

Letters to the Editor

## Genetically Engineered Plants Resistant to Soil Drying and Salt Stress: How to Interpret Osmotic Relations?

We call your attention to "Overexpression of  $\Delta^1$ -Pyrroline-5-Carboxylate Synthetase Increases Proline Production and Confers Osmotolerance in Transgenic Plants" by Kavi Kishor et al. [(1995) *Plant Physiol.* **108**: 1387–1394]. This is a report on a proline (Pro)-accumulating transgenic tobacco, which the authors claim expresses "osmotic adjustment" (last paragraph, p. 1393), "osmoprotection" (abstract), and "osmotolerance" (title). We are not aware of any accepted definitions of "osmotolerance" and "osmoprotection" with respect to drought. We would, however, like to address the well-defined phenomenon of osmotic adjustment, as treated in this paper.

We would like to point out that the interpretation of plant water relations and osmotic adjustment in this paper is in serious error. Osmotic adjustment involves the net accumulation of solutes in a cell in response to a fall in the water potential of the cell's environment. As a consequence of this net accumulation, the osmotic potential of the cell is lowered, which in turn attracts water into the cell and tends to maintain turgor pressure. Osmotic adjustment must be distinguished from the lowering of osmotic potential (increase in solute concentration) that accompanies any loss of water from a cell, and therefore must be measured in cells of defined water status, preferably full turgor.

It is totally wrong to say that osmotic adjustment "facilitates the maintenance of osmotic potential during water stress . . ." (p. 1390). Osmotic adjustment facilitates the maintenance of *turgor* by *lowering* osmotic potential. The authors compound this fallacy during their discussion of table I (p. 1391), which shows that the osmotic potential of their transgenic plants was unaffected or even perhaps increased by the stress treatment. The data show that the transgenic plants were probably even less able to adjust osmotically than were the wild-type plants.

If the authors wish to claim that overexpression of Pro does confer resistance to drought or salinity through osmotic adjustment, then they must measure osmotic adjustment correctly [e.g. J.M. Morgan (1992) *Aust. J. Plant Physiol.* **19**: 67–76], they must demonstrate a link between osmotic adjustment and growth under stress, and they must demonstrate that the overexpression of Pro accounts for any osmotic adjustment. The authors seem to have been so enthralled by their expectations that overexpression of Pro would enhance "osmotolerance" that they have

completely misunderstood their own data in the context of osmotic adjustment.

Transgenic plants have the potential to be powerful and to aid in helping us understand and manipulate the responses of plants to stress, but they can only be so when studied with the help of a sound background in stress physiology.

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This letter concerns the paper "Overexpression of  $\Delta^1$ -Pyrroline-5-Carboxylate Synthetase Increases Proline Production and Confers Osmotolerance in Transgenic Plants" by Kavi Kishor et al. [(1995) *Plant Physiol.* **108**: 1387–1394]. The paper describes the production of transgenic tobacco plants that overproduce proline (Pro), and it examines whether these plants have improved tolerance to soil drying and salinity. Evidence is presented of dramatic improvements in growth of transgenic versus wild-type plants exposed to either stress treatment. Unfortunately, the role of Pro overproduction in these responses is unclear, since the interpretations and conclusions regarding leaf osmotic relations are incorrect.

An accumulation of Pro in plants exposed to low-water-potential environments has often been reported. However, the importance of this accumulation for plant adaptation is uncertain; most commonly, Pro has been proposed to act as a "compatible" solute that contributes to osmotic adjustment of the cytoplasm. Osmotic adjustment describes a lowering of osmotic potential (excluding the concentrating effect of dehydration) in tissues of plants exposed to low-water-potential conditions. The use of transgenic plants modified in Pro production is a powerful approach to this question.

In the study in question, the authors measured leaf osmotic potentials of the plants subjected to soil drying, and they obtained, in our opinion, surprising results. In wild-type plants leaf osmotic potential fell

from around  $-0.7$  MPa before stress to  $-1.15$  MPa after stress, which is indicative of leaf dehydration and/or osmotic adjustment. In the transgenic plants, however, leaf osmotic potentials were unaffected by soil drying, remaining at around  $-0.7$  MPa. Thus, the data indicate that *osmotic adjustment did not occur in the leaves of the transgenic plants* despite the overproduction of Pro.

The authors suggest from these results that "Pro accumulation in transgenic plants helped the cells to maintain osmotic potential and thus enhanced the ability of the plants to tolerate water stress" (p. 1391). This statement is erroneous, since maintenance of osmotic potential would render the plants more susceptible to wilting and thus reduce water-stress tolerance.

Interestingly, despite the lack of osmotic adjustment in the transgenic plants, the text states that these plants exhibited delayed and less-severe wilting than the wild-type plants. This combination of results can only mean that the leaf water potential declined much less in the transgenic plants after water was withheld from the soil (unfortunately, leaf water potentials were not presented). This fact is critical, since it is the slower rate of leaf water potential decline and not the difference in leaf osmotic relations that conferred the improved tolerance to soil drying in the transgenic plants. The explanation for the slower decline in leaf water potential is not apparent, but could involve factors (e.g. leaf area, root distribution, stomatal behavior) that are not necessarily related to Pro overproduction.

Indeed, because this study does not include water content or water potential data, it is not possible to evaluate the extent of Pro overproduction during soil drying. The Pro results were based on a gram of tissue fresh weight. Therefore, comparison of Pro concentrations between wild-type and transgenic plants is confounded by likely differences in tissue water contents. Methods are available that could avoid this problem and could make the extent of overproduction clear.

We are puzzled that leaf osmotic relations were not also studied in the salinity-stressed plants, since figure 7A (p. 1393) illustrates wild-type and transgenic plants with seemingly turgid leaves after a 3-week exposure to  $0.4$  M NaCl. This concentration would have had a water potential of around  $-2.0$  MPa, and it is unavoidable that the leaf water potentials also decreased by about this magnitude. For the leaves to have maintained turgidity under these conditions, their osmotic potentials also must have decreased by at least  $2.0$  MPa. We wish to emphasize that maintenance of osmotic potential, as proposed by the authors to enhance water-stress tolerance, would have resulted in severe desiccation under these conditions. Unfortunately, because leaf osmotic relations were

not measured, it is not possible to assess whether the improved growth of the transgenic plants under salinity stress was associated with greater osmotic adjustment.

We believe that the contribution of the paper is considerably weakened by the lack of understanding of osmotic adjustment and poor appreciation of the water-relations measurements that are necessary to document and interpret whole-plant responses to low-water-potential environments. It is apparent from the conclusion of the article that the authors believe they have demonstrated that Pro overproduction promoted osmotic adjustment. Disappointingly, the paper does not provide any evidence that this was accomplished.

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#### A Reply from the Authors:

Many plants accumulate high levels of proline (Pro) in response to osmotic stress. In our recent report [Kavi Kishor et al. (1995) *Plant Physiol.* **108**: 1387-1394], we tested the hypothesis that Pro accumulation may help plants overcome osmotic stress. This was achieved by overexpression of  $\Delta^1$ -pyrroline-5-carboxylate synthetase (P5CS), the first enzyme in Pro biosynthesis, and we were able to produce transgenic tobacco plants that synthesize about 20-fold more Pro than control plants. When subjected to salinity and drought stresses, the transgenic plants were able to maintain a significant degree of sustained growth compared to the wild-type plants. These results, for the first time (to our knowledge), clearly demonstrated that the ability of plants to tolerate osmotic stress can be raised by overproduction of Pro. The term "osmotic adjustment" was not strictly used in our paper to define water relations, but was used simply to describe the fact that Pro-overproducing plants adjusted better to osmotic stress.

In an attempt to interpret how overproduction of Pro in transgenic plants confers osmotolerance, we followed the changes in osmotic potential in plants subjected to osmotic stress. Osmotic potentials of leaf saps were measured with a vapor pressure osmometer. The results obtained were unexpected and did not seem to fit the conventional concept on water relationship. Contrary to what one might have ex-

pected, osmotic potentials under unstressed conditions were about the same in the transgenic plants as in the controls (table I, p. 1391), despite the fact that the former produced almost 20-fold more Pro. When subjected to drought stress, the transgenic plants seemed to maintain similar osmotic potentials in their leaves, whereas these values declined significantly in the control plants (table I). Based on these data, we conclude that "the osmotic potentials of leaf sap from transgenic plants were less decreased under water-stress conditions compared to those of control plants" (abstract, p. 1387).

We appreciate the interest in our work from the authors of the two letters (published above) and their critical discussion of this subject. However, we feel it is too early for a detailed discussion of water relationships in transgenic plants that produce high levels of Pro. First, as pointed out by Sharp et al., it is difficult to evaluate the role of Pro in the transgenic plants without water content and water potential data. We intend to pursue these measurements and hope that the data will provide further insight on this topic. Second, the data in table I were obtained by using squeezed leaf sap and they did not represent the real osmotic potentials in the cytosol of leaf cells. The leaf water system is mainly composed of cytosol, vacuole, and cell-wall space. Pro apparently accumulates only in the cytosol and may also have a direct impact on the metabolism of the cell. Third, ions such as potassium are the major component of osmotic potentials in plants and play an important role in osmotic adjustment during stress [J.M. Morgan (1992) *Aust. J. Plant Physiol.* 19: 67-76]. It is not known whether Pro accumulation affects ion balance in the transgenic plants. Overall, we believe that further study on these transgenic plants will help us understand this question. Although Pro accumulation is often suggested to function directly in osmotic adjustment [A.J. Delauey and D.P.S. Verma (1993) *Plant J.* 4: 215-223], at this stage we do not exclude any

possibility that Pro accumulation may have other functions that, in turn, affect osmotic potentials.

The criticism in the first letter that "it is totally wrong to say that osmotic adjustment 'facilitates the maintenance of osmotic potential during water stress . . .'" quotes the statement out of context. The original sentence (p. 1390) reads "proline accumulation facilitates the maintenance of osmotic potential during water stress." This conclusion was drawn from the data presented in table I. As discussed above, it remains an open question how Pro accumulation affects osmotic adjustment.

In agreement with the interpretation offered by the authors of the second letter, it would have been more appropriate to state that "proline accumulation in transgenic plants helped the cells to maintain *water potential* and thus enhanced the ability of the plants to tolerate water stress" (p. 1391). This interpretation also explains the observation that the transgenic plants maintained a higher osmotic potential and exhibited less-severe wilting. We noticed that the transgenic plants developed larger root systems and showed higher overall vigor under osmotic stress conditions, but we have not investigated other factors that may affect rate of water loss in plants during osmotic stress.

The genetic engineering approach has proven to be successful in creating transgenic plants that accumulate high levels of compatible osmolytes such as Pro and mannitol [M.C. Tarczynski, R.G. Jensen, H.J. Bohnert (1993) *Science* 259: 508-510], and more studies on these plants will further our understanding of water-stress physiology. The power of this approach was acknowledged by the authors of both letters.

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