Romanian Biotechnological Letters *Vol. 15, No.2, Supplement, 2010*

ORIGINAL PAPER

Can lessons learned from resurrection plants be extended over crop plant species?

Received for publication, December 12, 2009
Accepted, January 9, 2010

OTTO TOLDI[1], ZOLTÁN TUBA[2,3] AND PETER SCOTT[4]
1 Hungarian Institute of Agricultural Engineering, H-2100 Gödöllő, Tessedik road 4. Hungary
2 Institute of Botany and Ecophysiology, Szent István University, H-2103 Gödöllő, Hungary
3 Plant Ecology Research Group of Hungarian Academy of Sciences at Department of Botany and Plant Physiology, Faculty of Agriculture and Environmental Sciences, Szent István University, Gödöllö, H-2103 Gödöllö, Hungary
4 School of Life Sciences, University of Sussex, Brighton BN1 9QG, UK

Abstract

A majority of plants possess stages in their life cycle at which specific tissues, mainly components of reproductive organs (mature pollen, seed) and dormant buds, can survive severe water loss. What is remarkable about resurrection plants is the ability of vegetative tissues (root, shoot, stem, leaves) to tolerate dehydration of the tissues and then return as functional units on rehydration. This phenomenon made resurrection plants exciting targets for molecular analysis of the poikilohydric ability and drought tolerance. Large-scale isolation of drought stress associated genes with unknown biological roles requires thorough functional analysis. Despite of the genetic and physiological complexity of desiccation tolerance, there are already examples where outcomes of targeted studies in resurrection plants are going to be directly utilized to engineer crop plants genetically. Here we show that conventional genetic transformation techniques, via in vitro plant regeneration systems, still represent an unavoidable part of the high-throughput technology chain of molecular breeding.

Keywords: desiccation tolerance, resurrection plant, molecular breeding, functional genomics, genetic transformation

Introduction

A vast majority of plant tissues are sensitive to dehydration. The tissue is damaged and will ultimately die once the water content of the tissue falls below a certain percentage. Ironically most plants possess at least one stage of their life cycle where at least some tissues or cells can survive severe dehydration. For many plant species this is limited to seeds, pollen or in dormant buds. During maturation, these tissues lose a large percentage of their water to enter a dormant state. These tissues can then remain apparently inactive for long periods. However, most plants lack this ability in other tissues such as leaves. Vegetative desiccation-tolerant plants are able to do this. Within this small group of plants the mature plant is poikilohydric, and leaves, roots and shoots can lose up to 95% of their water. This results in a shrivelled dried plant, which is actually still alive. Losing beyond 50% of their water content is enough to kill the tissue of most plants, but desiccation-tolerant, or in other words poikilohydric plants possess mechanisms to protect them in the dried state. These plants are also referred to as resurrection plants, because they can be resurrected by rehydration (Figure 1).

Fig. 1 Resurrection plants like *Craterostigma plantagineum* do not require any attention during brakes between experimental periods (**A**), but upon starting irrigation they are ready within a few days (**B**).

In addition, the wide occurence of desiccation tolerance has been remarked upon already. Desiccation-tolerance is also common among microorganisms, such as many algae, rotifers, nematodes and tardigrades and in *Crustacea* of seasonal water bodies. It is thus a widely expressed potentiality of living organisms.

Desiccation-tolerance occurs widely also in the plant kingdom. It is commonplace among *Bryophytes* and lichens, and is found sporadically among vascular plants from a range of families. In vascular plants desiccation tolerance of the vegetative tissues has been demonstrated in some 350 species, making up less than 0.2% of the total flora [1] but the list is constantly being extended. Desiccation tolerance is thus widely, but thinly and unevenly, scattered amongst vascular plants. Virtually all vascular plants have desiccation tolerant spores (including pollen) or seeds, so the potentiality for desiccation tolerance is probably universal.

It has been postulated that although the initial evolution of vegetative desiccation tolerance was a crucial step required for the colonization of the land by primitive plants, it came at a cost [2]. Metabolic rates, biomass production and competitive abilities, as interrelated features, are usually lower in such plants as compared to plants that do not possess extra energy-requiring protection and repair mechanisms [3]. The most serious consequence of this conclusion is that a fully desiccation tolerant crop plant would have little agricultural value. However, this should not deflect us from asking two important questions: what can we learn from desiccation tolerant plants, and to what extent we will be able to utilize this knowledge for bioengineering increased transient drought tolerance in crop plants? For that we need to select essential components of desiccation tolerance that are readily transferable to non-tolerant systems.

Desiccation-tolerance is a qualitatively different phenomenon from drought tolerance as ordinarily understood in vascular-plant physiology; indeed desiccation tolerance could be seen as a drought-avoiding mechanism. Desiccation tolerant plants are not simply an odd sideline from mainstream homoihydry [4]. They are an adaptive optimum in particular ecological situations, and (like 'normal' vascular plants) can be understood fully only in the context of a wide and multidimensional field of physiological and ecological possibilities. Most vascular desiccation-tolerant plants function as normal ('homoihydric') vascular plants until water becomes limiting. Like winter annuals and desert ephemerals (and other mesophytes), they then dry out within a few hours or days. The difference is that instead of dying and re-establishing from seed, their fall-back is to survive in a desiccated, but still viable vegetative state.

Wild species have been chosen as crops because of their high productivity, and bred to make them more so. Because crops are in general desiccation-avoiding, they must either be watered or grown where there is not drought. Aridity thus remains the greatest enemy of agriculture. In addition, there is a necessary functional conflict between high productivity and desiccation tolerance. Mechanisms proposed to explain the ability of vegetative desiccation tolerant plants to survive desiccation include sucrose and trehalose accumulation [5, 6], accumulation of stress proteins [7] and raffinose family oligosaccharides [8, 9], increased folding ability of cell wall structures [10] and accumulation of membrane stabilizing specific polyphenols, in particular galloylquinic acids, which have been shown to act as antioxidants and membrane protectants [11]. However, any of the above mechanisms may not have the same effect in not-desiccation-tolerant plants, like crops. The utility of using genes from the desiccation tolerant plants lies in the prospect of finding novel ways to maintain productivity of crop plants during drought by either broadening the limits of cell maintenance to encompass lower water potentials and/or repairing damage as it occurs allowing for broadening of the sensitivity range.

Despite of the genetic and physiological complexity of desiccation tolerance, there are already examples where outcomes of targeted studies in resurrection plants are going to be directly utilized to engineer crop plants genetically [9]. For example, as a part of an ongoing project, some desiccation tolerance-related genes of *Xerophyta viscosa* are to be expressed in maize. However, it is not known today whether the transfer of protective proteins, osmolyte sugars, signal metabolites or antioxidants from desiccation-tolerant species will result in crop plants with increased abiotic stress tolerance?

Similarities and differences in tissue culturing and genetic transformation of resurrection plants

To explore novel insights into gene function and the regulatory control of biological processes that are associated with responses to abiotic stress, cDNA microarrays offer a high-throughput approach to obtaining comprehensive gene expression profiles [12]. Large-scale parallel gene expression monitoring, using cDNA microarray-based methods, have been used to examine gene expression patterns in tissues including root, leaf and flowers at two different stages of development, and under dark and light conditions in genetic model systems (*Arabidopsis*, rice). cDNA microarray analyses of the expression profiles of genes that respond to abiotic stresses are also underway in tolerant systems like the CAM plant *Mesembryanthemum crystallinum* [13] and in desiccation tolerant plants [14, 15]. However, mRNA abundances may only represent putative function since there is still a questionable correlation between mRNA and protein levels [16, 17]. This is further highlighted by a survey of the proteomes of the model legume *Medicago truncatula* [18]. In this study, it was revealed that approximately only 50% of the proteins appear to be correlated with their corresponding

mRNA levels. In contrast, proteomics provides a more physiologically accurate snapshot of biochemical processes by revealing the actual protein constituents performing the enzymatic, regulatory, and structural functions encoded by the genome and transcriptome at a given point in time [19]. To further increase the clearness of the physiological snapshot, the transcriptome and proteome level monitoring could be complemented by large-scale analysis of the metabolome based on GC/MS technology. Using gas chromatography/mass spectrometry (GC/MS), 326 distinct compounds were quantified from *Arabidopsis* leaf extracts. Given metabolite levels can be regarded as the ultimate response of biological systems to genetic and environmental changes. It has the potential not only to provide deeper insight into complex regulatory processes but also to determine phenotype directly [20]. As such, metabolomics means a link between genotype and phenotype and the interplay between high-throughput analysis of the transcriptome and metabolome would result in candidate genes with more realistic potential being used in molecular breeding program. In addition, because of their unique metabolism, resurrection plants also have a potential to serve as renewable sources of novel bioactive compounds [21]. On this basis, it is not an exaggeration to predict the above combination of state-of-art technologies would generate enormous progress also in exploring the physiology of resurrection plants.

Although databanks have already provided information about putative function on the basis of sequence similarities, but *in vivo*-proofs can be obtained only after examining transgenic plants possessing upregulated or downregulated expression of the genes associated with desiccation tolerance [22, 23]. For this purpose, *in vitro* plant regeneration protocols had been established in resurrection plants such as *Craterostigma plantagineum* [24, 25], *Ramonda myconi* [26], *R. serbica* [27, 28], *Haberlea rhodopensis* [29] and *Lindernia brevidens* [30] of which *C. plantagineum*, *R. myconi* and *L. brevidens* have been successfully transformed [24, 25, 31, 30]. Similarities and differences in tissue culturing and genetic transformation between these resurrection plants, as a consequence of their common physiological specialization, are discussed.

Vegetative desiccation-tolerant plants possessed an extreme sensitivity during the establishment of their plant regeneration and genetic transformation systems in a similar manner. These tissues all displayed frequent evidence of tissue necrosis, development of hyperhydrated leaves and secretion of polyphenols into culture media under suboptimal conditions. However, this similarity in the *in vitro* behavior was just partial, because they required essentially different basal media for micropropagation, callus induction and plant regeneration. *C. plantagineum*, as a desert plant, developed a life strategy for fast and effective utilization of environmental resources, because it has only a few wet weeks to complete its whole ontogenetic program between the 2-3 years-long dry periods [32]. As it is a genetically fixed complex feature, *C. plantagineum* possesses a fast metabolism and rapid growth also under *in vitro* conditions that requires the application of a high salt containing MS [33] or B5 medium [34] throughout the whole tissue culture procedure (**Table 1**).

Table 1. Concentrations of some major ions in media used in tissue cultures of resurrection plants

Ion (μM/L)	MS	B5	CMS[(1)]	RA[(2)]	WPM[(3)]
NO_3	39.41	25.00	39.41	15.76	9.70
NH_4	20.62	2.00	20.62	8.25	5.00
Total N	60.03	27.03	60.03	24.01	14.70
Total P	1.25	1.08	1.25	0.50	1.25
Total K	20.05	25.00	20.05	8.02	11.36

(1) CMS media used in tissue cultures of *Craterostigma plantagineum* (Toldi *et al.* 2002)
(2) RA media used in tissue cultures of *Ramonda myconi* (Tóth *et al.* 2004)
(3) WPM media used in tissue cultures of *Haberlea rhodopensis* (Djilianov *et al.* 2005)

Whereas, a slow metabolism and ontogenesis make the survival of *H. rhodopensis*, *R. myconi* and *R. serbica* possible under the harsh alpine environment resulting in an alternative, but successful life strategy. The slow development means slow metabolism that does not require high concentrations of nutrients during tissue culturing. Even, the high salt containing MS medium was toxic for any kind of *R. myconi* and *H. rhodopensis* explants cultured *in vitro*. Only a limited number of plantlets incubated on this medium were relatively fast growing and developed large, healthy leaves without necrotic spots or hyperhydricity. Adequate results were obtained in micropropagation, callus induction and plant regeneration, only when the concentrations of macro and microelements of MS medium were drastically decreased, or when the culturing temperature was lowered [27]. We have no information on the growing characteristics of *L. brevidens* in its natural habitat (the fourth tissue cultured desiccation-tolerant plant), but the *in vitro* growth of this plant was satisfactory also in tissue culture media with lowered macro and microelement concentrations [30].

Except salt concentrations, there was a remarkable similarity between *C. plantagineum* and *R. myconi* in the other important constituents of their tissue culture media. This might be a result of their common physiological specialization and the fact that they both were regenerated via a callus phase. First, the determinative role of the synthetic auxin Picloram in the induction of dedifferentiated calluses in both plants was unparalelled, because there was no other growth regulator that could approximate its efficiency. The unavoidable application of cytokinins at low concentration also had a determining impact on the frequency of callus growth not only in this two species [25], but also in *L. brevidens*, where the combination of the cytokinin, BAP, and the synthetic auxin, napthalene acetic acid (NAA), gave the best results [30]. An additional common feature of these three resurrection plants was that a further significant increase in the efficiency of micropropagation, callus induction and plant regeneration could be achieved, when the given media were supplemented with antioxidants. The hyperhydricity and polyphenol secretion of calluses and regenerated plantlets of desiccation tolerant plants could be supressed by the use of the antioxidant glutathione [25, 26, 31] and by an antioxidant mixture consisting of ascorbic acid and citric acid [24, 30].

An alternative procedure has been developed for the *in vitro* plant regeneration in *H. rhodopensis* and *R. serbica*. The time consuming and laborious callus induction phase was omitted and the *de novo* developed regenerants were initiated directly on the leaf segment explants. For this purpose growth regulator-free MS [33] and WPM media [35] were used. Studies are in progress to show whether this system could serve also as an alternative basis for genetic transformation of *H. rhodopensis* [29] and *R. serbica* [27] or not?

The fact that *H. rhodopensis* and *R. serbica* are endangered plant species listed in the European and Bulgarian Red Books points toward an additional important application of tissue culture. The establishment of *in vitro* micropropagation systems ensures the preservation of such valuable plants and could facilitate the biodiversity studies [28, 29]. In this respect, it is important to note that resurrection plants propagated in tissue culture still possess identical poikilohydric behaviour after various periods of desiccation treatments to that of plants from the wild.

In contrast to *C. plantagineum*, *R. myconi* and *H. rhodopensis* displayed a sort of duality in their *in vitro* responses, probably as a result of slow metabolism and growth. Rapid and dramatic reactions to negative effects were paralelled with extended and moderate reaction to positive effects [26, 29]. This can make the genetic transformation of these species difficult, because most steps in a transformation procedure generates various types of physiological stresses that decrease survival rates of both transgenic and non-transgenic tissues. Indeed, the low survival rates of those plant explants that were made ready for genetic

transformation by cutting off all edges of leaf blades impeded the recovery of transgenic plants in *R. myconi*. At the same time, in detached leaves where the *Agrobacterium* penetration was promoted by microwounding, leaves survived the transformation procedure at a satisfactory frequency, which, in turn, allowed an efficient production of trangenic *C. plantagineum* [24], *R. myconi* [31] and *L. brevidens* [30] plants.

Another important component of so called 'low stress' transformation was the development of a non-lethal selection strategy for *R. myconi*. Most of the toxic drugs that are used as inhibitors of bacterial growth or as selective agents have side effects that can decrease or prevent plant regeneration, not only in non-transgenic explants, but also in transgenic ones [36]. This fact meant an additional risk knowing the *in vitro* behavior of *R. myconi*. Therefore, concentrations of both *Agrobacterium* eliminating and selective agents were optimized with the intention to accomplish the essential preconditions of a non-lethal selection strategy. That is, on the one hand, survival rates of plant explants have to be as high as possible in the presence of antibacterial drugs to make successful genetic transformation possible. On the other hand, the optimal concentration of the selective agent has to suppress, but not totally inhibit morphogenesis in non-transformed explants to allow a simple visual pre-selection of transgenic regenerants. In other words, we were in search of such sublethal concentrations of the applied selective agent, in which the main distinctive features of transgenic plants might be the green colour, morphologically normal phenotype and a more intense growth, while non-transgenic regenerants are likely to be bleached showing morphological abnormalities and retarded growth. The moderate tissue toxicity of kanamycin applied at a sublethal concentration (1.0 mg/l) made the differentiation of two types of regenerants possible in its presence. Examining all the abnormal regenerants no transgenic one was found among them, while normal regenerants carried the transgenes with no exceptions. We have no direct experience with *Lindernia* transformation, but the 15 mg/l hygromycin used as selective agent during its transformation [30] also does not seem to reach the usual effective concentration, which can be as high as 100-150 mg/l in other species.

While the development of a non-lethal selection system was found to be essential in the succesful genetic transformation in *R. myconi*, the lethal selection – based on the application of kanamycin at high concentrations (up to 150 mg/l) – was necessary to ensure efficient recovery of real transformants in *C. plantagineum*. It seems that the vigorously growing tissues required and tolerated a much harsher selection procedure in this latter case than in the slow growing and sensitive *R. myconi*, where 1 mg/l kanamycin could fulfill the duty of a selection agent. Additional differences between the two successfully transformed resurrection plants was that in contrast to *C. plantagineum* where the physical enhancements of bacterial penetration (microwounding) had negative rather than positive effects [25], such a treatment had primary importance in the success of genetic transformation of *R. myconi*. An additional two-fold increase in the transformation frequency of both plants was obtained when the above physical treatments were supplemented (or substitued in the case of *C. plantagineum*) with biochemical treatments. This included *in vitro* preinduction of the *Agrobacterium* *vir* genes by application of low pH liquid medium consisting of acetosyringone, aldose-type sugar source and organic nitrogen during the infection phase. These modifications together with the appropriate tissue culture system resulted in a reliable method for genetic transformation of the resurrection plants *C. plantagineum* and *R. myconi*. By using these special protocols, such R0 transgenic plants could be recovered that contained the transformation casettes with copy numbers varying between one and three copies in the lines tested. After growing the R0 generation of transgenic *C. plantagineum* and *R. myconi* lines, self-pollinated R1 and different R0 x wild type crosses were analyzed by germinating seeds on growth regulator-free CMS or RA medium containing kanamycin. Progenies from

selfed primary transformants (R0) in all cases showed a typical 3:1 (KmR:KmS) Mendelian segregation when seeds were germinated in the presence of kanamycin [31]. In a set of experiments, 21 plantlets from a transgenic x wild type cross were also analyzed. The progeny showed a 11:10 KmR:KmS segregation, also indicating a monogenic dominant fashion for the transmission of the *npt*II gene. All R0 and R1 transgenic plants developed fertile flowers, and no morphological abnormalities were observed at the whole plant-level.

Conclusion and future aspects

Genes with potential role in triggering desiccation tolerance can be identified by trancriptome and proteome analyses, metabolic processes that are involved in the functioning stress responses can be encompassed by metabolome analysis in an ideal world. We suppose that performing such complete analyses is still a complicated task both technically and intellectually. However, partial profiling of the transcriptome and proteome has already resulted in a provisional hierarchy among stress avoiding mechanisms and thus candidate genes for molecular breeding programs. Being realistic about our intellectual and technical capacities, only single traits can be transferred into a recipient genome by genetic engineering today. Therefore, those resurrection plant-originated protection agents can provide practical value in molecular breeding of crop plants (**Table 2**), which have simple metabolism, highly specific, and which is not interfering with essential primary metabolic fluxes and signaling processes (for more detailed information see [23]).

Table 2. Advantages and disadvantages of the most abundant protective agents associated with vegetative desiccation tolerance in terms of the practical application.

Protection agent	Metabolism	Advantage	Disadvantage
sucrose	carbohydrate	carbon and energy source, osmoprotectant	possible interference with growth and osmoregulation
trehalose	carbohydrate	osmoprotectant, signal metabolite	possible interference with sugar sensing and photosynthetic performance
RFOs	carbohydrate	osmoprotectant, antistress agent, metabolically inactive, synergistic effects	none
LEAs	protein	hydration buffer, protein and membrane protectant	the most specific and most determinative LEAs are still missing
glutathione	tripeptid	antioxidant, integrated sensor, signal metabolite, synergistic effects	none
ABA	mevalonate	growth regulator, signal metabolite	possible interference with plant development and growth, contrasting effects

Now we have signaling and metabolic processes to modify and we will have genes by which these modifications can be achieved. The question is how, because the weakest point within the whole high-throughput technology is the limited capacity of conventional genetic transformation techniques. Therefore, the development of an alternative transformation system called *in planta* transformation appears promising, at least in the case of some model plants [37] and is currently available for both monocotyledonous and dicotyledonous plants.

The advantage of this technique is that the time consuming and laborious *in vitro* tissue culture procedures can be totally replaced by immersing plants at a suitable developmental stage directly into *Agrobacterium* suspension cultures, which is followed by screening F1 generation for transgenic individuals. The problem with this method is that it works only in a few species. Until *in planta* transformation techniques are available, the conventional ways of genetic transformation through *in vitro* tissue culture systems are still vital in terms of functional genetics [22]. However, stable transformants are not always needed for analyzing gene functions. Large-scale transient gene expression assays, based on inoculation of plants by viral vectors, can be also performed on both protoplast and whole plant levels [38]. This method could also mean a reasonable alternative to conventional transformation approaches and thus can accelerate cataloging genes associated with vegetative desiccation tolerance.

Acknowledgements

This project has been funded by grants of OTKA (The Hungarian Scientific Research Fund) T-043444 and OECD (Organisation for Economic Co-operation and Development) Co-operative Research Programme to OT, and are gratefully acknowledged.

References

1. M.J. OLIVER, A.J. WOOD, P. O'MAHONY, *Plant Growth Reg.* 24, 193-201 (1998).
2. M.J. OLIVER, Z. TUBA, B.D. MISHLER, *Plant Ecol.* 151, 85-100 (2000).
3. D.I. PĂCURAR, *Romanian Biotechnological Letters* 14, 4087-4094 (2009).
4. D.F. GAFF *Structural and functional responses to environmental stresses*, K.H. KREBB, H. RICHTER, T.M. HINKLEY eds., SPB Academic The Hague Publisher, 1989., pp. 255-268.
5. N. SMIRNOFF, *J. Bryol.* 17, 185-191 (1992).
6. P.M. DRENNAN, M.T. SMITH, D. GOLDSWORTHY, J. van STADEN, *J. Plant Physiol.* 142, 493-436 (1993).
7. S. CHAKRABORTEE, C. BOSCHETTI, L.J. WALTON, S. SARKAR, D.C. RUBINSZTEIN, A. TUNNACLIFFE, *Proc. Natl. Acad. Sci. USA*, 104, 18073-18078 (2007).
8. M. NORWOOD, O. TOLDI, A. RICHTER, P. SCOTT, *J. Exp. Bot.* 54, 2313-2321 (2003).
9. S. PETERS, S.G. MUNDREE, J.A. THOMSON, J. M. FARRANT, F. KELLER, *J. Exp. Bot.* 58, 1947-1956 (2007).
10. Y.B. LIU, G. WANG, J. LIU, X. ZHAO, H.J. TAN, X.R. LI, *Journal of Arid Environments* 70 (2007) 183-194.
11. J.P. MOORE, K.L. WESTALL, N. RAVENSCROFT, J.M. FARRANT, G.G. LINDSEY, W.F. BRANDT, *Biochem J.*, 385, 301–308 (2005).
12. M. ISHIANTI, I. XIONG, B. STEVENSON, J.K. ZHU, *Plant Cell* 9, 1935-1949 (1997).
13. M.A. CUSHMAN, D. BUFFORD, M. FREDERICKSON, A. RAY, I. AKSELROD, D. LANDRITH, L. STOUT, J. MAROCO, J. CUSHMAN, *Plant Physiol.* 120S, 145 (1999).
14. H. COLLETT, A. SHEN, M. GARDNER, J.M. FARRANT, K.J. DENBY, N. ILLING, *Physiol. Plantarum* 122, 39-53 (2004).
15. G. ITURRIAGA, M.A.F. CUSHMAN, J.C. CUSHMAN, *Plant Sci.* 170, 1173-1184 (2006).
16. B. FUTCHER, G.I. LATTER, P. MONARDO, C.S. McLAUGHLIN, J.I. GARRELS, *Molecular and Cellular Biology* 19, 7357-7368 (1999).
17. S.P. GYGI, Y. ROCHON, B. R. FRANZA, R. AEBERSOLD, *Molecular and Cellular Biology*, 19, 1720-1730 (1999).
18. B.S. WATSON, V.S. ASIRVATHAM, L. WANG, L.W. SUMNER, *Plant Physiol.* 131, 1104–1123 (2003).
19. G. JIANG, Z. WANG, H. SHANG, W. YANG, Z. HU, J. PHILLIPS, X. DENG, *Planta* 225, 1405-1420 (2007).
20. O. FIEHN, *Plant Mol. Biol.* 48, 155-171 (2002).
21. D. DJILIANOV, S. IVANOV, T. GEORGIEVA, D. MOYANKOVA, S. BERKOV, G. PETROVA, P. MLADENOV, N. CHRISTOV, N. HRISTOZOVA, D. PRESHEV, M. TCHORBADJIEVA, V.

ALEXIEVA, A. TOSHEVA, M. NIKOLOVA, I. IONKOVA, W. van den ENDE, *Biotechnol. & Biotechnol. Eq.* 23, 1414-1416 (2009).
22. O. TOLDI, D. DJILIANOV, P. SCOTT, *Floriculture, Ornamental and Plant Biotechnology Vol. III*, J.A. TEIXEIRA da SILVA ed. Global Science Book, UK., 2006., pp. 209-217.
23. O. TOLDI, Z. TUBA, P. SCOTT, *Plant Sci.* 176, 187-199 (2009).
24. A. FURINI, C. KONCZ, F. SALAMINI, D. BARTELS, *Plant Cell Rep.* 14, 102-106 (1994).
25. O. TOLDI, S. TÓTH, T. PONYI, P. SCOTT, *Plant Cell Rep.* 21, 63-69 (2002).
26. S. TÓTH, P. SCOTT, S. SORVARI, O. TOLDI, *Plant Sci.* 166, 1027-1034 (2004).
27. A. SABOVLJEVIC, M. SABOVLJEVIC, T. RAKIC, B. STEVANOVIC, *Belg. J. Bot.* 141(2): 178-184 (2008)
28. S. DONTCHEVA, E. DASKALOVA, G. YAHUBYAN, I. DENEV, I. MINKOV, V. TONEVA, *Biotechnol. & Biotechnol. Eq.* 23, 369-372 (2009).
29. D. DJILIANOV, G. GENOVA, D. PARVANOVA, N. ZAPRYANOVA, T. KONSTANTINOVA, A. ATANASOV, *Plant Cell Tissue Organ Cult.* 80, 115-118 (2005).
30. C. SMITH-ESPINOZA, D. BARTELS, J. PHILLIPS, *Plant Cell Rep.* 26, 1681-1688 (2007).
31. S. TÓTH, C. KISS, P. SCOTT, G. KOVÁCS, S. SORVARI, O. TOLDI, *Plant Cell Rep.* 25, 442-449 (2006).
32. P. SCOTT, *Ann. Bot.* 85, 159-166 (2000).
33. T. MURASHIGE, F. SKOOG, *Physiol. Plantarum* 15, 473-497 (1962).
34. O.L. GAMBORG, R.A. MILLER, K. OJIMA, *Exp. Cell Res.* 50, 151-158 (1968).
35. G.B. LLOYD, B.H. McCOWN, *Proc. Intl. Plant Prop. Soc.* 30, 421-427 (1980).
36. A.S. OREIFIG, G. KOVÁCS, B. JENES, E. KISS, P. SCOTT, O. TOLDI, *Plant Cell Rep.* 22, 490-496 (2004).
37. A.F. BENT, *Plant Physiol.* 124, 1540-1547 (2000).
38. O.V. VOINNET, Y.M. PINTO, D.C. BAULCOMBE, *Proc. Natl. Acad. Sci. USA* 96, 14147-14152 (1999).