DROUGHT AND SALT TOLERANCE CONFERRED BY OVEREXPRESSION OF SPLICING FACTORS IN TRANSGENIC PLANTS

Bourgon L, B Amorós, MA Naranjo, O Vicente

Instituto de Biología Molecular y Celular de Plantas, Ciudad Politécnica de la Innovación, Edificio 8E. Universidad Politécnica de Valencia, Camino de Vera s/n. 46022 Valencia, Spain
Correspondence to O. Vicente, ovicente@ibmcp.upv.es

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Abstract. Abiotic stress conditions, especially drought and soil salinity, are the major causes for the reduction of crop yields and the loss of arable land world-wide. These problems will probably worsen in the next decades, according to climate change models, which predict longer, more frequent and more intense drought periods and the spread of desertification in many temperate and subtropical regions. In this situation, breeding of stress tolerance in crop plants has become an urgent need for the future of agriculture and food production. Over the last years, many research groups have isolated and characterised different genes, involved in mechanisms of plant responses to stress, to be used as biotechnological tools to reach this goal through genetic engineering techniques. Despite the fact that many of these genes actually confer variable levels of tolerance to different types of abiotic stress when expressed in transgenic plants, their practical usefulness has been questioned. In fact, no crop cultivars with sufficient tolerance levels, from an agronomic point of view, have yet been obtained by molecular breeding. In this paper, we will discuss briefly the present situation and future perspectives in this field. Concerning our own work, we will describe the strategy used in our laboratory for the isolation of additional, putative "stress tolerance" genes, based on the functional screening of plant cDNA libraries by expression in yeast. We will focus on two of the isolated Arabidopsis genes, *SRL1* and *RCY1*, which encode proteins belonging to the family of "SR-like" splicing factors, and that, when over-expressed in transgenic Arabidopsis plants, markedly increase their tolerance to water and salt stress, during seed germination and vegetative growth as well as during the phase of reproductive development.

ABIOTIC STRESSES AND CROP PRODUCTION

Different abiotic stress conditions, such as extreme temperatures (too cold or too hot), acid or alkaline soils, oxidative stress or, especially, drought and soil salinity, are the major causes for the reduction of crop yields world-wide, as well as for preventing the extension of farming to not cultivated, marginal soils [4, 5, 7, 12, 14, 24, 25]

Large areas of our planet are subjected to frequent drought periods, sometimes lasting several years, which cause a drastic reduction of agricultural production or, quite often, a complete crop loss. On the other hand, the most productive lands, those cultivated under irrigation in arid and semi-arid regions, suffer a progressive salinisation, mostly due to accumulation of the salts dissolved in irrigation water; approximately half of the total area of irrigated land is already affected by salt stress, in a lesser or a larger extent. These regions, which represent less than 20% of the total arable land but produce more than 40% of the world food, include, for example, large parts of California, Southeastern Asia and Australia, and all the Mediterranean basin.

If the current trends do not change in the near future, it is estimated that up to 50% of the land cultivated at present will have been completely lost for agriculture by 2050 [26]. The

situation could become even worse, considering additional factors such as the progressive limitation of water for irrigation, the loss of farming land due to urbanisation, the difficulties to extend the present-day cultivated land to zones not yet used (because of their low soil fertility or high ecological value), or the foreseeable effects of global climate change: among other extreme phenomena, longer, more frequent and more intense drought periods and "heat waves" are predicted in temperate and sub-tropical regions (e.g., [6]), causing an accelerated loss of arable land and the spread of desertification. In this situation, the increase in crop production that will be necessary to feed a still growing human population is clearly at risk.

BIOTECHNOLOGY TO THE RESCUE?

Although no simple solution can be envisaged for such a complex problem, it is clear that the genetic improvement of stress tolerance in crop plants will help to alleviate it, allowing, for example, a more efficient use of water resources by the plants, irrigation with lower quality (e.g., salty) water, to continue cultivation of soils affected by drought and/or salinity, to reclaim land already lost for agriculture by these reasons, or even to extend farming to marginal soils not used before. All in all, it should be possible to maintain or even increase crop productivity despite the negative effects of drought and salt stress.

Improvement of stress tolerance has been one of the major goals of traditional breeding programmes, but with a very limited success, except for some specific cases (e.g., [2, 27]). This has been due, not only to the intrinsic limitations of classical breeding techniques, but also to the genetic and physiological complexity of tolerance traits, which depend on a large number of genes, most of them with very small individual effects as compared to the influence of the environment [5, 7]. These difficulties have created a wide interest on the possibilities of genetic engineering as a rapid and general method for the transfer to crop plants of genes which could confer increased levels of stress tolerance. This approach, however, requires an in-depth understanding of the molecular mechanisms of plant responses to abiotic stress and, obviously, the previous isolation and characterisation of putative "stress-tolerance genes". An important part of today's research in plant molecular biology and biotechnology is focused on this field of work.

Over the last decade, a large number of papers have been published, describing how transformation with different genes confers to transgenic plants variable (but generally modest) levels of tolerance to different abiotic stresses, such as cold, high temperatures, water stress or salt. In most cases, these genes have been selected because of their likely or demonstrated participation in the mechanisms of cellular response to stress in plants; they can be involved in regulation of ion transport [1, 19, 28], in the synthesis of osmolytes ("compatible solutes") in the cytoplasm [11, 16, 17, 20], or in signal transduction and transcriptional regulation [10, 15] (see also: [18, 21, 22, 26], for reviews with additional examples).

However, the real usefulness of these genes for molecular breeding of stress tolerance in crop plants has been questioned [7], for various reasons. First, in many cases the tolerance phenotypes have been determined in *in vitro* systems, which generally do not represent the natural physiological conditions of the plants. In other reports, differences in stress tolerance between the transgenic plants and the wild-type controls have been registered photographically, but have not been quantified. Quite often, the plants are evaluated only during seed germination, or by biomass accumulation during vegetative growth, without taking into account that, for the same species, stress tolerance may be very variable in different developmental phases. Moreover, constitutive over-expression of some genes, in the

absence of stress, leads to reduced growth or abnormal development of the transgenic plants. Nevertheless, the most common criticism is that almost all these experiments have been performed in model species, mostly in *Arabidopsis thaliana*, and there are hardly any data allowing the extension of these results to crop species, where stress tolerance must be considered in the context of their agronomic characteristics: an increase in tolerance is of no value if the crop's yield or product (fruits, seeds, etc.) quality are significantly affected.

STRATEGIES TO ISOLATE PLANT STRESS TOLERANCE GENES

As our knowledge of the molecular mechanisms of plant responses to stress increases, novel putative tolerance genes will, no doubt, be identified, but different strategies can be used for the active search for such genes. Modern genomic techniques, such as DNA microarrays, allow the analysis of gene expression patterns under different conditions, at the whole genome level. All genes transcriptionally activated by a particular stress can be identified and could be considered as possible "stress-tolerance" genes, since many genes participating in defence mechanisms are indeed induced by that specific stress... but not necessarily: many other induced genes will not play any important role in the response mechanisms, and their over-expression will not confer any tolerance to transgenic plants. On the other hand, *bona fide* tolerance genes will not be detected by this method if their expression is regulated at a postranscriptional level.

In our laboratory, during the last years, we have been using and alternative, or rather complementary approach for the isolation of novel stress tolerance genes, through the functional screening of plant cDNA libraries by expression in yeast [18]. This strategy is based on the conservation between yeast and plant cells of many metabolic pathways and basic cellular processes, and in the fact that stress tolerance at the whole plant level depends, to a large extent, on cellular mechanisms of tolerance. Yeast and plant cells share, particularly, the mechanisms of regulation of ion transport and ion homeostasis and several targets of salt toxicity; indeed, this approach was initially used for the isolation of halotolerance genes, defined as those increasing salt tolerance upon their expression in yeast, but obviously can be, and has been extended to other types of stress affecting yeast growth, such as oxidative conditions, acidic pH, etc. The expression in *Saccharomyces cerevisiae* of cDNA libraries from *Arabidopsis thaliana* and from salt-treated *Beta vulgaris* plants, has led to the isolation of a number of novel halotolerance genes [8, 18], encoding putative transcription factors, metabolic enzymes, a protein kinase, a translation initiation factor or, interestingly, several proteins apparently involved in mRNA processing, including splicing factors and RNA binding proteins (see below); some of these clones have also been expressed in transgenic Arabidopsis plants, confirming the salt tolerance phenotypes observed in yeast.

SPLICING FACTORS AS STRESS TOLERANCE DETERMINANTS

As mentioned above, an *Arabidopsis thaliana* cDNA library, cloned in a yeast expression vector, was screened to isolate plant genes conferring salt tolerance when expressed in *Saccharomyces cerevisie*. Surprisingly, the three only independent clones isolated from about $7,5 \times 10^5$ initial transformants, were all apparently involved in the process of pre-mRNA splicing, since they encoded a previously characterised splicing factor (the U1A protein, a component of the U1 snRNP) and two putative proteins, SRL1 and RCY1, belonging to the family of "SR-like" or "alternating arginine-rich" factors [8]. These proteins are defined by the presence of a carboxi-terminal domain with a high content in Arg residues

alternating with Ser, Asp and/or Glu (RS domain); all characterised members of this family are components of the spliceosome and are involved in constitutive and/or alternative splicing, or in other steps of pre-mRNA processing, such as coupling of transcription with postranscriptional RNA modifications or mRNA transport to the cytoplasm [9, 13, 23]. Once the possibility that the salt (LiCl and NaCl) tolerance phenotype was due to regulation of ion transport in the yeast cells was ruled out, we proposed that pre-mRNA splicing represents a target of salt toxicity in eukaryotic cells, which had not been previously described [8]: salt stress (and probably other stresses too) inhibits pre-mRNA processing, and the expression of RS-domain proteins may stimulate this process, probably in an unspecific manner, thus partially counteracting the toxic effect of the salt. We have been able to demonstrate that this is indeed the case, at least with some specific introns of yeast and plants: their splicing is inhibited *in vivo*, in the presence of LiCl, but this inhibition is partially blocked by over-expression of the SRL1 protein [3, 8]. We have also studied the expression patterns of the *SRL1* and *RCY1* genes (both are activated by NaCl, LiCl, and under drought and other stress conditions), and are working on the full biochemical and functional characterisation of the proteins. But, from the biotechnological point of view, it is important to mention that the constitutive over-expression of *SRL1* or *RCY1* (under control of the CaMV 35S promoter), confers to transgenic Arabidopsis plants a marked increase in their tolerance to salt, (as expected from our previous results in yeast)... but they are also quite tolerant to water stress, so that *SRL1* and *RCY1* can be define as more general "stress tolerance" genes and not only as specific "halotolerance" genes. As an example of our results, figure 1 shows the tolerance phenotypes of one of the transgenic lines transformed with *SRL1*.
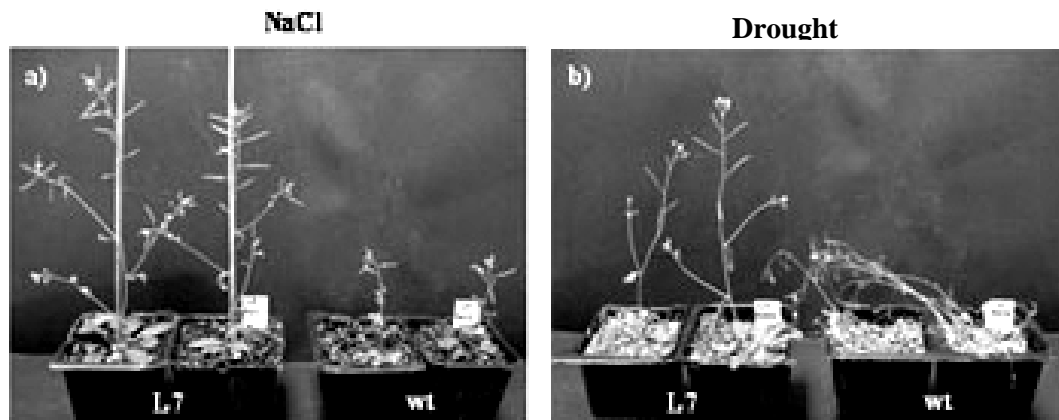

Fig. 1. Phenotypes of tolerance to salt (250 mM NaCl) **(a)** and to drought **(b)** of *Arabidopsis thaliana* transgenic plants (L7 line) overexpressing the SRL1 splicing factor, under control of the CaMV 35S promoter, as compared to control, non-transformed plants (wt) (taken from ref. [3])

Although, as for most other putative stress tolerance genes, we have not yet checked the effects of the expression of SRL1 and RCY1 in crop species (we are planning to transform tomato with the two cDNAs), the results obtained in Arabidopsis look quite promising concerning the possible use of these genes (and maybe also other genes of "SR-like" proteins) as additional tools for molecular breeding of tolerance to abiotic stress in crop species. Even more considering that we have taken into account most of the criticisms to this kind of approach, which were discussed above. Thus, we did not limit the assessment of salt and drought tolerance to photographic records. Transgenic plants and the controls were grown

under continuous salt stress (addition of NaCl to the watering solution, to a final concentration of 250 mM) or water stress (no watering at all) conditions, from two weeks after sowing to the end of their biological cycle. We have quantified different parameters for vegetative growth (fresh weight and dry weight of the plants, number of rosette leaves) and for reproductive development (length of the reproductive stem, number of flowers, silique formation), using a number of plants statistically significant (between 20 and 50 per measurement), considering possible individual differences in the response to the stresses applied. The transgenic plants retained more water than the controls during both treatments and, despite the drastic conditions used, were able to complete their biological cycle, producing a similar number of flowers, siliques and seeds than the non-treated controls. On the contrary, a high percentage of wild-type plants did not survive the treatments, and those that did showed a clear inhibition of their reproductive development (which is more sensitive to stress than vegetative growth): there was a clear reduction in the average length of the reproductive stem, in the number of flowers formed and in the number of siliques with seeds. On the other hand, over-expression of the splicing factors did not affect growth and development of the transgenic plants in the absence of stress ([3]; B Amorós and O Vicente, unpublished results).

PERSPECTIVES

Transgenic crops with improved levels of tolerance to drought, salt and other abiotic stresses, should be one of the basis of agriculture in a not too distant future, if we are to produce enough food to feed the growing human population. At present, no commercial crop variety has been developed with sufficient tolerance levels, from an agronomic point of view, that is, maintaining its productivity and quality under stress conditions. In fact, one of the few examples [28] of transformation of a crop (tomato, with the *AtNHX1* gene from Arabidopsis, which encode a vacuolar Na^+/H^+ antiporter) showing an increase in the tolerance to stress (NaCl, in this case) without much affecting yield or other agronomic characteristics, has been questioned by lack of reproducibility [5, 7]. *Arabidopsis thaliana* will remain as a useful model for the pre-selection of putative stress tolerance genes, but the development of efficient biotechnological tools for molecular breeding of tolerance to drought, salinity or other stresses will require the analysis of the effects of their expression in transgenic crops.

A large collection of possible stress tolerance genes has already been isolated (even if their precise biological functions and mechanisms of action are not yet understood for many of them). More candidate genes will be identified in the future, by standard molecular biological methods, by genomic technologies or by the functional approach described here and used by our laboratory (and now also by other groups), screening plant libraries in yeast cells (*S. cerevisiae* **or** *S. pombe*) to isolate clones conferring tolerance to specific stresses.

Regarding the "SR-like" proteins, they seem to be promising candidates for stress tolerance determinants, but this should be confirmed by transformation of some crop plant. In any case, it is important to have a wide array of available genes conferring tolerance by different and independent mechanisms, for example by stimulation of pre-mRNA processing, ion transport and osmolyte biosynthesis. Co-expression of two or more of these genes may then have additive effects, leading to higher tolerance levels.

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