos. Os genótipos supracitados foram induzidos à embriogênese somática em meio suplementado com ácido naftalenoacético e kinetina. Corroborando com o resultado obtido via RT-qPCR, embriões somáticos formaram-se apenas na Coker 312, BRS Seridó e BRS Rubi. Nesta última, o percentual de plantas férteis obtidas foi maior (50%) do que o Coker 312 (41,6%). A aplicabilidade seletiva do *GhSERK1* foi validada em quatro genótipos top lines (CNPA 286, CNPA BA 139, CNPA BA 1366 e CNPA BA 2247) com comportamento *in vitro* desconhecido. Simultaneamente, realizamos ensaios de indução à embriogênese somática para comprovar os resultados de expressão. Nos genótipos em que a expressão relativa foi maior (Coker 312 e CNPA BA 139) houve a formação de embriões somáticos. Uma sonda

não radioativa foi então desenvolvida a partir do fragmento de 186 pb e atestada sua eficiência via Southern Blot nos genótipos top lines, manchas robustas foram observadas em Coker 312 e CNPA BA 139. Com base nestes resultados, este último foi selecionado como genótipo receptor em futuros estudos de transformação. Além disso, a sequência utilizada permitiu identificar os genótipos responsivos à embriogênese somática a partir de tecidos zigóticos, otimizando o tempo e os custos. Este trabalho fornece colaborações para a compreensão de fatores que podem estar envolvidos na aquisição de competência embriogênica gerando perspectivas de novos estudos para fortalecer os programas de melhoramento genético da cultura.

Palavras-chave: *Gossypium hirsutum L.*; Transgenia; Regeneração; Regulação gênica.

GENERAL ABSTRACT

Soares, T. da C. *Somatic Embryogenesis Receptor-Like Kinase (SERK)*: applicability as an embryogenic marker in cotton. 2018. 87f. Thesis (PhD) - Postgraduate course in Biotechnology, Federal Rural University of Pernambuco, Recife.

The release of transgenic cultivars of recalcitrant species such as cotton can be delayed due to the difficulty in regenerating the transformed tissue. Existing regeneration protocols are limited to a few responsive varieties, such as Coker, commonly used as recipients during genetic transformation. One of the factors that may be related to recalcitrance is the inefficiency in the activation of different genes during in vitro culture that may be associated with the acquisition of embryogenic competence. Among the genes mentioned in the literature, the *Somatic Embryogenesis Receptor-Like Kinase (SERK)* is shown to be responsible for the transition from the somatic cell state to the embryogenic in different plant species. The potential of this gene as a marker of somatic embryogenesis has been investigated. In this work, the relationship of the levels of *GhSERK1* expression with the acquisition of embryogenic competence in cotton genotypes was analyzed. For this, a short sequence (186 bp) of this gene was selected from the conserved region between cotton and five other dicotyledons (*Theobroma cacao, Citrus sinensis, Vitis vinifera, Glycine max* and *Phaseolus vulgaris*) in order to allow the probe can be applied in the selection of other cultures. Initially, the RT-qPCR gene expression tests were performed in six genotypes with known embryogenic capacity (embryogenic - Coker 312, BRS Rubi and BRS Seridó and non-embryogenic - BRS 201, CNPA Precoce 1 and BRS Topázio), from RNA extracted from meristematic zygotic tissues. The aforementioned genotypes were induced to somatic embryogenesis in medium supplemented with naphthaleneacetic acid and kinetin. Corroborating with the result obtained via RT-qPCR, somatic embryos were formed only in Coker 312, BRS Seridó and BRS Rubi. In the latter, the percentage of fertile plants obtained was higher (50%) than Coker 312 (41.6%). The selective applicability of *GhSERK1* was validated in four genotypes top lines (CNPA 286, CNPA BA 139, CNPA BA 1366 and CNPA BA 2247) with unknown in vitro behavior. Simultaneously, we performed induction tests on somatic embryogenesis to verify the expression results. In the genotypes in which the relative expression was higher (Coker 312 and CNPA BA 139), somatic embryos were formed. A nonradioactive probe was then grown from the 186 bp fragment and attested for its efficiency via Southern Blot in the top line genotypes, robust spots were observed

in Coker 312 and CNPA BA 139. Based on these results, the latter was selected as the recipient genotype in future transformation studies. In addition, the sequence used allowed to identify genotypes responsive to somatic embryogenesis from zygotic tissues, optimizing time and costs. This work provides collaborations for the understanding of factors that may be involved in the acquisition of embryogenic competence generating perspectives of new studies to strengthen the programs of genetic improvement of the culture.

Keywords: *Gossypium hirsutum* L .; Transgenic; Regeneration; Gene regulation.

LISTA DE ILUSTRAÇÕES

CAPÍTULO I: REVISÃO DE LITERATURA

CAPÍTULO II: Validating a probe from *GhSERK1* gene for selection of cotton genotypes with somatic embryogenic capacity

LISTA DE ABREVIATURAS

ACT – Actina

KT – Kinetina

GM - Geneticamente modificado

LRR - Leucine-rich repeat

NAA - Naphthalene acetic acid

PP2A - Protein phosphatase 2A

ES – Embriogênese Somática

EZ – Embriogênese Zigótica

SERK - Somatic Embryogenesis Receptor Kinase

SUMÁRIO

CAPÍTULO I

REVISÃO DE LITERATURA

1. INTRODUÇÃO GERAL

Cultivado em diversos países, inclusive no Brasil, o algodão (*Gossypium hirsutum L. var. latifolium Hutch*) é uma das principais culturas de representatividade comercial mundialmente. Seus produtos são empregados em diversos setores industriais, como na produção de óleo, ração animal, cosméticos, e principalmente na indústria têxtil (Beltrão et al., 2010; Sousa, 2010).

A representatividade dessa cultura no cenário agrícola mundial suscita a busca pelo desenvolvimento de cultivares com características comercialmente competitivas, como precocidade, produtividade e resistência. Muitas cultivares cultivadas comercialmente e com características superiores foram obtidas através do melhoramento convencional. Entretanto, com o advento da engenharia genética, surgiram técnicas que viabilizam a inserção de genes exógenos ao genoma da planta, por um processo de transgenia, possibilitando a expressão de características que não seriam impetradas através de cruzamentos devido às barreiras reprodutivas.

Diversos genes que conferem tolerância a estresses bióticos e abióticos, ou melhoram o rendimento e a qualidade da fibra têm sido isolados, caracterizados e utilizados em protocolos de transformação genética (Zeng et al., 2006; Coutinho et al., 2016). Entretanto, a maioria dos métodos de transformação requer um processo de regeneração celular dependente da cultura de tecidos.

Através da cultura de tecidos uma célula ou tecido transformado pode ser regenerado em uma planta completa e funcional por organogênese ou embriogênese somática (ES). Sendo esta última, a principal via de regeneração utilizada nos protocolos de transformação, por proporcionar a formação de uma estrutura bipolar sem ligação vascular com o explante (Sidorov, 2013)

A ES envolve mecanismos fisiológicos complexos e de reprogramação do padrão genético celular. As células do explante devem adquirir potencial para expressar totipotência e responder a sinais exógenos, induzindo a formação de células competentes por sinais específicos, geralmente reguladores de crescimento. Assim, perdem a

especificidade e tornam-se células meristemáticas capazes de originar embriões somáticos (Elhiti et al., 2013).

A compreensão dos fatores chave que iniciam a ES é crucial para reverter problemas ligados à recalcitrância. Ao se tratar desta problemática, variedades de algodão 'elite' são, majoritariamente, recalcitrantes e não passíveis de manipulação genética quando submetidas a protocolos de regeneração *in vitro* (Kumar et al., 2013). Na literatura poucas cultivares são mencionadas como responsivas à ES, é o caso das variedades Coker, utilizadas há décadas nos estudos de transformação (Davidonis e Hamilton, 1983; Trolinder e Goodin, 1987; Haq, 2005; Pandey e Chaudary, 2014).

Esforços têm sido feitos para desvendar a base genética dos processos que envolvem a aquisição de competência embriogênica durante a morfogênese *in vitro*, resultando em evidências do envolvimento de alguns genes que podem estar intrinsicamente ligados a transição de célula somática à embrionária, como o *BBM* (*Baby boom*) (Boutilier et al., 2002); *LEC* (*Leafy Cotyledon*) (Kwong et al., 2003); *AGL15* (*Agamous-Like15*) (Zuo et al., 2002); *WUS* (*Wuschel*) (Zeng et al., 2006); *PKL* (*Pickle*) (Karami et al., 2009) e o *SERK (Somatic Embryogenesis Receptor-Like Kinase)* (Hecht et al., 2001).

Dentre estes, o gene *SERK* vem se destacando nos estudos acerca da regulação gênica da embriogênese somática. Inicialmente isolado a partir de células de *Daucus carota* (Schmidt et al., 1997), este gene quando super expresso incrementa a competência embriogênica, promovendo a transição de células somáticas para o estado embriogênico, podendo ser considerado um marcador do início do processo embriogênico tanto em monocotiledôneas quanto em dicotiledôneas (Hecht et al., 2001; Obembe et al., 2011; Pandey e Chaudhary, 2014). Somando-se a isso, os genes *SERK* são positivamente regulados durante a embriogênese zigótica (EZ) em várias espécies (Hecht et al., 2001; Hu et al., 2005).

A similaridade no processo de desenvolvimento pelas vias, somática e zigótica, pode servir como argumento para que o mesmo conjunto de genes atue nos dois casos para especificar o desenvolvimento embrionário (Jin et al., 2014). Dessa forma, a expressão de um gene que atue tanto na ES quanto na EZ, como o *SERK*, pode representar uma importante vantagem na identificação de genótipos com competência embriogênica

somática, a partir da utilização de tecidos zigóticos, já que estes demandam menor tempo e custos para obtenção.

Dessa forma, desenvolveu-se uma sonda não radioativa a partir de região conservada do gene *GhSERK1* para ser utilizada na identificação de genótipos responsivos à ES, por um procedimento diferenciado que permite a análise a partir de RNA extraído de tecidos zigóticos meristemáticos, de modo a reduzir o tempo e os custos de seleção. Ao identificar genótipos competentes, técnicas de transformação poderão ser viabilizadas, possibilitando maior eficiência dos protocolos de regeneração *in vitro* e obtenção de organismos transgênicos.

2. REFERENCIAL TEÓRICO

2.1. A Cultura do Algodoeiro: Aspectos gerais e importância econômica

O algodoeiro é uma dicotiledônea pertencente à família Malvaceae. O gênero *Gossypium* mais cultivado comercialmente compreende 50 espécies com dois níveis de ploidia, diplóides (2n = 2x = 26) e alotetraplóides (2n = 4x = 52) distribuídas em todo o mundo nos continentes asiático, americano, africano e oceânico (Fryxell et al., 1992; Freire, 2000).

A espécie alotetraplóide *G. hirsutum L*. também denominada de algodoeiro "herbáceo" ou "anual", é responsável por mais de 90% da produção mundial de algodão e deu origem à maioria das variedades cultivadas no mundo, sendo representada no Brasil pelas raças: *G. hirsutum L.r. latifolium* Hutch (algodoeiro herbáceo), e *G. hirsutum var. marie-galante* (Watt) Hutch (algodoeiro-mocó) (Freire, 2000; Busoli et al., 2011).

A importância econômica desta cultura pode ser percebida em diversos setores, com destaque para a fibra que é utilizada em diversos setores produtivos e, absorvida principalmente pela indústria têxtil mundial. As sementes são utilizadas de forma in natura, para alimentação animal, ou esmagada, permitindo a elaboração de subprodutos importantes, tais como a torta para ração animal e óleo utilizado pela indústria de gêneros alimentícios, de combustíveis, cosméticos, entre outras (CONAB, 2015; Buainain e Batalha, 2007).

No Brasil o cultivo do algodão foi marcado por ciclos de expansão e declínio. No final da década de 80, após o extraordinário avanço pelo Nordeste, Sudeste e Centro-oeste, a cotonicultura brasileira apresentou uma mudança considerável no seu perfil, passando de exportador a importador. Dentre os fatores que contribuíram para isso, destacam-se a propagação de pragas e doenças, a falta de incentivos financeiros e o despreparo dos agricultores (Silva et al., 2005).

No que diz respeito ao ataque de pragas, o declínio da cotonicultura naquela época foi marcado pelo surgimento e estabelecimento do bicudo do algodoeiro (*Anthonomus grandis*) que persiste até os dias atuais causando prejuízos a pequenos e grandes produtores. Essa praga ataca a planta durante a frutificação, causando prejuízos

consideráveis, pois compromete a produtividade, a qualidade das fibras e eleva os custos de produção (Bastos et al., 2005).

A inserção da cultura em programas de melhoramento genético foi crucial para a reestruturação da cotonicultura, marcada pelo desenvolvimento das primeiras variedades geneticamente modificadas. Segundo Buainain e Batalha (2007), a pesquisa e transferência de tecnologia realizada pela Embrapa foram relevantes para viabilizar o processo de reorganização da produção do algodão. O programa tem sido bem sucedido e, o plantio de algodão no cerrado brasileiro é um dos mais competitivos do mundo.

A região dos Cerrados brasileiros é responsável por 93,7% do total do algodão produzido no país (CONAB, 2017). O Cerrado apresenta vantagens para o estabelecimento do plantio comparativas às demais regiões, destacando-se a topografia, que permite a mecanização completa das atividades, e o clima, marcado por estação chuvosa e seca, o que proporciona maior qualidade à fibra. A produção em grande escala atende altos padrões de tecnologia que garantem maior produtividade, melhor aproveitamento e qualidade para atender o mercado nacional e internacional movidos pela competitividade (CONAB, 2017; Vidal Neto e Freire, 2013).

Os investimentos tecnológicos e científicos tem feito o Brasil destacar-se entre os maiores exportadores mundiais e o cenário interno também é promissor, pois está entre os maiores consumidores de algodão em pluma do mundo. É o quinto maior produtor de algodão conforme projeção dos dados da safra 2017/ 18 (USDA, 2018), com destaque para os estados de Mato Grosso e Bahia que serão responsáveis por 89% (Safra 2017/ 2018) da produção nacional e se destacam pelo investimento em biotecnologia, gerenciamento do setor e novas técnicas de manejo (CONAB, 2018).

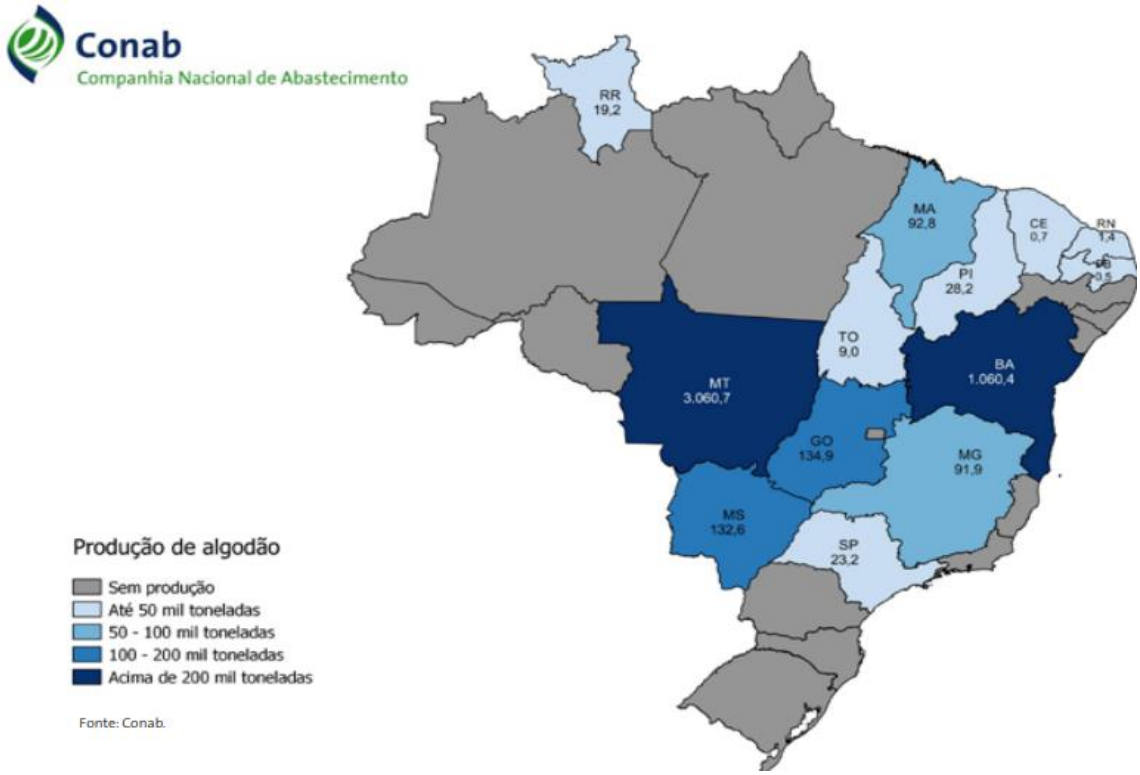

Figura 1. Mapa de produção do algodão. Fonte: Conab, 2018.

2.2. Melhoramento Genético do Algodoeiro no Brasil

As pesquisas envolvendo o melhoramento do algodoeiro datam de 1921 com a reativação do Ministério da Agricultura, mas as primeiras sementes melhoradas só foram lançadas em 1926 pelo Instituto Agronômico de Campinas (IAC). Em 1976, a Embrapa (Empresa Nacional de Pesquisa Agropecuária) criou o Centro Nacional de Pesquisa do Algodão (CNPA) dando início às pesquisas de melhoramento com a espécie herbácea, *G. hirsutum* L. (Freire et al., 2007).

O investimento em pesquisas possibilitou o desenvolvimento das primeiras variedades de algodão obtidas a partir de técnicas de melhoramento convencional como mencionado em Freire (2000): seleção direta em germoplasma nativo de algodoeiro mocó; cruzamento inter-racial entre os algodoeiros mocó (*G. hirsutum var. mariegalante*) e herbáceo (*G. hirsutum latifolium*); desenvolvimento da população de base ampla SRI3 da qual se originaram muitas cultivares plantadas no Cerrado do Brasil; algodoeiro anual com a participação de *G. herbaceum var. africanum* e *G. hirsutum var. yucatanense*;

populações híbridas entre *G. mustelinum, G. barbadense, G. hirsutum var. marie-galante* e algodoeiros anuais.

O programa de melhoramento do algodão, conduzido pela Embrapa Algodão, vem desenvolvendo variedades importantes a partir de cruzamentos entre diferentes genótipos de algodoeiro. É o caso das cultivares de fibra colorida como a BRS 200, BRS Verde, BRS Safira, BRS Rubi e BRS Topázio, adotadas principalmente pela agricultura de base familiar por meio do cultivo orgânico. E outras importantes cultivares de fibra branca, como a CNPA 8H, BRS Araripe, BRS Seridó, BRS 293, BRS 286, BRS 336, dentre outras (Freire, 2000).

Entretanto, para atender as exigências e demandas da cadeia produtiva, além de produtividade, precocidade e qualidade da fibra, outras características como resistência às principais pragas, doenças e outros estresses bióticos e abióticos tornaram-se imprescindíveis. Nessa perspectiva, houve um avanço significativo na cotonicultura do país a partir da introdução das lavouras geneticamente modificadas (GM) com a liberação para o cultivo comercial e pesquisa (Parecer Técnico Conclusivo, CTNBio nº.513/2005). A inserção das técnicas de transgenia tornou possível incorporar características de interesse oriundas de outros organismos, em cultivares e linhagens "elites" de algodão, o que seria impossível pelo melhoramento convencional devido às barreiras reprodutivas (Araújo et al., 2013).

Os principais avanços no desenvolvimento de cultivares transgênicas foi impulsionado pelo surgimento da tecnologia *Bt.* A bactéria *Bacillus thuringiensis* é um microrganismo de solo, aeróbico, Gram positiva com propriedades inseticidas em virtude da síntese de uma proteína denominada *Cry* ou delta toxina (Pinto e Fiuza, 2008). Essa proteína em formato de cristal é produzida durante a fase de esporulação da bactéria, apresenta especificidade e ação entomopatogênica. Plantas transformadas com genes *Cry* provocam paralisia da musculatura intestinal de insetos-alvo, inibindo a absorção de alimentos e a formação de poros nas membranas do intestino acarretando a morte. Entretanto, não oferece riscos toxicológicos em humanos e animais (Costa e Queiroz, 2015).

Dentre as principais cultivares de algodão transgênicos comercializados no Brasil, estão o MON 88913 - tolerante ao glifosato; TwinLink - resistente a insetos e tolerante

ao herbicida glufosinato de amônio; GHB614 - Algodão GlyTol® - tolerante a herbicidas; MON 531 x MON 1445 - tolerante a herbicida e resistente a insetos da Ordem Lepidoptera; Bollgard II - resistente a insetos; Widestrike - resistente a insetos e tolerante ao Glufosinato de Amônio; Roundup Ready - confere às plantas de algodão o atributo que possibilita o uso em pós-emergência do herbicida glifosato, para manejo de plantas daninhas, sem causar injúria à lavoura; LLCotton25 - tolerante ao glufosinato de amônio e Bollgard evento 531 - resistente a insetos da Ordem Lepidoptera. E cultivares transgênicas lançadas pela Embrapa como, BRS 368RF, BRS 369RF, BRS 370RF, BRS 371RF, BRS 430 B2RF, BRS 432 B2RF e BRS 433 FL B2RF que apresentam algum tipo de resistência a herbicidas e/ou pragas (RNC - MAPA, 2017; Embrapa, 2017).

Figura 2. Algodão branco BRS 433 FL B2RF.
Fonte: Embrapa Algodão.

As variedades transgênicas de algodão lançadas até o presente momento são, normalmente, oriundas de transformação mediadas por *Agrobacterium tumefaciens* ou biobalística. Estes métodos dependem que os tecidos transformados sejam posteriormente regenerados *in vitro* e usualmente por ES. Por esta razão, variedades da cultivar Coker são normalmente usadas como receptoras durante a transformação, por não apresentarem recalcitrância à regeneração *in vitro*, característica que limita a transformação na maioria

das espécies de algodão (Davidonis e Hamilton, 1983; Zeng et al., 2006; Obembe et al., 2011; Kumar et al., 2013).

2.3. Embriogênese Somática

A ES constitui uma valiosa ferramenta biotecnológica na produção de novas cultivares, isso porque, além de constituir-se importante via de regeneração de explantes, também permite estudos relacionados à morfologia, bioquímica, genética e mecanismos moleculares que envolvem o desenvolvimento embrionário. Trata-se de um mecanismo baseado na totipotência celular em que uma única célula pode desenvolver a habilidade de dividir-se até formar uma planta completa e funcional (Nic-Can et al., 2015; Loyola-Vargas et al., 2008).

As células totipotente, ao adquirirem capacidade embriogênica, sofrem um processo de transição do estado diferenciado do explante para o estado embriogênico, como resultado de diferentes estímulos químicos e físicos que desencadeiam a expressão de genes específicos (Von et al., 2002; Elhiti et al., 2013).

Trata-se de um evento complexo que envolve fases distintas que compreendem a desdiferenciação do explante, reativação celular, divisão celular e várias etapas de reprogramação gênica e metabólica que poderão resultar na formação de embriões somáticos, estruturas bipolares que se desenvolvem com ligações vasculares independentes do explante, passam pela maturação, dessecação até regenerar-se em plantas completas (Zheng e Perry, 2014; Obembe et al., 2011; Karami et al., 2009; Von et al., 2002).

Existem duas formas de indução da ES: direta ou indireta. Na forma direta, os embriões se desenvolvem a partir do tecido organizado do explante, enquanto que, na indireta são originados a partir das células de calos provenientes da Desdiferenciação de células do explante. Willemsen e Scheres (2004) sugeriram que na ES direta já existe células competentes proembriogênicas e a expressão das características embriogênicas depende meramente de condições favoráveis e de reprogramação mínima de genes envolvidos neste processo.

Na ES indireta, é necessária a reprogramação de vários genes para que células desdiferenciadas adquiram o estado embriogênico. Apesar da complexidade deste processo, é a via de regeneração mais utilizada nos programas de melhoramento genético na maioria das culturas, incluindo o algodão, pois permite a regeneração de tecidos mais diferenciados e maduros em meio de cultura apropriados (Sidorov, 2013).

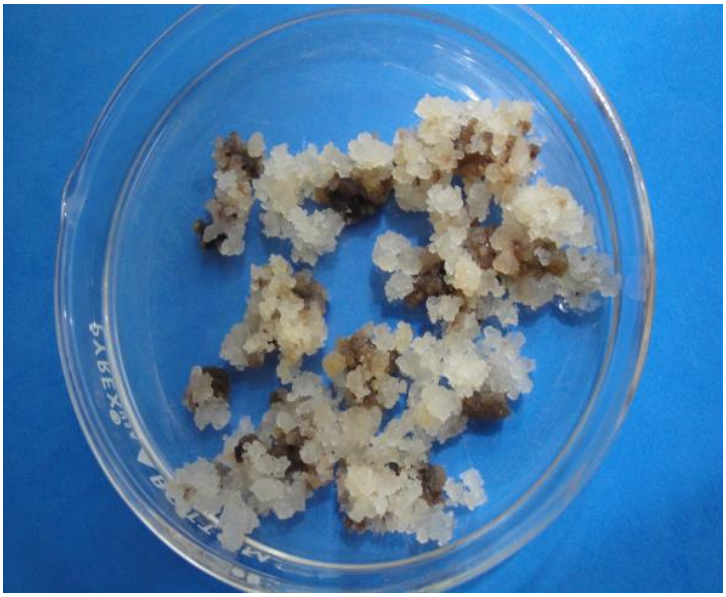

Figura 3. Formação de calos durante a embriogênese somática indireta em algodão.

As auxinas são utilizadas como principais indutores da ES, pois mediam à transição do estado somático para embriogênico, favorecendo a aquisição de competência embriogênica pelas células somáticas (Chugh e Khurana, 2002). Entretanto, o balanço hormonal entre auxinas e outros reguladores de crescimento, como as citocininas pode otimizar a regulação da divisão e reprogramação celular. Em algodão, combinações entre as auxinas ácido 2,4-Diclorofenóxiacético (2,4-D), ácido indol-3-butírico (AIB), e ácido naftalenoacético (ANA) e a citocinina Kinetina (KT) são frequentemente utilizadas (Sun et al., 2006).

Os reguladores de crescimento são cruciais a desdiferenciação celular que ocorre após a indução. Subsequentemente, deve ocorrer a fase de expressão que é marcada pelo momento em que as células exibem sua competência embriogênica e se diferenciam para

formar embriões somáticos (Jiménez, 2001). Nesse momento, o genótipo, a origem do explante, as condições de cultura, os efeitos hormonais, os estresses fisiológicos e a manipulação *in vitro* ainda podem interferir na resposta embriogênica por influenciar na ativação e nos padrões de expressão gênica que atuam na aquisição da competência embriogênica, bem como na formação dos embriões somáticos (Neelakandan e Wang, 2012).

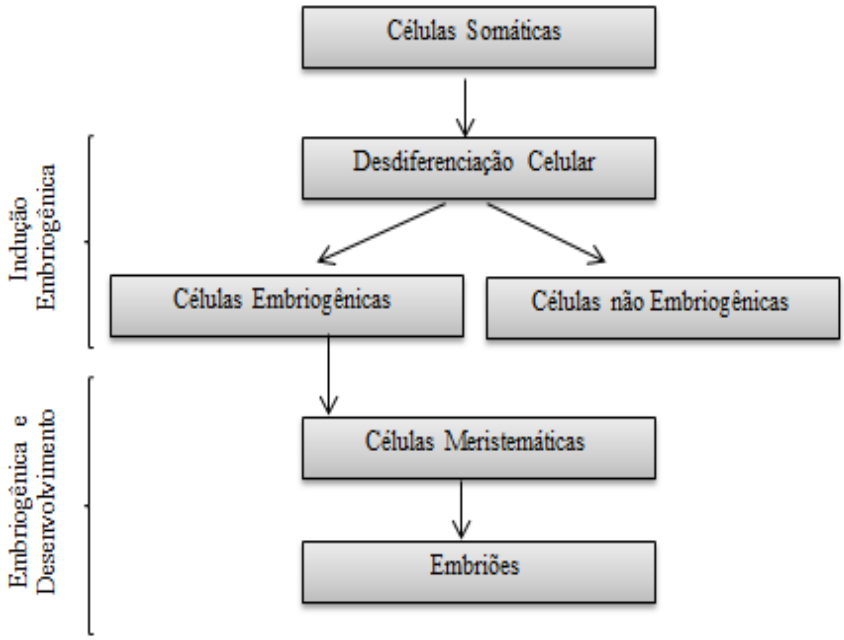

Figura 4. Esquema simplificado do desenvolvimento embrionário *in vitro* a partir de células somáticas.

Com intuito de compreender a complexidade dos eventos que envolvem a indução a ES, principalmente em genótipos poucos responsivos, como o algodão, estudos focados na caracterização funcional de genes especificamente expressos descrevem algumas categorias proteicas envolvidas neste processo, como os fatores de transcrição e proteínas relacionadas ao ciclo celular, à formação da parede celular, envolvidas em vias de sinalização e em resposta a ação dos reguladores de crescimento (Elhiti et al., 2013; Yang e Zhang, 2010). A indução e a ativação de uma variedade de genes são requisitos para a aquisição de competência embriogênica e formação do embrião.

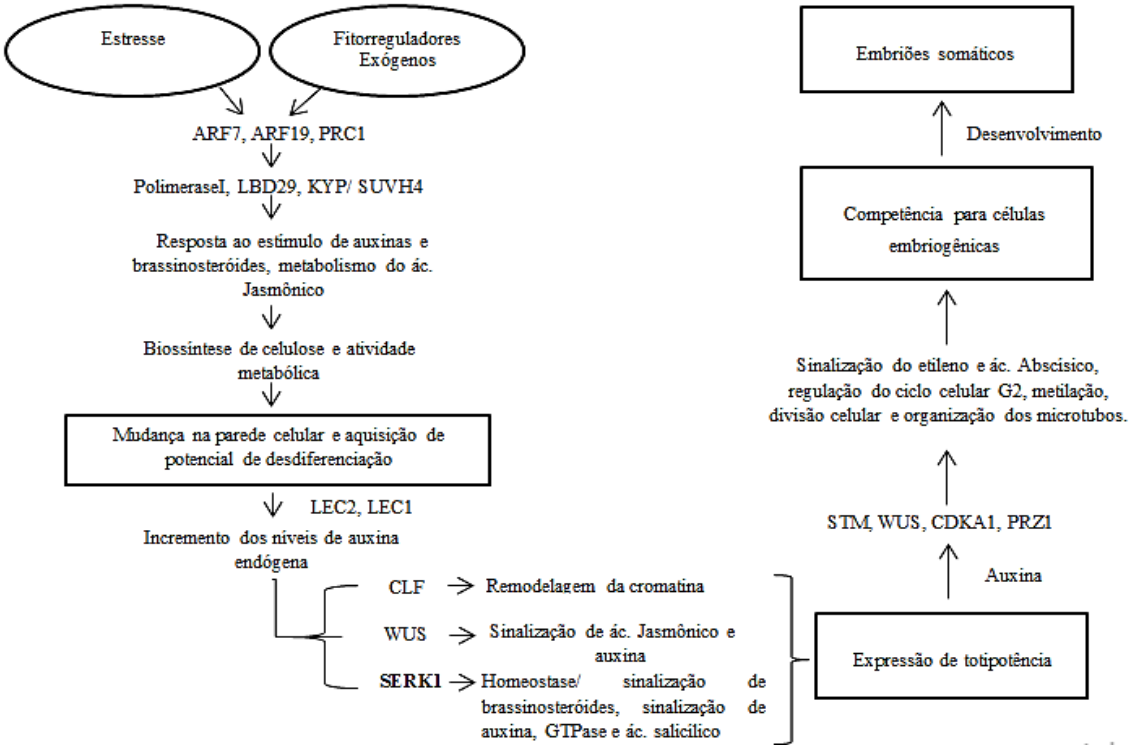

Figura 5. Regulação molecular da embriogênese somática vegetal. Fonte: Elhiti et al., 2013.

2.3.1. Embriogênese Somática x Embriogênese Zigótica

A EZ é uma das etapas mais importantes no ciclo de vida das plantas. Em angiospermas, esse processo se inicia com a fertilização, seguido pela determinação dos três eixos embrionários (longitudinal, lateral e radial), e uma sequência de alterações na morfologia do embrião, passando pelos estádios globular, cordiforme, torpedo e cotiledonar. Os processos que ocorrem ao longo da EZ são regulados por diversos fatores, incluindo fitormônios, enzimas e outras substâncias importantes para esta rota morfogenética (Ikeda et al., 2006).

Embora desencadeadas por mecanismos diferentes, a ES se assemelha em muitos aspectos a EZ como morfológicos, anatômicos, bioquímicos e moleculares, incluindo a expressão proteica, gênica e diferentes metabólitos. Culminando numa série de estádios de desenvolvimento, que se aproximam da sequência de eventos observados na EZ, como polaridade e assimetria da divisão celular, formação de meristemas, fases da maturação (globular, cordiforme, torpedo e cotiledonar) e germinação (Dodeman et al., 1997; Ikeda et al., 2006). Em virtude dessas similaridades, suscitou-se a busca por marcadores comuns

dos diferentes estádios do desenvolvimento da ES e EZ, já que provavelmente o mesmo conjunto de genes controla o desenvolvimento em ambos os casos (Domoki et al., 2006; Sghaier-Hammami et al., 2008).

Uma análise comparativa entre ES e EZ realizada em algodão descreve que mais de 50% dos genes superexpressos durante o desenvolvimento embrionário são compartilhados entre as duas vias morfogênicas. Este conjunto comum de genes atua de diversas formas entre as quais, na metilação, resposta fisiológica a estresses, regulação da via embriogênica, divisão celular e polaridade (Jin et al., 2014). Nesse contexto, uma das descobertas mais importantes foi o gene *SERK*, positivamente regulado durante os estágios iniciais tanto da ES quanto da EZ (Hecht et al., 2001; Hu et al., 2005).

2.4. Recalcitrância e Regulação Gênica da Embriogênese Somática: Gene *SERK (Somatic Embryogenesis Receptor-Like Kinase)*

Recalcitrância pode ser definida como a incapacidade de células, tecidos ou órgãos vegetais para responder à regeneração *in vitro* pelo declínio e/ou perda da competência morfogenética e capacidade totipotente (Benson, 2000). Uma espécie vegetal pode ser considerada como recalcitrante quando são utilizados procedimentos de cultura de tecidos e, frequentemente não ocorre resposta satisfatória à ES ou organogênese (Benson, 2000; Bonga et al., 2010).

O algodão é uma das espécies de plantas notoriamente recalcitrante ao cultivo *in vitro*, principalmente a ES. Somente alguns genótipos, como a Coker foram utilizados com sucesso na transformação genética. Além disso, geralmente requer um tempo de cultura muito longo, e desenvolve alta frequência de embriões anormais. Estudos surgem na tentativa de entender os mecanismos moleculares subjacentes a ES do algodão e identificar genes que em resposta a auxinas atuam nesse processo promovendo a aquisição de competência embriogênica (Zeng et al. 2006; Yang et al. 2012; Xu et al. 2013).

Nesse aspecto, compreender os fatores chave que iniciam a ES é crucial para reverter problemas ligados à recalcitrância. A identificação dos principais genes envolvidos na adaptação e no desenvolvimento *in vitro* é fundamental para o avanço de

estratégias a fim de aprimorar a morfogênese em espécies recalcitrantes. Vários estudos teem sido voltados para identificação e descrição das funções de genes envolvidos no processo de aquisição da competência celular (Kikuchi et al., 2013; Zhu et al., 2014; Zheng e Perry, 2014).

Dentre estes genes estão o *BBM* (*Baby boom*) que codifica um fator de transcrição da família AP2/ ERF (APETALA2/ ethylene response fator) (Karami et al., 2009) e é capaz de ativar vias de transdução de sinal que conduzem à indução do desenvolvimento de embriões a partir de células somáticas diferenciadas, podendo ser um importante regulador do desenvolvimento embrionário em plantas (Boutilier et al., 2002). O *LEC* (*Leafy Cotyledon*) e genes relacionados a ele como, *LEC1*, *LEC2* e *FUSCA3* foram identificados como reguladores necessários para o desenvolvimento embrionário normal e desempenham um papel central no controle de muitos aspectos da ES (Kwong et al., 2003).

Os genes *AGL15* (*AGAMOUS-LIKE15*) e *WUS* (*Wuschel*) que são expressos em resposta ao tratamento com auxina e promovem a transição vegetativa para embrionária de diferentes órgãos de plantas de *Arabidopsis* (Zuo et al., 2002; Zeng et al., 2006). E o *PKL* (*PICKLE*), mencionado como importante regulador durante as fases da embriogênese somática (Karami et al., 2009).

Dentre os genes envolvidos na transdução de sinais que regulam a ES um dos mais estudados é o *SERK*, por sua expressão transiente nas fases iniciais tanto da embriogênese somática quanto zigótica. Este gene foi identificado pela primeira vez ao ser isolado de *Daucus carrota* (*DcSERK*) (Schmidt et al., 1997). Desde então, inúmeros homólogos tem sido caracterizados em diferentes monocotiledôneas e dicotiledôneas, incluindo *Gossypium hirsutum* (Shi et al., 2012), *Arabidopsis thaliana* (Hecht et al., 2001), *Medicago truncatula* (Nolan et al. 2003), *Zea mays* (Baudino et al., 2001), *Citrus unshiu* (Shimada et al., 2005), *Ananas comosus* (Ma et al., 2014), *Momordica charantia* L. (Talapatra et al., 2014), *Oryza sativa* (Ito et al., 2005).

O produto do gene *SERK* é uma proteína quinase transmembrana que desempenham papéis importantes nas vias de sinalização das plantas. A proteína prevista contém um domínio extracelular com repetições ricas em leucina (Leucina N-terminal seguido por cinco LRRs – Leucin Rich Repeat), um domínio rico em serina e prolina (SPP), um

domínio transmembrana (TM) e um domínio serina/ treonina quinase intracelular (STK). Estas proteínas pertencem à superfamília de receptores do tipo quinase (RLK – Receptor Like Kinases) (Saze et al., 2003; Pérez-Nunez et al., 2009).

O domínio SPP é uma característica intrínseca dos genes da família *SERK* de receptores quinases (Saze et al., 2003). As LRR quinases transmitem seu sinal formando homodímeros ou heterodímeros com outros RLKs. Esta dimerização induzida por um ligante provoca a fosforilação dos domínios quinases intracelular, ativando as fases seguintes da via de transdução de sinal, esse mecanismo pode estar ligado à aquisição de competência ES (Santos e Aragão, 2009).

Variantes do gene *SERK* foram mencionados como componentes da via de sinalização de brassinosteróides, tais como *Brassinosteroid-Insensitive1* (*BRI1*) e seu co-receptor *BRI1-Associated Receptor Kinase 1 (BAK1)/ SERK.* Brassinosteróide é uma classe essencial de reguladores de crescimento de plantas envolvidos na transdução de sinal durante diferentes eventos relacionados à resistência da planta à estresses bióticos e abióticos e, consequentemente quando ativada a sua via de sinalização auxilia na aquisição de competência à ES. (Karami et al., 2008).

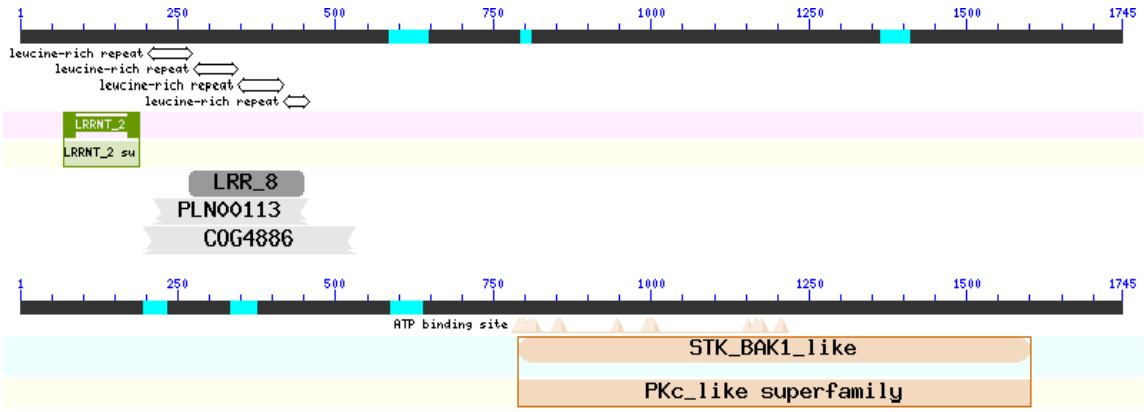

Figura 6. Domínios conservados em estrutura protéica de *SERK1* em *G. hirsutum*.

A alta expressão de *SERK* na superfície celular está relacionada à elicitação da auxina, um conhecido indutor da embriogênese somática que desencadeia a ativação de outros sinais moleculares que se ligam aos receptores da superfície celular. Quando isso

ocorre, o domínio extracelular de *SERK* mediado pela região LRR induz a cascata de sinalização no interior da célula, que além de estar relacionada a processos-chave no crescimento da planta, esta sinalização através de diferentes etapas, altera o padrão de expressão de genes indutores da ES como *LEA*, *LEC* e *BBM*. Estudos remetem que a resposta mediada por variações no padrão de expressão gênica de *SERK* pode ser a chave para a transição da célula vegetal somática para o estádio embrionário (Albrecht et al., 2008; Sharma et al., 2008; Pandey e Chaudhary, 2014).

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CAPÍTULO II:

Molecular basis of cotton recalcitrance – Review

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Molecular basis of cotton recalcitrance – Review

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Abstract

The current tools of modern biotechnology have provided excellent resources to aid in the general procedures of genetic improvement of plants. The isolation and transference of genes and the subsequent steps of plant regeneration have allowed spectacular feats in the biotechnology area, with the insertion of specific traits, eliminating the incorporation of undesirable genes, which often occurs through conventional breeding. Despite the advances, some barriers are still found in the processes of plant regeneration, especially recalcitrance of some species to somatic embryogenesis, whose issue involves a series of cascades of biochemical and molecular events. According to the literature, several biotic and abiotic factors may be related to the limitation of regenerative competence, acting positively or negatively on the activation of genes, regardless of the route of regeneration (organogenesis or somatic embryogenesis). In this revision we will make an approach about some factors related to plant regeneration, focusing on recalcitrance to somatic embryogenesis, especially in cotton. The processes related to the physiological, biochemical and molecular pathways will be reported here, based on current information available in the literature.

Keywords *Gossypium hirsutum*; regeneration *in vitro*; somatic embryogenesis; gene regulation.

Abbreviations

AGL15 - AGAMOUS-LIKE15

AGPs - Arabinogalactan proteins

BAK1- BRI1-ASSOCIATED RECEPTOR KINASE 1

BBM -Baby boom

BRI1- BRASSINOSTEROID-INSENSITIVE 1

CLV - CLAVATA

LEC - Leafy Cotyledon

LTP- Lipid transfer protein

PKL –PICKLE

PLA1- PHYTOCYANIN-LIKE ARABINOGALACTAN-PROTEIN1

SERF - Somatic Embryo-Related Factor

SERK - Somatic Embryogenesis Receptor-Like Kinase

STK-BAK - Serine/Threonine Kinase

WIND1- WOUND-INDUCED DEDIFFERENTIATION1

WUS - Wuschel

Introduction

Recalcitrance in plants refers to inability of cells, tissues and organs to respond to *in vitro* regeneration. This physiologic phenomena takes place at all stages of a culture and concerns the time related decline and/or loss of morphogenetic competence and totipotent capacity (Benson 2000). In mono and dicotyledons, genotypic dependence is one of the main factors associated with recalcitrance, sometimes more decisive than the explant (Benson 2000; Neelakandan and Wang 2012). In dicotyledons, the distribution of meristematic regions occurs in different parts of the plant, which would make them more responsive to *in vitro* crop due to wider totipotent capacity exhibited in these regions.

Explant tissue is excised from the intact plant after dissecting that involves wounding, and is incubated under aseptic, artificial conditions with an exogenous nutrient source provided by the media on which the tissue is cultured. In some cases, like protoplast culture, chemical/ mechanical treatment is required to eliminate plant cell walls thereby contributing to additional stress. Other physical factors such as the reduction–oxidation (redox) environment, temperature, light quality, photoperiod and presence of especific hormones, may to influence the ability of the tissue to adjust to these conditions and initiate developmental transitions for survival (Gaj 2004).

Oxidative stress have an important role in recalcitrance, because free radical formation is a generic stress response in all aerobic organisms. The metabolic disturbances induced through crop tissues promote the formation of free radicals and secondary products, weakening the protection system of cells against free radical damage, depending on the period of exposition. The duration of disturbance leads to molecular dysfunction, and a progressive and time related decline in culture competence ensues, which eventually leads to a loss in totipotency (Benson 2000).

According to Pandey and Chaudhary (2014), the somatic embryogenesis (SE) is directly influenced by the activity of antioxidant molecules in tissues of embryonic callus, and some metabolites, such as glutathione, are used as a marker of oxidative stress in plants. The monitoring of the activity of such metabolites during *in vitro* crop may provide evidence on transition through somatic to embryonic cells. Pandey and Chaudhary (2014) studied the dynamics of cellular redox during SE in cotton using isogenic two lines of cv. Coker 310 (fully regenerating and non-regenerating), using Glutathione Detection Kit (Clontech Inc.). The activity antioxidant of glutathione was analyzed in different developmental stages including cotyledonary leaf, calli from different stages of regeneration of both lines. The authors found that the activity of glutathione was substantially up regulated in embryogenic calli of fully regenerating line, indicating the potential of glutathione as early biomarkers for somatic cell-to-embryo transition in cotton.

The conditions of the culture media produce expressive differences in regeneration procedures, especially the growth regulators, such auxin and cytokinin, because trigger important metabolic pathways and regulate gene activity during induction

of *in vitro* morphogenesis. In organogenesis, auxins and cytokinins are used in combination, whereas in SE, auxins act as major inducers (Obembe et al. 2011).

After acquiring embryogenic competence, the cells continue to proliferate and form proembryogenic masses. Auxin is required for proliferation of proembryogenic masses but is inhibitory for the development of somatic embryos, which justifies the absence of growth regulators and the insertion of nitrogen compounds in the dedifferentiation media (Filonova et al. 2000). In pumpkin (*Cucurbita pepo* L.), Mihaljević et al. (2011), reported that cell proliferation increased and differentiation was inhibited when ammonium supply (1.0 mM) was reduced, which was reversed by replenishing glutamine (10.0 mM), because the supply of nitrogen improved the response to oxidative stress. Thus, the balance of nitrogen may be critical for SE of crops.

In cotton, Wang et al. (2006) optimized a protocol for SE and regeneration of two recalcitrant genotypes, cv. CCRI521 and Zhongzhi86-6, and found high rate of calli and embryogenic tissues in MSB media (Murashige and Skoog (MS) medium and B5 vitamins), supplemented with 0.1 $mg.L^{-1}$ of Kinetin (KT) and 0.01 $mg.L^{-1}$ of auxin (2,4-dichlorophenoxyacetic acid - 2,4-D). The transition from embryogenic calli to somatic embryos was successfully obtained in MSB supplemented with 0.5 $g.L^{-1}$ of glutamine and 0.5 $g.L^{-1}$ of asparagine. The rate of regeneration was about 19% to both genotypes. In other work, Han et al. (2009) developed a protocol that allowed consistent SE and plant regeneration from five recalcitrant cotton cultivars. The authors found that MSB media supplemented with indole-3-butyric acid (IBA) (0.3 $mg.L^{-1}$), KT (0.05 $mg.L^{-1}$), NH_4NO_3 (825 $mg.L^{-1}$), KNO_3 (3800 $mg.L^{-1}$), glutamine (2.0 g/L) and asparagine (0.5 g/L) provided 18% a 30% of somatic embryos and regenerated plants were successfully induced (18.5 at 30%).

Auxin has been reported to be involved with regulation of cell-signaling and antioxidant molecules during *in vitro* morphogenesis, such as Glutathione Reductase (GR), Glutathione S-Transferase (GST), Superoxide Dismutase (SOD), Catalase (CAT) and Peroxidases (POXs), which protect cells against oxidative damage caused by production and accumulation of Reactive Oxygen Species (ROS). Increased ROS accumulation results in oxidative stress and phytotoxicity, that leads to self-perpetuating

peroxidation of lipids, damage nucleic acids and proteins and ultimately cause cellular dysfunction and cell death (Papadakis and Angelakis 2002).

ROS are involved in signaling pathways leading to alternations in ion fluxes, activation of kinases and changes in gene expression, can interact with other signal molecules, including phytohormones indispensable to cellular competence. ROS also directly activate mitogen-activated protein kinases, which is, among other factors, linked to the control of root hair development (Pitzschke et al. 2006). In cotton, Cheng et al. (2016) identified several unigenes differentially expressed, from a RNA library of non-embryogenic calli, embryogenic calli and somatic embryos. Those genes were involved in processes such as, hormone homeostasis, stress and ROS responses, and metabolism of polyamines. In order to confirm the involvement of some genes associated to SE pathways, authors performed experiments using hormones (IBA, KT), polyamines, H_2O_2 and stresses on SE. They found that exogenous application of IBA and KT positively regulated the development of embryogenic calli and somatic embryos, and that polyamines and H_2O_2 promoted the conversion of embryogenic calli into somatic embryos. Additionally, genes encoding enzymes of the protective system, Calcium-dependent Protein Kinase (CDPK), Heat Shock Protein 90 (HSP90) and Glycosyltransferase (GST), including CAT, SOD, POX and Ascorbate peroxidase (APX), were upregulated in embryogenic calli and somatic embryos.

Under oxidative stress, the elevation of auxin in the cell can alter the transcription of signaling genes responsible for the onset of SE-cascade events, such *SERF* and *SERK*. The high expression of these genes is reported as essential for SE since it determines the ability of cells to withstand the physiological disorders caused by environmental stress (Pandey and Chaudary 2014). Free radicals lead to changes in development, generating a negative effect on cellular morphogenesis, influencing on recalcitrance of tissues grown in vitro (Papadakis and Angelakis 2002).

Auxin is also involved in different biochemical Indole-3-acetic acid (IAA)-pathways, such as *Tryptophan biosynthesis 1 (TRP1)*, *Anthranilate Synthase 1 (ASB1)*, *Tryptophan Synthase β subunit 2 (TSB2)*, *Nitrilase 4 (NIT4A)*, Chorismatemutase (CM1), Cytochrome P450, Family 79, Subfamily C, Polypeptide 1 (CYP79C1), *YUCCA* (*YUC)* and Flavin-containing Monooxygenase (FMO), among others. Yang et al. (2012)

performed a broad survey of transcriptome during cotton SE, through differentiation and dedifferentiation, during which the cells were stimulated by exogenous levels of auxins in culture media. A total of 5,076 differentially expressed genes was found, with significant transcriptional complexity during SE process, associated with morphological, histological changes and endogenous indole-3-acetic acid (IAA) alteration. The complex pathways of auxin abundance genes revealed that the auxin-related transcripts belonged to IAA biosynthesis, IBA metabolism, IAA conjugate metabolism, auxin transport, Aux/IAA, ARF, small auxin-up RNA, Aux/IAA degradation, and other auxin-related proteins, which allow an intricate system of auxin utilization to achieve multiple purposes in SE. According to authors, *TRP1* and *ASB1* transcripts were up-regulated throughout SE, while *NIT4A* was down-regulated. *NIT4A* and *CM1* were restricted to dedifferentiating cells, while and *CYP79C1* and *YUC* were restricted to embryogenic tissues; *AUX1* and *IAA/AUX* were down-regulated during dedifferentiation and up-regulated during somatic embryo development.

The cytokinins have also a fundamental role in the differentiation and regeneration of plants, since they are involved in induction of cell division, proliferation and morphogenesis of vegetative tissues, cell proliferation in calluses and, still can act to inhibit the formation of roots in seedlings (Gaspar et al. 1996). In the *in vitro* procedures, the most used synthetic cytokinins are KT, 6-Benzylaminopurine (BAP), Zeatin (ZT) and Thidiazuron (TDZ). Many aspects of cell growth of the tissue and organ cultures, such as cell differentiation, organogenesis and SE, are controlled by cytokinin and auxin interaction.

The concentration of cytokinin plays an important role in the induction of multiple shoots in several plant explants. In cotton, Pathi and Tuteja (2013) developed a fast protocol for recalcitrant genotype using embryonic apices containing only the meristematic region. The authors obtained 16 shoots/explant in media supplemented with 2.0 mg/ L of BAP and 2.0 mg/ L of KT, whose value had not been achieved with this same genotype in previous protocols. In other work, Khan et al. (2010) tested the ability of two cotton genotypes to regeneration via SE, using a recalcitrant (Narasimha) and non-recalcitrant (Coker 310) germplasm. The authors found embryogenic calli in recalcitrant genotype using both hypocotyls and cotyledonary leaves, grown in MS media containing

high concentration of KT (1.0 $mg.L^{-1}$) with low levels of 2,4-D (0.1 $mg.L^{-1}$). In Coker 310 assays, calli were obtained from 0.5 $mg.L^{-1}$ KT and 0.1 $mg.L^{-1}$ 2,4-D.

Citology of *Gossypium*

Cotton plant is a dicotyledon of the Malvaceae family. The genus *Gossypium* comprises 50 species with two ploidy levels: diploid (2n = 2x = 26), whose species came from America, Africa, Asia and Australia continents, and are cytogenetically differentiated into eight genomic groups (A, B, C, D, E, F, G and K), and alotetraploids (2n = 4x = 52), whose species came from Americas (Fryxell 1992).

The diversity centers of *Gossypium* include southern Mexico (genome D), Australia (genomes C, G and K), and northeast Africa and Asia (A, B, E and F) (Cronn et al. 2002). Based on the most number of associated diploid genomic groups, Saunders (1961) proposed that Mid Africa is the origin center of the genus. The alotetraploid species were originated from interspecific hybridization between ancestors of African-genome A and American-genome D species (Wendel and Cronn 2003).

Commercially only four species are cultivated at worldwide, two alotetraploids (*G. hirsutum* and *G. barbadense*) and two diploids (Freire 2000). In Brazil, three species are found, all alotetraploids: *G. hirsutum*, *G. barbadenese* and *G. mustelinum*. The first is classified in two varieties, G. *hirsutum* var. latifolium Hutch (Upland type), and *G. hirsutum* var. Marie-Galante (Watt) Hutch (annual type, also known as Mocó type) (Freire 2000). The variety latifolium is responsible for up to 90% of the all world cotton production and responsible by majority of cultivars available in seed marker.

Several breeding programs at worldwide have made use of various molecular and cytological tools in order to assist in the development of new cultivars or to recovery non-viable germplasm. The most crucial aspect in plant propagation is the maintenance of genetic integrity of the donor plant, in the new lines. This is because in vitro procedures can induce to variability due to somaclonal variations (Larkin and Scowcroft 1981; Soares et al. 2017). Somaclonal variation, although is not a frequent event, is manifested as cytological abnormalities that takes place in cells, leading to phenotypic mutation, activation and silencing of gene, among others events. In cotton, Jin et al. (2008) found somaclonal variation through modifications on MSB media to SE induction. Twenty-eight embryogenic cell lines via SE and 67 regenerated plants from these embryogenic calli were selected and used to RAPD, SSR, chromosomal number counting, and flow

cytometric analysis in order to find somaclonal variations. The authors found more somaclonal variation in embryogenic calli and regenerated plantlets obtained from cell lines grown in 2,4-D (0.1 mg/L) and KT (0.1 mg/L) than in IBA (1.0 mg/L) and KT (0.5 mg/L) combinations. The chromosome counting and flow cytometry analysis revealed that the number of chromosomes and ploidy levels were nearly stable in all regenerated plants except to two regenerated plantlets, that lost 4 and 5 chromosomes, respectively.

Although 2,4-D and KT are widely used in crop tissue procedures of several species, the effects of 2,4-D conducive to genome changes has been reported. Li et al. (1989) used 2,4-D (0.1 mg/L) and KT (0.1 mg/L) during calli induction of two Coker lines, and found that only 1 of 80 cells from Coker 312 and 7 of 109 cells from Coker 315 had normal number of chromosomes.

Physiological and molecular basis of recalcitrance in cotton

Since 1996, cotton biotech has evolved at worldwide, mainly with the commercial production of genetically modified (GM) cultivars, obtained with support of genetic engineering tolls that play a prominent role in improvement programs of several important commodities. Cotton biotechnology hinges on two tightly inter laced processes – genetic transformation and regeneration (Mishra et al. 2003). Despite to the broad contribution that the biotechnological processes have generated to cotton agribusiness, the efficiency and genotype-dependence of regeneration are the two most limiting factors in the development of GM cotton, due to high level of recalcitrance of genotypes.

In *G. hirsutum,* the cultivars derived from Coker have been widely exploited in studies involving SE (Zhang et al. 2000; Khan et al. 2010; Vinodhana et al. 2013; Kumar et al. 2013), however with others genotypes the difficulties to SE have been reported. Some few cultivars have been reported as able to SE, such as Acala (Mishra et al. 2003), Simian-3 (Wu et al. 2008) and ZhongMian-35 (Zhao et al. 2006; Miao et al. 2010). Differentiated procedures were adopted in order to promote improvement to SE. Mishra et al. (2003) achieved regeneration potential in commercial cultivars derived from Acala, by using selection procedures of calli grown in induction media, containing MS supplemented with a unique combination of two synthetic auxins. All cultivars tested (Maxxa, Ultima and Riata) produced a high quality, friable calli, with regeneration rate of 17%, 44% and 80%, respectively. As to authors, genotypic

differences between cultivars for regeneration were reflected by supplemental kinetin. The selection pressure through two cycles of selection resulted in development of advanced highly regenerable lines, suitable for breeding and biotechnology applications.

Although the most reports on SE in cotton has been found with *G. hirsutum*, successful procedures involving others species have been reported in *G. arboreum (*Sakhanokho et al. 2004) and some wild diploid cotton species (*G. davidsonii, G. klotzschianum, G. raimondii,* and *G. stocksii*) (Sun et al. 2006). In *G. arboreum*, Sakhanokho et al. (2004) obtained embryogenic calli submitting them to 3 days of desiccation. According to the authors, 49% of the embryogenic calli were converted into plantlets. I*n G. barbadense*, Sakhanokho et al. (2001) obtained approximately 92 somatic embryos/g of embryogenic calli and 27% of regenerated plants using an adjustment in initiation, selection and proliferation protocols. With *G. klotzschianum* Anderss, Sun et al. (2003) obtained 9% of normal plantlets using a protocol containing a balance with 2,4-D, KT and IBA in MSB media.

It is known that the media components have a deep contribution in the response to SE, but possibly intrinsic physiological factors in some cultivars also contribute to the regenerative capacity. This aspect has been little explored in the articles, whose focus has been deeply exploited on media composition, especially on balance of phytoregulators. In works involving transformation practices, the understanding of the intrinsic factors must be well established since the production of cotton transgenes is relatively time consuming. Since onset of blooming in regenerated plants varies considerably, seed production to produce genetically stable transgenic lines requires an additional 6–8 months, meaning that each transgenic plant may take 2 years or more to develop (Mishra et al. 2003). This is the reason that some transformation procedures that require SE are so long and the lack of information on regenerative ability of the genotype may contribute to further delays in obtaining of transformants. Soares et al. (2018) validated a probe, obtained from *GhSERK1*, that identify previously contrasting genotypes with different capacity to SE, including cv. Coker 312 that was used as no recalcitrant- control. Authors used *in vitro* procedures and molecular assays (qRT-PCR and RT-PCR blotting) in order to confirm the results. They found that recalcitrant genotypes expressed low expression

of *GhSERK1* and recommended this probe as a reliable marker to estimate SE in cotton plants.

Gene regulation of cell competence

Plant regeneration via *in vitro* procedures involves a reprogramming of gene expression in explant cells, through a signaling cascade, which turns on and off the expression of specific genes, independent of the morphogenic route of organogenesis or SE processes (Fehér et al. 2008; Karami et al. 2009). The process of SE is generally divided into induction and expression phases. During induction, undifferentiated somatic cells acquire the capacity for embryogenesis and proliferate into embryogenic cells. Latter, during expression phase, the embryogenic cells display their capacity for embryogenesis and differentiate into somatic embryos (Jimenez and Bangerth 2001). These processes require a signal that induces a somatic cell to dedifferentiate and gain embryogenic competence as well as the expression of an appropriate cellular environment for the response of the inductive signal (Braybrook and Harada 2008).

Jin et al. (2014) used molecular tolls in order to find cellular differences between SE and Zygotic embryogenesis (ZE) using globular, torpedo and cotyledonary-stages in cotton embryos. A total of 4242 *differentially* expressed genes (DEGs) were identified in at least one developmental stage. Expression pattern and functional classification analysis based on these DEGs reveals that SE development exhibits a transcriptional activation of stress responses. In stress assays induced by NaCl and ABA, SE development was accelerated and the transcription of genes related to stress response was increased, in parallel with decelerated proliferation of embryogenic calluses under stress treatment. As to authors, SE development involves the activation of stress responses, which may regulate the balance between cell proliferation and differentiation.

Most of the processes associated with recalcitrance are related to the activation of genes that act during *in vitro* and *in vivo* morphogenesis, whose are involved in transition path of explant to the embryogenic or organogenic state (Kikuchi et al. 2013; Zhu et al. 2014; Zheng and Perry 2014). A large number of genes related to the acquirement of embryogenic competence have been characterized. A brief description of the most reported in the literature will be made below, focusing in cotton.

SERK

It was firstly characterized as a marker gene which transits cells from somatic to the embryogenic state in carrot (*Daucus carota*) (Schmidt et al. 1997). Homologous genes have also been reported from *G. hirsutum* (Guo et al. 2010; Guo et al. 2011; Zhang et al. 2011); *A. thaliana (*Hecht et al. 2001), *Cocos nucifera* (Perez-Nunez et al. 2009), *Citrus unshiu* (Shimada et al. 2005), *Dactylis glomerata* (Somleva et al. 2000), *Helianthus annus* (Thomas et al. 2004), *Medicago truncatula* (Nolan et al. 2003), *Oryza sativa* (Hu et al. 2005), *Solanum tuberosum* (Sharma et al. 2008), *Theobroma cacao* (Santos et al. 2005), *Triticum aestivum* (Singla et al. 2008), *Vitis vinifera* (Maillot et al. 2009) and *Glycine max* (Yang et al. 2011), suggesting the ubiquity of a small family of *SERK* in all species of plants, in addition to the functional conservation of a specific role in embryogenesis (Solís-Ramos et al. 2012).

The *SERK* gene encodes a membrane-located leucine-rich repeat receptor-like protein kinase (LRR-RLKs) that play important roles in plant signaling pathways. The LRR-RKL complex is formed from a signal that LRR kinases transmit, performing homodimers or heterodimers with other RLKs. This association is induced by a ligand and causes phosphorylation of the intracellular kinase domains, activating the subsequent phases of the signal transduction pathway (Santos and Aragão 2009).

The predicted protein contains an N-terminal Leucine zipper domain followed by five LRRs, a serine and proline rich SPP domain, a transmembrane domain, and an intracellular serine/threonine kinase domain. The SPP domain is a unique feature of the *SERK* family of receptor kinases (Hecht et al. 2001).

The homologous of *SERKL1* were shown in protein complexes that include components of the brassinosteroid signaling pathway such as BRI1 and its co-receptor BAK1/SERK3. Receptors such as BRI1 and SERK1 are synthesized in the endoplasmic reticulum, from where they pass through the Golgi network to be inserted into the plasma membrane. BAK1 functions in various signaling pathways, such as modulates pathways involved in plant resistance to pathogen infection (pattern-triggered immunity, PTI) and herbivore attack. The STK_BAK1-like is part of a larger superfamily that includes the catalytic domains of other STK proteins (Karami et al. 2008).

SERK1 is involved in brassinosteroid signaling as well as in the acquisition of embryogenic capacity somatic and zygotic (Karami et al. 2008). In *Arabidopsis*, overexpression of *SERK1* (*AtSERK1*) was related to enhanced embryogenic competence (Hecht et al. 2001). *AtSERK1* was highly expressed during embryogenic cell formation and during early embryogenesis. The path of expression involved the megasporogenesis in the nucleus of developing ovules, the functional megaspore, and all cells of the embryo sac. Further, high expression was also found during developing embryo until the heart stage, although low expression was found in adult vascular tissue. Seedlings that overexpressed *AtSERK1* exhibited a 3-to4-fold increase for initiation of SE.

In *M. truncatula, MtSERK1* expression was related to both SE and organogenesis and, possibly, other forms of cellular reprogramming (Nolan et al. 2003), while in *Araucaria,* Steiner et al. (2012) reported that *AaSERK1* is preferentially expressed in embryogenic cell cultures, which transcripts initially accumulate in groups of cells located at the periphery of embryogenic calli and then are restricted to the embryo development.

AGPs

AGPs are a class of hyperglycosylated macromolecules in the Hydroxyproline-rich glycoprotein (HRGP) superfamily and widely distributed from simpler organisms to higher plants. They belong to a heterogeneous group of proteoglycans, involved in differentiation of tissues. The AGPs are present in cell membrane and wall, in intercellular spaces of tissues and are secreted into cell culture media (Fincher et al. 1983). Findings in literature have reported that AGPs are involved in the growth and development processes, such as cell development and differentiation, root and seed regeneration, gametogenesis, somatic embryogenesis, hormone signaling, apoptosis, among others (Van Hengel et al. 2001; Park et al. 2003; Qin and Zhao 2006; Liu et al. 2008; Wiszniewska and Piwowarczyk 2014). Cells with embryogenic capacity can secrete more AGPs (Poon et al. 2012).

In protoplast culture, the activity of the AGPs is essential to the formation of cell wall and may be directly related to the recalcitrance during cropping. This is because cell wall renewal is a fundamental step in reprogramming and dedifferentiation of protoplasts,

which are stimulated by AGPs. Wiszniewska and Piwowarczyk (2014) reported that AGPs play a role in the proper binding of components in the new wall and could be markers of cells entering the apoptosis pathway. According to Lamport et al. (2006), AGPs act as cell wall plasticizers, acting on the pectic network to increase wall porosity and enhance cell expansion/extension.

In legumes, Wiszniewska and Piwowarczyk (2014) studied the cell wall regeneration in protoplast culture, based on occurrence of cellulose, callose and AGPs during 15 days of culture. Protoplasts were cultured in different media to test the effect of culture environment on the cell wall regeneration. In chitosan (2.0 mg/l) enriched media AGPs were localized in cell walls of different types of cells: dividing, elongating, but predominantly in degenerating ones.

In cotton, AGPs are involved in development of fibers, and as contributors to plant stem strength, fiber development (Li et al. 2010). Poon et al. (2012) reported that cotton calli undergoing SE secrete an AGP fraction that promotes SE when incorporated back into the growth medium. High levels of total AGPs were found in embryogenic calli compared with non-embyrogenic calli. According to authors, when these AGPs were partly or fully deglycosylated, SE promoting activity was not diminished.

WUS

WUS belong to a class of the plant-specific WUS homeobox-containing *(WOX)* protein family. The WOX family members fulfill specialized functions in key developmental processes in plants, such as embryonic patterning, stem-cell maintenance and organ formation. These functions may be related to either promotion of cell division activity and/or prevention of premature cell differentiation (Van der Graaff et al. 2009). *WUS* encodes to a protein that is a bifunctional transcription factor, which plays a vital role in the maintenance of stem cell populations in shoot apical and floral meristems (Laux et al. 1996; Zuo et al. 2002; Ikeda et al. 2009). The protein contains three functional domains at the conserved C-terminal region, which have a significant role in the dual biological functions of *WUS* and is remarkably essential for the maintenance of stem cell identity (Ikeda et al. 2009).

WUS was firstly described in *Arabidopsis* by Endrizzi et al. (1996), using gene mutations, whose found defective and premature development of meristematic shoots. As

to authors *WUS* is necessary for the proper functioning of meristematic cells and their transition to differentiated state.

In shoot apical meristems, the expression of *WUS* is limited to a few cells of the organizing center, which is regulated by *CLV* signaling pathway (Mayer et al. 1998). As both SE and shoot regeneration require the establishment of shoot apical meristems, *WUS* is essential for SE and shoot regeneration (Xiao et al. 2018). Gallois et al. (2002) reported that *WUS* interacts with Shoot meristemless (STM) by activating a subset of meristem functions, including cell division, *CL*V expression, and finally organogenesis. According to the authors, *WUS* produces an autonomous signal that activates cell division in combination with STM and that WUS/STM functions can initiate the transition from totipotent cells to organ initiation.

The ability of *WUS* to stimulate organogenesis and/or SE appears to be dependent on the cellular context (Xu et al. 2005) or on the exogenous hormonal regime (Gallois et al. 2004). Leibfried et al. (2005) found a connection between *WUS* and cytokinins in the regulation of stem cells in the shoot apical meristems. In *Arabidopsis*, the function of the proper apical meristem requires the interaction between *WUS* and *Arabidopsis response regulators (ARR)* genes, which act in the negative feedback loop of cytokinin signaling, with *WUS* repressing the transcription of several *ARRs* and facilitating high cytokinin activity (Shani et al. 2006). *WUS* also function as an activator when involved in the regulation of the *AGAMOUS* gene (Ikeda et al. 2009). The ectopic expression of *WUS* increases the size of shoot meristems and induces ectopic accumulation of stem cells, with resultant formation of adventitious shoots and somatic embryos in root tissues (Zuo et al. 2002). According to Karami et al. (2009), *WUS* can reprogram cell fate, bypassing the auxin requirement, or simply taking advantage of the endogenous auxin flux. In *Arabidopsis*, *WUS* promoted the vegetative to embryonic transition of different plant organs (Zuo et al. 2002; Zeng et al. 2006).

In *G. hirsutum*, Xiao et al. (2018) studied the effect of *GhWUS* on shoot regeneration and SE, based on two homologues isolated from *AtWUS*, and found that *GhWUS* is an important regulator in both processes. Ectopic expression of *GhWUS* in *Arabidopsis* induced somatic embryo and shoot formation from seedling root tips. According to authors, in the absence of exogenous hormone, overexpression of *GhWUSs* in *Arabidopsis* promoted shoot regeneration from excised roots, and in the

presence of exogenous auxin, excised roots expressing *GhWUS* was induced to produce somatic embryo. In addition, expression of the chimeric *GhWUS* repressor blocked the formation of embryogenic callus, suggesting that *GhWUS* is a key regulator during cotton SE. Bouchabké-Coussa et al. (2013) showed that overexpression of the WUS enhanced the expression of embryogenic competence and triggered organogenesis from some cells of the regenerated embryo-like structures of cotton. Somatic embryogenesis was improved in *WUS* expressed calli, as the percentage of explants giving rise to embryogenic tissues was significantly higher (×3) when *WUS* was over-expressed than in the control. The authors suggest that targeted expression of the *WUS* gene is a promising strategy to improve gene transfer in recalcitrant cotton cultivars.

BBM

BBM was originally identified as a marker for embryogenic cells in microspore-derived tissue of *Brassica napus*. *BBM* encodes an APETALA2/ethylene response factor (AP2/ERF) transcription factor involved with root, seed, basal embryo, and shoot meristem development (Boutilier et al. 2002). The AP2/ERF is a DNA binding domain of approximately 60 to 70 amino acids that regulates the response to environmental stresses and control of growth and development, in plants (Okamuro et al. 1997). While members of the AP2 subfamily regulate embryogenic and developmental processes, the members of the ERF subfamily are related to biotic and abiotic stress responses (Silva et al. 2015).

In *Arabidopsis*, Iwase et al. (2011) showed that the AP2/ERF-type transcriptional regulator (WIND1 and its homologs WIND2, WIND3 and WIND4) are induced upon wounding and promote callus formation at cut sites. The callus induced by transient overexpression of WIND1 regenerates shoots and roots when transferred to non-inducible media, indicating that WIND1 can reprogram somatic cells to confer pluripotency. The ability of *BBM* to promote organogenesis and embryogenesis in the absence of exogenously applied growth regulators suggested that *BBM* may act by stimulating the production of plant hormones and/or increasing the sensitivity of the cell to these substances (Karami et al. 2009). Ectopic expression of *BBM* in *Arabidopsis* primarily

induces spontaneous somatic embryo formation from seedlings, although ectopic shoots and callus also develop at a lower frequency (Boutilier et al. 2002).

In *Populus tomentosa* (Deng et al. 2009) and *Theobroma cacao* (Florez et al. 2015), *BBM* overexpression promoted SE. In *Gossypium*, Yang et al. (2012) characterized the molecular events of SE, by using transcriptome analysis, in combination with biochemical and histological approaches. Genome-wide profiling of gene expression allowed the identification of novel molecular markers characteristic of this developmental process. RNA-Seq was used to identify 5,076 differentially expressed genes during cotton SE, among them 466 transcription factors associated with functions in cell differentiation, embryogenic patterning and embryo maturation processes (Zinc finger, Basic Leucine Zipper Domain (b-ZIP), *basic helix-loop-helix* (bHLH), B3 and MYB), meristem maintenance or identity (NAC, YABBY (YAB) and GRAS), while others had roles in hormone-mediated signaling by auxin (Aux/IAA, ARF) or ethylene (AP2/ERF). Expression profile and functional assignments of these genes indicated significant transcriptional complexity during this process, associated with morphological, histological changes and endogenous IAA alteration.

LEC

LEC1 is a critical regulator required for normal development during the early and late phases of embryogenesis that is sufficient to induce embryonic development in vegetative cells (Kwong et al. 2003). *LEC1* is required to maintain suspensor cell fate, to specify cotyledon identity in the early morphogenesis phase, and to initiate and/or maintain the maturation phase and inhibit precocious germination late in embryogenesis (Lotan et al. 1998; Kwong et al. 2003).

LEC1, which encodes subunit B9 of a nuclear factor Y protein (NF-YB9), and the B3 domain protein LEC2 are part of a larger network of "LAFL" proteins (LEC1/LEC1-LIKE [L1L], Abscisic Acid Insensitive 3 (ABI3), FUSCA3 (FUS3) and LEC2) that regulate embryo identity and maturation (Jia et al. 2013). *LEC1* is expressed in the octant stage embryo onwards in all cells, including the suspensor, but also in the endosperm. Expression was found to be gradually restricted towards the periphery of the embryo after the transition stage, and then uniformly again in the bent cotyledon stage embryo. No

expression was reported in any other plant tissue beyond the late torpedo embryo stage (Vries 1998). As to Lotan et al. (1998), ectopic postembryonic expression of *LEC1* is sufficient to confer embryonic characteristics to seedlings and to induce somatic embryo formation from vegetative cells. It is a central regulator that acts far upstream in the regulatory hierarchy that controls embryogenesis, establishing a cellular environment that promotes embryo development.

In *Gossypium*, the articles with *LEC* are scarcely exploited. Min et al. (2015) described the regulatory network of *CKI*, which is a unique key regulatory factor that strongly affects SE. Overexpressing *GhCKI* halted the formation of embryoids and plant regeneration because of a block in the transition from non-embryogenic callus to somatic embryos. *LEC1* was identified to be targeted to a cis-element, CTTTTC, in the promoter of *GhCKI*. Moreover, *GhCKI* interacted and phosphorylated cotton CINCINNATA-like TEOSINTE BRANCHED1-CYCLOIDEA-PCF transcription factor by coordinately regulating the expression of cotton *PHYTOCHROME INTERACTING FACTOR4 (PIF4)*, finally disrupting auxin homeostasis, which led to increased cell proliferation and aborted somatic embryo formation in *GhCKI* overexpressing somatic cells. Based on the analysis of upstream and downstream regulatory networks involving *GhCKI*, the authors found that a unique gene network regulates auxin homeostasis to affect the fate of callus cells during SE.

Zhu et al. (2018) performed comparative proteomics analysis using samples of non-embryogenic callus, embryogenic callus and somatic embryo and found 5892 proteins amongst the three samples. The majority of these proteins (93.4%) have catalytic, binding, transporter or structural molecular activities. Of these proteins, 1024 and 858 were differentially expressed in non-embryogenic callus X embryogenic callus and embryogenic callus X somatic embryo, respectively. As to authors, *SERK* and *LEC* were positively regulated in embryogenic callus and somatic embryo when compared to non-embryogenic callus. These two types of proteins are multifunctional regulators in both zygotic and somatic embryos acting on hormone signaling and stress responses and can be used as totipotency markers.

AGL15

*AGL15 b*elongs to a family of transcriptional regulatory factors and was initially identified as an embryo-expressed gene in *Arabidopsis* (Heck et al. 1995). In soybean (*Glycine max*), *GmAGL15* is preferentially expressed in developing embryos, and ectopic expression enhances somatic embryo development (Thakare et al. 2008). *AGL15* encodes a MADS-domain regulatory protein and is known to play vital roles in diverse plant developmental and promotes somatic embryogenesis in many species (Parenicova et al. 2003). Constitutively expressed *AGL15* enhances production of secondary embryos from cultured zygotic embryos, and promotes somatic embryo formation (Harding et al. 2003).

In *Arabidopsis*, *AGL15* accumulates in the nuclei of cells in the embryo at early development and remains at relatively high levels throughout morphogenesis and into maturation stage (Perry et al. 1996). It is expressed after germination, in the vegetative shoot apical meristem and bases of lateral organs such as leaves and floral organs. The level of expression in these tissues is generally at least 10-fold lower than found in the embryo (Fernandez et al. 2000). Ectopic expression of *AGL15* induces embryogenic callus that expresses maturation genes and enhances apical meristem competence to undergo somatic embryogenesis in response to auxin treatment (Harding et al. 2003). Braybrook et al. (2006), state that the *LEC2* may be directly linked to the control of *AGL15* expression in response to auxin.

AGL15 is a component of the SERK1 protein complex that controls ethylene biosynthesis and regulates the proportion of gibberellin / abscisic acid. The reduction of gibberellin levels during the maturation phase is related to the acquisition of embryogenic competence (Zheng et al. 2013).

In *Gossypium*, Yang et al. (2014) isolated three genes homologous to *AGL15*, named *GhAGL15-1*, *GhAGL15-3*, and *GhAGL15-4*, all localized to the nucleus, that play a predominant role in cotton embryogenic callus formation. The putative proteins of these three genes contained a highly conserved MADS-box DNA-binding domain and a less conserved K domain. Over-expression of these genes in cotton callus improved quality and significantly increased the embryogenic callus formation rate, while *GhAGL15-4* had the highest positive effect on the embryogenic callus formation rate (an increase from 38.1 to 65.2%). As to authors, *GhAGL15-1* and *GhAGL15-3* were significantly induced by 2,4-D and kinetin, whereas *GhAGL15-4* was only responsive to 2,4-D treatment.

GST

GSTs belong to a multigene superfamily with three major subcellular localization patterns such as cytosolic, mitochondrial and microsomal. Based on their genomic organization, sequence similarity and functions, GSTs could be categorized into several distinct classes (Islam et al. 2017). In plants, tau (U) and phi (F) classes are the most represented, as to function in phytohormone physiology, especially auxin metabolism. *GST* genes are also involved in plant defense and can be induced by various exogenous factors such as pathogen attack, heavy metals, heat shock, wounding or auxins (Galland et al. 2007; Lallement et al. 2015).

In chicory, Galland et al. (2007) found.*GST*-mRNAs and proteins colocalized in small veins, particularly in young protoxylem cell walls, via situ-hybridization. As to authors, the presence of GST transcripts and proteins in reactived cells and multi-celled embryos indicated that anti-oxidation mechanisms were active during precocious phases of SE. In maize, Salvo et al. (2014) found *GST* coexpressed with *BBM*, *WUS*, and *SERK* in embryogenic tissues, but not in non-embryogenic tissues.

In *G. hirsutum*, Zhou et al. (2016) found several GST-related proteins differentially abundant throughout SE. In a study involving proteomics approach based on 2-DE combined with MALDI-TOF/TOF, authors found 149 unique differentially expressed proteins at different stages of cotton SE. The expression profile and functional annotation of these DEPs revealed that SE activated stress-related proteins, including several ROS scavenging enzymes. ROS homeostasis was important for dedifferentiation, which was maintained by the activity of ROS-related proteins, such as APX, GST, SOD, and thioredoxin. The suppression of *GhGSTL3*, identified during SE, had no significant effect on dedifferentiation, but redifferentiation was accelerated, and increased H_2O_2 levels were observed compared with wild-type tissues. According to literature, plant GSTs also act as ROS-scavenging enzymes associated with responses to stresses, hormone signaling, and developmental changes (Kampranis et al. 2000; Gong et al. 2005).

LTPs

LTPs are small peptides, each with eight highly conserved cysteine residues, which form the internal hydrophobic cavity of the three-dimensional structure, and an N-terminal hydrophobic signal peptide (Kader 1996). In plants, LTPs were discovered in 1975 and reported through ability to facilitate the transfer of phospholipids between membranes *in vitro* (Kader 1996). LTPs are mainly involved in plant defense, in adaptation to stressful environmental conditions, and in SE (Potocka et al. 2012; Sterk et al. 1991).

In cotton, Zeng et al. (2006) identified 671 differentially expressed cDNA fragments and 242 unigenes significantly up-regulated during cotton SE, from embryogenic and non-embryogenic calli, through cv. Coker 201. In embryogenic calli, many genes that encode enzymes involved in lipid metabolism and wax biosynthesis were significantly activated during early SE, such as LTP and acyltransferase. LTPs were absent in hypocotyls, non-embryogenic tissues, and plantlets but markedly activated in embryogenic calli/ preglobular embryo, through transitional proembryogenic masses, but showed a sharply minimal level of expression in all postglobular-stage somatic. The authors suggested that the increased activity of LTPs may facilitate membrane biosynthesis, cell expansion, and polar differentiation, which are likely to be limiting factors during the SE period.

PLA

PLA belong to AGP protein family due to the existence of AGP protein-like region and Plastocyanin-like domain (Li et al. 2013). Previous studies have also proposed that PLAs play a role in cell wall reorganization and organ differentiation (Yoshizaki et al. 2000). These authors isolated the *Pn14*, a gene family of PLAs, by differential screening of an apical bud cDNA library of *Pharbitis nil*. The levels of the *Pn14* transcript were significant in apical buds, roots and flower buds, but undetectable in cotyledons, stems and mature leaves. In situ hybridization analysis showed that the *Pn14* transcript is localized in meristems, procambia, trichomes of leaf primordia around shoot apices and procambia of root tips. These results suggest that members of *PLA*s is involved in proliferation and/or differentiation of cells. The cell wall plays an intrinsically important role in the proliferation of plant cells, and its reorganization must occur during plant organ

differentiation. The Pn14 protein is likely to function in the extracellular matrix and the expression of phytocyanin-related proteins can be involved in the control of cell wall reorganization and organ differentiation in plants.

In cotton, Poon et al. (2012) cloned a full-length complementary DNA (*GhPLA1) and reported that it* has a chimeric structure comprising an amino-terminal signal sequence, a phytocyanin-like domain, an AGP-like domain, and a hydrophobic carboxyl-terminal domain, encoding a protein backbone of an AGP in the active fraction with 175-aminoacid that promotes SE. *GhPLA1* shares sequence similarity to the phytocyanins, whose phytocyanin-domain is responsible for SE-promoting activity, through a signaling or messenger role. *GhPLA1* may function through an interaction between its PL domain and another protein. As *GhPLA1* is most likely located in the extracellular space, it is probably cleaved from the plasma membrane, which allows its diffusion or transport through the cell wall.

Conclusion

The advances made in the molecular area in the last two decades were essential to elucidate several biological processes in the animal and plant kingdoms. In plants, these advances have been faster due to cycle and lower legislative requirements in terms of ethics. Cotton is a great world commodity, due to expressive value of products traded in the textile, oil and derivatives markets. Plant regeneration through somatic embryogenesis provides a useful tool to accelerate cotton breeding, both via conventional procedures and gene transfer. Most biotechnological practices in cotton are widely established, but there are still some bottlenecks to be clarified, such as the genotype-dependence in accesses with limitation to *in vitro* regeneration, especially when dealing with recalcitrance to somatic embryogenesis.

Despite the advances, studies have shown that many aspects of gene regulation in nonresponsive recalcitrant genotypes have not yet been fully elucidated. The understanding of these processes involves physiological, biochemical and molecular routes, whose elucidation brings practical benefits for crop improvement, since rescue of accessions in germplasm banks, as well as progress in procedures of plant regeneration.

The topics covered in this review show the advances made, especially in molecular area. New genes are available for use as tool in genotyping selection, contributing to improve the regeneration procedures. The understanding of the molecular mechanisms involved in *in vitro* morphogenesis of genotypes with regenerative capacity has minimizing the time and costs required in *in vitro* practices. Positive results have been presented in this segment, resulting in indication of probes to identify recalcitrant accessions. These advances have allowed adjustments in protocols, resulting in better rates of plant transformation and regeneration, besides reduction of time and costs that are often expensive in these segments.

Author contribution statement

RCS, LML, CRCS, PAMF and TCS conceived and designed research. TCS and RCS wrote the manuscript. RCS, PAMF, LML and CRCS made the manuscript revisions.

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Conflict of interests

The authors declare no conflict of interest in the conduction of this study.

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CAPÍTULO III:

Validating a probe from *GhSERK1* gene for selection of cotton genotypes with somatic embryogenic capacity

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Validating a probe from *GhSERK1* gene for selection of cotton genotypes with somatic embryogenic capacity

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Abstract

Substantial progress is being reported in the techniques for plant transformation, but successful regeneration of some genotyp remains a challenging step in the attempts to transform some recalcitrant species. *GhSERK1* gene is involved on embryo formation, and its overexpression enhances the embryogenic competence. In this study we validate a short *GhSERK1* probe in order to identify embryogenic cotton genotypes using RT-qPCR and blotting assays. Cotton genotypes with contrasting somatic embryogenic capacity were tested using *in vitro* procedures. High expression of transcripts was found in embryogenic genotypes, and the results were confirmed by the RT-PCR-blotting using a non-radioactive probe. The regeneration ability was confirmed in embryogenic genotypes. We confirmed that *GhSERK1* can be used as marker for estimating the somatic embryogenesis ability of cotton plants.

Keywords: *Gossypium hirsutum* L., somatic embryogenesis, gene expression, blotting.

1. Introduction

Plant regeneration through somatic embryogenesis (SE) provides a useful tool to speed up cotton breeding via screening or gene transfer. SE is an asexual process that depends on the ability of competent cells to become somatic embryos (Williams and Maheswaran, 1986). It is a complex process involving the transition from somatic cells to embryogenic state and its development into somatic embryo (Karami et al., 2009). Such developmental switching involves differential gene expression which confers to the somatic cells the ability to activate the embryogenic potential (Chugh and Khurana, 2002).

Many intrinsic and extrinsic factors affect the efficiency of a regeneration procedure, such as genotype, origin of explants, culture medium, physiological stresses, *in vitro* manipulation, and hormonal effects (Neelakandan and Wang, 2012). Hormones are the most important factor because the endogenous and exogenous auxin levels are associated with regulation of signaling processes in competent somatic cells (Fehér et al., 2003; Yang et al., 2012; Pandey and Chaudhary, 2014).

As observed in the zygotic embryogenesis (ZE), the formation of somatic embryo involves the same phases of development: globular, heart-shaped, torpedo, and cotyledonary; however, the mechanisms that induce each phase are different between the two types of embryo formation (Middleton et al., 2007; Pandey and Chaudhary, 2014). The development of zygote embryos is prompted by fertilization, whereas SE results from somatic cells that acquire embryogenic competence when chemical and physical stimuli reprogram the gene expression (Gray et al., 1995; Karami et al., 2009; Obembe et al., 2011; Elhiti et al., 2013). Many elements were shown to enhance embryo formation from vegetative cells, immature microspores, or zygotic embryos, such as *LEAFY COTYLEDON1* (*LEC*1), BABY BOOM (BBM), *AGAMOUS-LIKE* 15 (*AGL*15), *Pickle (PKL)*, and *Somatic Embryogenesis Receptor Kinase (SERK)*, among others (Schmidt et al., 1997; Harding et al., 2003; Srinivasan et al., 2007; Alemanno et al., 2008).

SERK is reported in the literature as a marker of the transition from somatic to embryogenic cells in several crops (Schmidt et al., 1997; Hecht et al., 2001; Talapatra et al., 2014; Toorn et al., 2015). The expression of *SERK* is observed from competent cell stage up to the globular stage of somatic embryos, although it is not detectable in non-embryogenic stages of embryogenic cultures (Chugh and Khurana, 2002). Based on the

number of copies found in some species, it is generally suggested that *SERK* consists of a gene family that is preferentially expressed during the induction of SE and probably in ZE. In *Arabidopsis thaliana*, the expression of *SERK* was observed in transgenic embryogenic calli from floral explants and in zygotic embryos (Hecht et al., 2001). In maize (*Zea mays*), *SERK* is expressed in different tissues, such as embryogenic cells, late embryo stages, ovules, and leaves (Baudino et al., 2001). In *Medicago truncatula*, the expression was observed in different stages of embryo development (Nolan et al., 2003).

SERK encodes a transmembrane protein that carries leucine-rich repeat (LRR) domains exposed to the cell membrane. This protein belongs to the receptor-like kinase-LRR (RLK) family that is responsible for the interaction with other signal molecules, external signal perceptions, and signal transduction during SE induction (Schmidt et al., 1994; Hecht et al., 2001). Although the precise expression pattern varies among species, findings in the literature have demonstrated that up-regulation of specific *SERK* can be associated with the induction of totipotency in different plant species (Pilarska et al., 2016). Chugh and Khurana (2002) suggest that *SERK* may serve as a molecular marker for distinguishing competent and non-competent cells.

Cotton (*Gossypium hirsutum* L.) belongs to the Malvaceae family, and it is the most important source of natural fiber for textile industry. The importance of cotton is reflected in the estimation that 180 million people depend directly or indirectly on the production of this fiber for living (Freire et al., 2015). Cotton breeding programs are continually demanded to improve fiber yield and quality, and a list of agronomic traits such as resistance to pests and diseases, nutrient use efficiency, and earliness of production are desired. Biotechnology has delivered important contributions to cotton improvement, particularly through genetic engineering and tissue culture procedures. The successful use of biotechnological tools depends on the availability of efficient plant regeneration protocols.

Most practices used in cotton transformation are based on SE protocols that have drawbacks such as requirement of long culture periods and limited numbers of cultivars with regeneration ability (Kumria et al., 2003; Obembe et al., 2011). According to Bairu et al. (2011), the long culture duration leads to potential problems with somaclonal variation, low rates of plant regeneration, and high frequency of plant infertility.

Regeneration of cotton plants is highly genotype-dependent despite the numerous reports of protocols found in the literature, and it is known that several commercial cotton

cultivars are recalcitrant to regeneration. Most transgenic cotton released to the market was obtained using Coker lines (Coker 310, Coker 201, and Coker 315) because this genotype is amenable to *in vitro* regeneration (Davidonis and Hamilton, 1983; Sakhanokho et al., 2001; Leelavathi et al., 2004; Sidorov, 2013). As few Coker lines have been used to generate most of the GM cotton cultivars offered to farmers, the genetic variability in cotton being cultivated worldwide has been potentially narrowed.

Procedures for identification of cotton with somatic embryogenic capacity using tissue culture are quite expensive and time-consuming because each genotype responds differently to the hormone formulation (Davidonis and Hamilton, 1983; Fehér et al., 2003; Yang et al., 2012). Considering that the overexpression of *SERK* is associated with embryogenic competence in many crops (Hecht et al., 2001; Baudino et al., 2001; Maillot et al., 2009), we hypothesize that a *GhSERK1* probe is a reliable marker for identification of embryogenic cotton genotypes. This study was made to test the hypothesis on cotton genotypes contrasting in their somatic embryogenic capacity.

2. Material and methods

2.1. Design of homologous primers from GhSERK1 sequences

Complete sequences (mRNA) of *SERK* from cotton and five crop species were used for primer design based on regions of high homology. The sequences were obtained from NCBI-GenBank (http://www.ncbi.nlm.nih.gov) and used in alignment analysis (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The sequences used were from *Theobroma cacao* (GenBank accession no. **XM_007042963.1**), *Citrus sinensis* (GenBank accession no. **XM_006493226.1**), *Vitis vinifera* (GenBank accession no. **XM_002270811.2**), *Glycine max* (GenBank accession no. **NM_001250980.1**), *Phaseolus vulgaris* (GenBank accession no. **XM_007148370.1**), and *G. hirsutum* (GenBank accession no. **KM_384767.1**). The primers were designed using the software Primer3 (http://frodo.wi.mit.edu/primer3) based on amplicon size (186 bp), absence of dimers, and percentage of GC: 45-50%. A combination was defined with high homology (F: 5' GCATGATCATTGTAACCCCAAG 3' and R: 5' GGTATTCAGGGGCTATATGACC 3') and further used in molecular assays.

2.2. Prospection of GhSERK1 in discriminant cotton genotypes

Six cotton genotypes contrasting in their embryogenic capacity were assayed in order to investigate the expression of *GhSERK1* by the RT-qPCR method. Based on the results published by Soares (2013), the cvs. BRS Rubi and BRS Seridó have embryogenic capacity while BRS 201, CNPA Precoce 1, and BRS Topázio, are not responsive. The variety Coker 312 was used as an embryogenic control. The cotton plants were grown in 20-L pots, fertilized with urea (20 kg ha^{-1}), single super-phosphate (60 kg ha^{-1}), and potassium chloride (30 kg ha^{-1}), and watered daily.

Floral primordia collected at 50 days after emergence were used for RNA extraction (Invitrogen, USA). This tissue was chosen because *GhSERK1* transcripts are more abundant in reproductive organs, although it is expressed in all organs of cotton plants in different stages (Shi et al., 2012). The cDNA of each genotype was obtained using ImProm-II™ Reverse Transcription System kit (Promega, Madison, USA) and 1 µg of RNA, according to the manufacturer's instructions. RT-qPCR was carried out using Eco Real-Time PCR System (Illumina, Inc, USA) in a total volume of 12 µL containing 6 µL of qPCR-SYBR-Green mix (Ludwig, Alvorada, RS, BR), 0.3 µM of each forward and reverse primers, and 1 µL cDNA (1 µg/ µL) of each sample. Two endogenous cotton genes (*GhACT* and *GhPP2A*) were used as endogenous controls (Ártico et al., 2010). The reactions were performed as follows: previous heating and denaturation at 50°C for 2 min and 95°C for 5 min, respectively, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 1 min, and extension at 72°C for 15 sec.

The graphics, Cqs and Melt curve were automatically generated through thermocycler software of the Eco™ Real-Time PCR System (Illumina, Inc., San Diego, CA, USA) based on the normalization method with a reference gene, ΔΔCq (Livak and Schmittgen, 2001). The assays were repetitiously performed for three times. The student's t-test was used to perform the statistical analyses of the data.

2.3. Blotting assay for GhSERK1 expression

A RT-PCR assay was performed using 2 µL of cDNA (1 µg), 0.04 U of *Taq* Polimerase (Fermentas), 0.28 µM of dNTP set (10 µM), 2.5 mM of $MgCl_2$ (25 mM), 1X kit buffer (10X), and 1.0 µL (10 mM) of each Forward (5' GCATGATCATTGTAACCCCAAG 3') and Reverse (5' GGTATTCAG GGGCTATATGACC 3') primer. The RT-PCR thermal condition was pre-denaturation

at 96°C for 5 min, followed by 35 denaturation cycles at 96°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min. A final extension was added at 72°C for 7 min.

The amplicons were analyzed in agarose gel (2%), photo-documented (Bio-Imaging Systems - Mini Bis Pro, Uniscience), and transferred onto a nylon membrane (Hybond N+, Healthcare Amersham) by capillary. A highly specific probe (186 bp) was generated by PCR and labeled with DIG High Prime DNA Labeling and Detection Starter Kit I (Roche Applied Science, Switzerland) for further using in hybridization assays at 42°C, according to the manufacturer's instructions. Immunological detection was performed using CDP-Star detection solution (Roche Applied Science). The membrane was then exposed to x-ray (Kodak) for 30 min and developed for further blotting analysis.

2.4. In vitro culture of genotypes through SE

Regeneration assays were carried out in order to validate the molecular results obtained with *GhSERK1* transcripts. Hypocotyl fragments (1 cm) were placed in petri dishes containing MS medium (Murashige and Skoog, 1962), supplemented with vitamins and glucose (30 g L^{-1}), and pH 5.8 (Soares et al., 2014). The medium was solidified with phytagel (5.7 g L-1) and autoclaved for 20 min at 115°C. Finally, sterile naphthalene acetic acid (NAA) (2 mg L^{-1}) and Kinetin (KT) (1 mg L^{-1}) were added to the medium. The experimental plot consisted of a petri dish containing nine hypocotyledonary explants.

The cultures were stored in a growth chamber for 30 days, at 28 ± 2°C, photoperiod 16:8, and light intensity of 40 μmol $m^{-2}s^{-1}$. The calli were transferred to proliferation medium using MS + vitamins + glucose + NAA (0.5 mg L^{-1}) and KT (0.1 mg L^{-1}), in the same environmental conditions, for 30 days. After 60 days, the calli were transferred to redifferentiation medium using MS + vitamins + glucose + glutamine (2 mg L^{-1}), and stored in the same conditions. Assay was entirely randomized with 10 replicates. Data were statically analyzed using software GENES, version 2013.5.1. (Cruz, 2013). Means were compared by Tukey test. The following traits were collected: embryogenic calli (%), somatic embryos in cotyledonal phase (%) and efficiency of regeneration (%). The cotyledonal embryos were cultivated in MS medium until the acclimation phase. The number of fertile plants was estimated based on plants that developed until the phase of fruit production.

2.5. Validation of GhSERK1 probe in cotton top lines

In order to validate the *GhSERK1* probe as a tool for identification of embryogenic cotton genotypes, four elite lines from Embrapa's Cotton Breeding Program (CNPA BA 139, CNPA BA 2247, CNPA 286, and CNPA BA 1366) were used in molecular and *in vitro* assays. Cultivars Coker 312 and BRS 201 were used as embryogenic and non-embryogenic controls, respectively. The procedures adopted for both assays were performed as previously described.

3. Results and Discussion

3.1. Embryogenesis in contrasting cotton genotypes

Somatic embryos were obtained from Coker 312 (Fig. 1c and 1d), BRS Rubi (Fig. 1e), and BRS Seridó (Fig. 1f). The rate of embryogenic calli (Fig. 1b) was statistically similar in Coker 312 and BRS Rubi (*c.a*: 65%), using a NAA and KT balance, with an average of efficiency of regeneration of 17.7% (Table 1). The other genotypes (BRS 201, BRS Topázio, and CNPA Precoce 1) did not develop embryogenic calli (Fig. 1a) and failed to regenerate corroborating the results reported by Soares (2013).

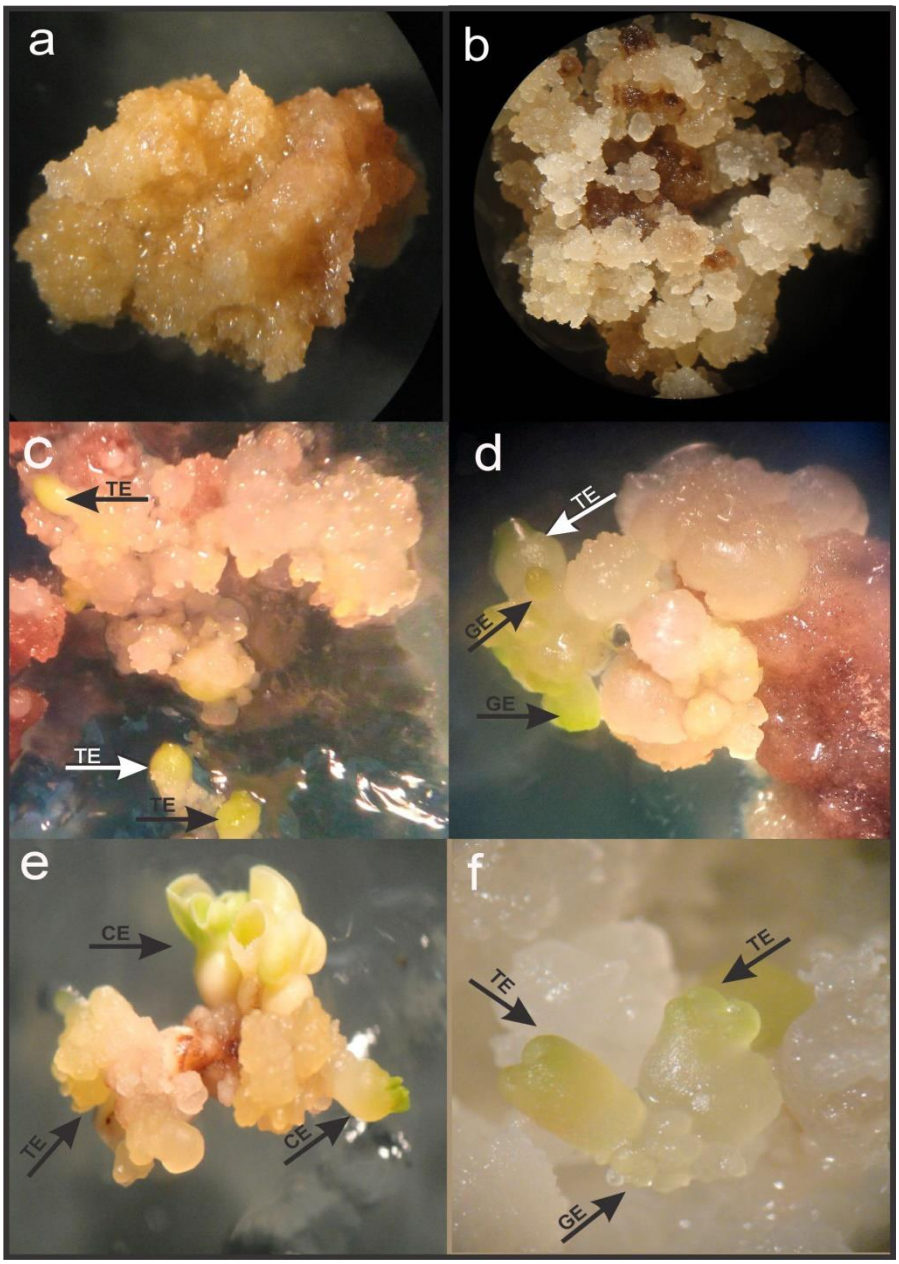

Fig. 1. Somatic embryogenesis in embryogenic and non-embryogenic genotypes at 60 days after cultivation: non-embryogenic calli in BRS 201 (a), embryogenic calli in Coker 312 (b), globular (GE) and torpedo (TE) embryos in Coker 312 (c, d), torpedo and cotyledonary (CE) embryos in BRS Rubi (e), and globular and torpedo embryos in BRS Seridó (f).

Table 1. Embryogenic data obtained from cotton genotypes and percent of fertile plants at final of cycle.

Genotype	EC (%)	SECP (N°)	ER (%)	FP (%)
BRS Rubi	70±2.6a	12±0.56a	17.14a	50,0
BRS Seridó	40±1.8b	5±0.26b	12.50b	8,30
Coker 312	60±2.1a	11±0.32a	18.30a	41,6
Mean	0.18	0.11	0.49	
F test	1.85	2.18	1.72	
Error	0.10	0.05	0.28	

EC- Embryogenic calli; SECP- Somatic embryos in cotyledonal phase; ER- efficiency of regeneration; Data transformed in arcsen$\sqrt{x}$; FP- Fertile plants. To: EC and SECP- means obtained from units verified in 10 plates. Each plate had nine hipocotyledonal fragments. Means with same letter do not differ at Tukey test ($p \leq 0.05$)

The plants obtained from Coker 312 and BRS Rubi were successfully acclimatized in greenhouse and grew normally up to full maturation (Fig. 2), with a rate of fertile plants of 41.6% and 50.0%, respectively (Table 1). The fertility rate of BRS Seridó was quite low (8.3%), because most of the embryos showed abnormal growth with limited radicular growth. On the other hand, this genotype also presented a high tendency of hyperhydricity, which refers to the accumulation of water in the cell and tissues interfering in the development of full plant.

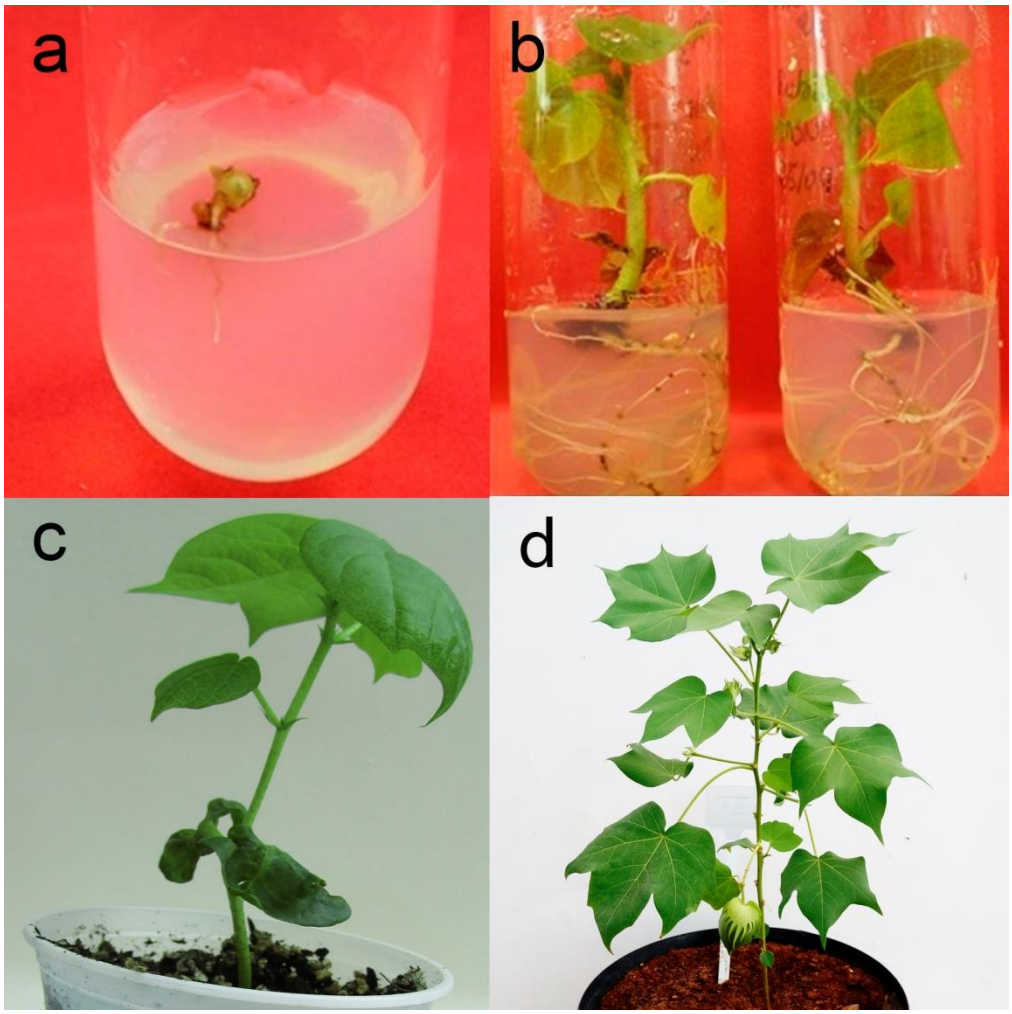

Fig. 2. Detail of BRS Rubi growth: germination of somatic embryo on MS media (a), seedling maturation (b), early acclimation in sterile substrate, in growth chamber (c) and full acclimation in greenhouse (d).

Discovering cotton cultivars with ability to be regenerated through *in vitro* procedures is an important achievement because the genetic modification can be performed in genotypes with outstanding agronomic traits. It is an advantage compared to the insertion of transgenes in lines with poor agronomic performance because they require a time-consuming breeding process to get rid of the undesirable traits.

The low efficiency of regeneration in cotton plants through SE is widely reported in the literature (Davidonis and Hamilton, 1983; Wu et al., 2004; Han et al., 2009). This barrier restricts the development of transgenic cotton because the improvement of a recalcitrant cultivar will require more cycles of backcrosses in order to recover the genetic quality of the matrix-plant (Pandey and Chaudhary, 2014; Wu et al., 2004). According to

Han et al. (2009), genotype dependence is one of the most important factors restricting SE and plant regeneration in *Gossypium* species, although the components of the medium, particularly the balance of phytohormones, plays an important role in the regeneration efficiency. In general, a balanced combination of NAA and KT is used in medium, supplemented with L-asparagine and L-glutamine.

Wu et al. (2004) developed a protocol for SE and plant regeneration using recalcitrant Chinese cotton cultivars. Calli and embryogenic calli were induced on MSB1 medium containing indole-3-butyric acid (2.46 μM) and KT (2.32 μM). According to those authors, up to 86.7% of embryogenic calli differentiated into globular somatic embryos after culture on MSB2 medium containing double KNO_3 and free of growth regulators. Up to 38.3% of the somatic embryos were converted into complete plants after 8 weeks on MSB3 medium, containing L-asparagine/L-glutamine (7.6/13.6 mM). The plants were successfully transferred to soil and grew to maturity. Zhang et al. (2000) obtained embryogenic calli and somatic embryos from Coker 201 and CRI 12 explants using MS medium supplemented with 0.1 mg L^{-1} Zeatin and 2 g L^{-1} activated carbon. An average of 28 matured somatic embryos were obtained from leaf explants, which were converted into normal plantlets when cultured on modified MS medium supplemented with 0.1 mg L^{-1} Zeatin. Those plants were regenerated within 60-80 days.

3.2. GhSERK1 transcripts expressed in cotton

Overexpression of *GhSERK1* transcripts obtained from floral primordia by RT-qPCR assays were found in BRS Rubi (20X) and Coker 312 (10X) (Fig. 3). The other genotypes showed low expression and also low embryogenic capacity. The expression of *GhSERK1* transcripts is associated with the regeneration ability as discussed in the previous section.

Overexpression of *SERK* has been reported as a signal of efficiency for SE due to the production of other molecules that act as ligand to the cell surface receptors (Pandey and Chaudhary, 2014). There are reports of *SERK* overexpression in several embryogenic species (Chugh and Khurana, 2002; Jin et al., 2014), such as *Arabidopsis thaliana* (Hecht et al., 2001), *Ocotea catharinensis* (Santa-Catarina et al., 2004), *Theobroma cacao* (Santos et al., 2005), *Vitis vinifera* (Schellenbaum et al., 2008), *Cocos nucifera* (Pérez-Núňez et al., 2009), and *Ananas comosus* (Ma et al., 2012).

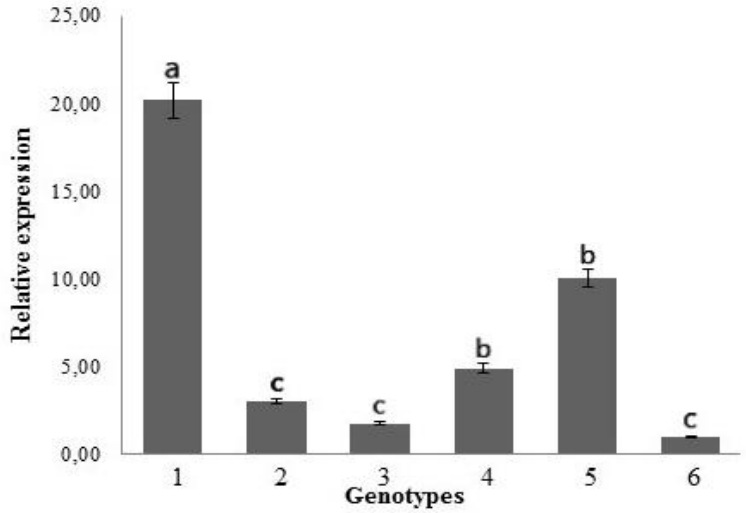

Fig. 3. Relative expression of *GhSERK1* in young cotton floral primordia: BRS Rubi (1), BRS Topázio (2), CNPA Precoce 1 (3), BRS Seridó (4), Coker 312 (5), and BRS 201 (6). The student's t-test was used to perform the statistical analyses of the data. Different letters indicate significant statistical difference at $p \leq 0.01$.

Based on these results, Coker 312 and BRS 201 were chosen as embryogenic and non-embryogenic controls, respectively, in order to validate the expression of *GhSERK1* in four cotton-bred lines in which the somatic embryogenic capacity had not been previously assessed. The genotypes were chosen from Embrapa's Cotton Breeding Program as elite lines and candidates to be genetically transformed. The line CNPA BA 139 overexpressed *GhSERK1* (65X) in qRT-PCR assays and the expression was significantly higher than in Coker 312 (10X) (Fig. 4).

In the blotting assay performed with cDNA using a non-radioactive probe labeled with chemiluminescent reagent (CDP-Star, Roche), two robust blots were hybridized with CNPA BA 139 and Coker 312 (Fig. 5), at same size of *GhSERK1* amplicons obtained through PCR assay. These results were also confirmed in *in vitro* assays carried out with other different bred-lines, following the same procedures previously described. Somatic embryos were obtained only from Coker 312 (Fig. 6a) and CNPA BA 139 (Fig. 6b) corroborating the positive association between regeneration capacity and *GhSERK1* expression. Steiner et al. (2012) reported *AaSERK1* expressing in embryogenic cells from Araucaria (*Araucaria angustifolia* (Bertol.) Kuntze 1898) with domains potentially conserved involving *SERK*, LRR (leucine-rich repeat), and RLK (receptor-like kinase) favoring the embryogenic process in several plant species.

The interaction of other genes involved in cell regeneration during SE is poorly understood. Yang et al. (2008) carried out studies related to gene expression during cell wall regeneration in cotton Coker 201 and found several up-regulated genes in protoplasts, such as proline-rich protein (*PRPL*), glycine-rich protein (*GRP*), extension (*EPR1*), fasciclin-like arabinogalactan protein (*FLA2*), and expensing-like protein (*EXLA* and *EXLB*). All of them might participate in primary or secondary cell wall construction and modification.

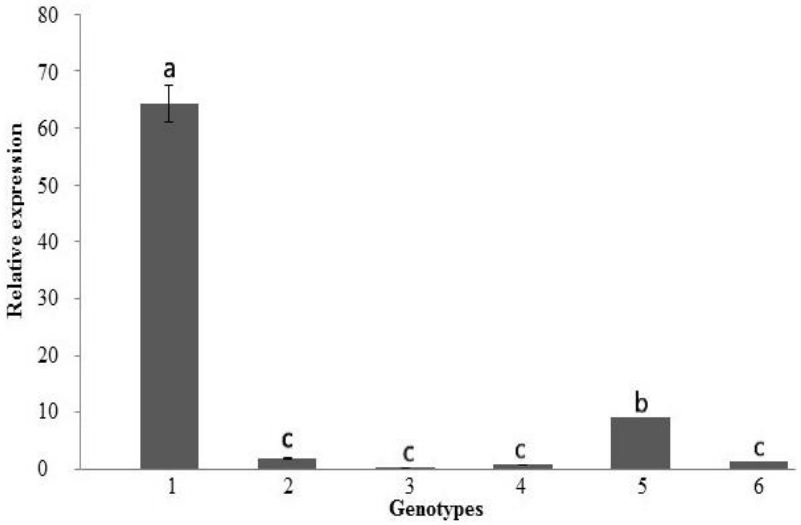

Fig. 4. Relative expression of *GhSERK1* in cotton genotypes of contrasting somatic embryogenic capacity: CNPA BA 139 (1), CNPA BA 2247 (2), CNPA 286 (3), CNPA BA 1366 (4), Coker 312 - embryogenic (5), BRS 201- non-embryogenic (6). The student's t-test was used to perform the statistical analyses of the data. Different letters indicate significant statistical difference at $p \leq 0.01$.

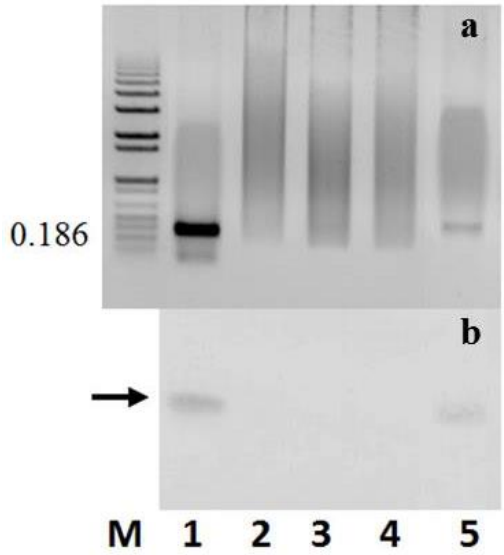

Fig. 5. Blotting of *GhSERK1* generated by RT-PCR using floral primordia-cDNA of cotton lines: Amplicons loaded in agarose gel (2.0%) (a), blot exposition in X-ray with a non-radioactive probe (186 bp) labeled with chemiluminescent reagent (b). 1Kb Plus DNA Ladder (Invitrogen) (M), CNPA BA 139 (1), CNPA BA 2247 (2), CNPA 286 (3), CNPA BA 1366 (4), Coker 312 (5).

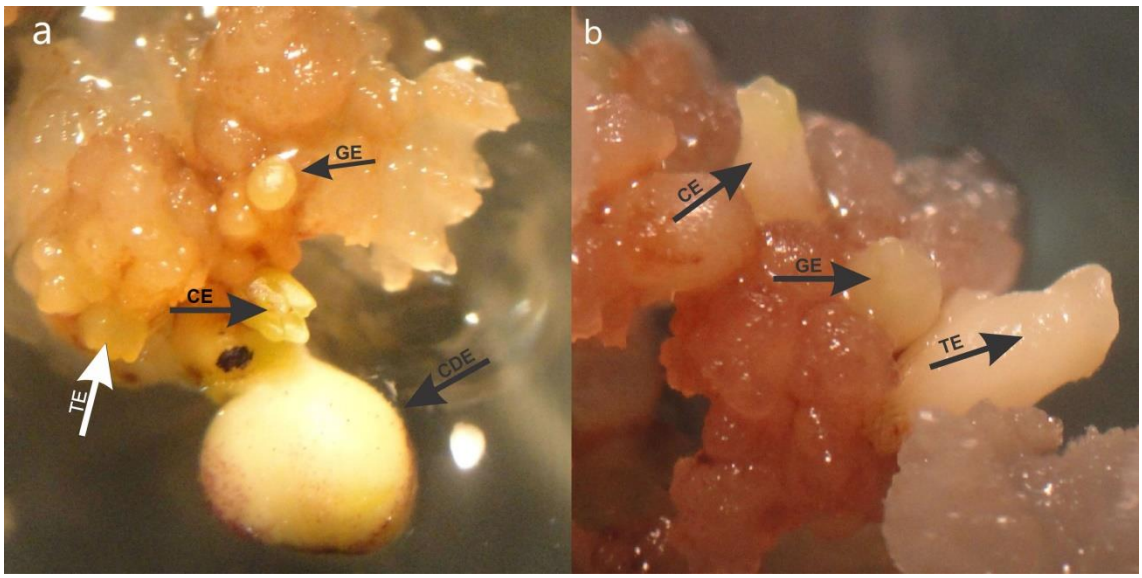

Fig. 6. Somatic embryos grown on redifferentiation media after 60 days. Cotyledon (CE), globular (GE), torpedo (TE) and cordiform (CDE) embryos in Coker 312 (a), globular, torpedo and cotyledon embryos (CE) in CNPA BA 139 (b).

The role played by *SERK* on cell-to-embryo transition and in the control of other fundamental aspects of plant development is reported in the literature. However, *SERK* orthology is not directly proportional to their functions. Pandey and Chaudhary (2014) measured the expression of *SERK* in some stages of regeneration in fully regenerating and non-regenerating tissues of Coker 310 lines and found high up-regulation of *SERK* in embryogenic calli of fully regenerating tissues. In contrast, calli obtained in early-stages of regeneration from both types of tissues had no significant influences on *SERK* regulation prior to acquisition of embryogenesis. The authors suggested that the positive regulation of *GhSERK1* during the formation of embryogenic calli indicates an increase in cell signaling kinase-mediated in competent somatic cells, which is required for SE induction. According to that result, up-regulation of *GhSERK1* only in embryogenic calli supports the hypothesis of association of this factor with the induction of SE in cotton. As the probe used in this work has homologous regions with other crops (*T. cacao, C. sinensis, V. vinifera, G. max* and *P. vulgaris*, it is probable that the association of *GhSERK1* expression and embryogenic capacity suggested in this work occurs in many other plant species.

Shi et al. (2012) cloned and characterized *GhSERK1* and reported that the transcript was present in all organs of plants at different developmental stages, but transcripts were most abundant in reproductive organs, particularly during the anther formation. In *Arabidopsis*, *AtSERK1* is expressed in oocytes, in cells of embryo sac, and in the cordiform phase of embryo development (Hecht et al., 2001). According to Schmidt et al. (1997), *SERK* has the same signaling pathway activated during both ZE and SE. In *Medicago truncatula*, *SERK1* was involved in some stages of plant development like lateral root formation (Nolan et al., 2011). This involvement of *SERK* with both ZE and SE favors the adoption of zygotic structures in molecular studies because it saves time and cost of analyses. The requirement of calli could make the technique unfeasible due to longer SE stages and low uniformity among materials during cell differentiation.

4. Conclusion

A short *GhSERK1* probe may be used to estimate the somatic embryogenic capacity in cotton genotypes. This probe is quite useful to cotton breeding because it is a reliable molecular tool to be used as marker. It replaces some time-consuming and expensive

procedures required in crop tissue assays. Despite the ease of adoption of the probe, we emphasize that the increased levels of GhSERK1 transcripts correlate with the greater ability of a genotype to go through somatic embryogenesis but this is not proportional to the rate of embryo formation. Moreover, it should be noted that this study did not define a threshold value of gene expression to indicate whether a cotton genotype is embryogenic or not.

Conflict of interests

The authors declare no conflict of interest in the conduction of this study.

Author Contributions Statement

RCS, JMFCC, LML, PAMF, and JJVC conceived and designed the experiments. TCS performed the experiments. TCS, CRCS, RCS, JMFCC analyzed the data. JMFCC, JJVC contributed reagents/materials/analysis tools. RCS, TCS, LSS wrote the manuscript. All authors read and approved the manuscript.

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ANEXOS

Confirmação de submissão do Artigo 1:

Plant Cell Reports (PCRE) - Editorial Office <em@editorialmanager.com> 14:07 (Há 4 horas)

para mim

Submission ID: PCRE-D-18-00345

Dear Mrs Soares,

We have received the submission entitled: "Molecular basis of cotton recalcitrance - Review" for possible publication in Plant Cell Reports, and you are listed as one of the co-authors.

The manuscript has been submitted to the journal by Dr. Liziane Maria de Lima who will be able to track the status of the paper through his/her login.

If you have any objections, please feel free to e-mail the Editorial Office by clicking on "Contact Us" in the menu bar at the top of the screen. If we do not hear back from you, we will assume you agree with your co-authorship.

Thank you very much.

With kind regards,
Abdul Hakkim
Plant Cell Reports
Springer Journals Editorial Office

Confirmação de publicação do Artigo 2:

Journal of Biotechnology (B1 – Biotecnologia)

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Validating a probe from *GhSERK1* gene for selection of cotton genotypes with somatic embryogenic capacity

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ABSTRACT

Substantial progress is being reported in the techniques for plant transformation, but successful regeneration of some genotypes remains a challenging step in the attempts to transform some recalcitrant species. *GhSERK1* gene is involved on embryo formation, and its overexpression enhances the embryogenic competence. In this study we validate a short *GhSERK1* probe in order to identify embryogenic cotton genotypes using RT-qPCR and blotting

Confirmação de publicação (Outras publicações):

Genetics Molecular Research (A2 – Ciências Agrárias/ B2 – Biotecnologia)

GMR

Investigation on possible occurrence of somaclonal effects in zygotic and embryonic cotton structures based on agronomical traits

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ABSTRACT. Zygotic and embryonic cotton structures were used in *in vitro* procedures to obtain stable plants. Zygotes (24, 48, and 72 h) and immature embryos (10 and 25 days after fertilization) were grown in MS medium supplemented with glucose, indoleacetic acid, kinetin, and activated charcoal. Regenerated seedlings were acclimatized and grown in 10-kg pots for further phenological evaluation, based on agronomic traits and possible occurrence of somaclonal effects. We found that zygotes and immature embryos at 10 days did not develop physiological