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Perturbation of the ubiquitin system causes leaf curling, vascular tissue alterations and necrotic lesions in a higher plant

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A ubiquitin variant with Lys48 changed to Arg acts *in vitro* as an inhibitor of ubiquitin dependent protein degradation. To assess the role of this proteolytic pathway in the life cycle of plants, we expressed the ubiquitin variant in *Nicotiana tabacum*. Expression of variant mono- or polyubiquitin leads to marked abnormalities in vascular tissue. In addition, overexpression of variant polyubiquitin induces discrete lesions on leaves. This indicates that perturbations of the ubiquitin system can induce a programmed necrotic response in plants.

Key words: Nicotiana tabacum transformation/proteolysis inhibition/ubiquitin dependent proteolysis/ubiquitin overexpression

Introduction

Ubiquitin is a small cytoplasmic protein which occurs either free, or covalently attached to other cellular proteins. In the latter case, the carboxyl terminus of ubiquitin forms an isopeptide bond with ε-amino groups of lysine residues in target proteins. This post-translational modification has been shown to be essential for a variety of cellular processes (for reviews, see Finley and Varshavsky, 1985; Rechsteiner, 1987; Finley *et al.*, 1988; Hershko, 1988; Varshavsky *et al.*, 1988; Jentsch *et al.*, 1990).

A major function of ubiquitin is to participate in ATP dependent proteolysis. Covalent attachment of several ubiquitin moieties in a branched chain (Chau *et al.*, 1989) marks substrate proteins for rapid proteolysis by a multienzyme protease complex (Waxman *et al.*, 1987; Eytan *et al.*, 1989; for review, see Hough *et al.*, 1988). The ubiquitin system is a pivotal component of the stress response of eukaryotic cells (Finley *et al.*, 1984; Bond and Schlesinger, 1985). Like some classical heat shock proteins, many components of the system are heat shock inducible, but are also expressed and essential during normal growth.

While much work focuses on the biochemistry and cell biology of processes involving ubiquitin, virtually no information is available on the contribution of the ubiquitin system to differentiation processes in plants or animals. A straightforward genetic analysis of the ubiquitin system, however, is complicated by the fact that it is not obvious on which possible phenotype a screen for mutants might be based. Moreover, many components of this system exist as multigene families. The enzyme catalysing the first step in the ligation of ubiquitin to target proteins occurs in three isoforms in wheat (Hatfield and Vierstra, 1989). In barley as well as in other plants, ubiquitin is encoded by a multiplicity of genes (Gausing and Barkardottir, 1986; for review, see Callis and Vierstra, 1989).

Most of these ubiquitin genes code for polyubiquitin, a protein consisting of a spacerless head-to-tail arrangement of several ubiquitin moieties. This precursor protein is cleaved into monomeric ubiquitin by specific cytoplasmic proteases (Jonnalagadda *et al.*, 1989; Wilkinson *et al.*, 1989).

Our approach to analyse ubiquitin dependent proteolysis in plants is based on recent findings concerning the substrate selection process for this proteolytic pathway (Bachmair *et al.*, 1986; Bachmair and Varshavsky, 1989; Chau *et al.*, 1989; Gonda *et al.*, 1989; Reiss and Hershko, 1990; for reviews, see Hershko, 1988; Varshavsky *et al.*, 1988; Ciechanover and Schwartz, 1989). Protein–ubiquitin conjugates that are metabolically stable usually carry a single ubiquitin moiety. Ubiquitin dependent proteolysis, on the other hand, requires the addition of several ubiquitin residues onto a presumptive substrate. After the initial attachment of one ubiquitin moiety to the target protein, additional ubiquitin moieties are added sequentially by linkage to the ε-amino group of Lys48 of the previously attached ubiquitin. As a result, a branched ubiquitin–ubiquitin structure is formed.

If ubiquitin is replaced by a variant protein in which Lys48 is changed to Arg (referred to as ubiquitin R-48 below), the first ubiquitin group is efficiently attached to substrate proteins in an *in vitro* system. However, due to the absence of the branch point Lys, no further ubiquitin residue can be added. Several proteolytic substrates have been shown to be completely stable in this *in vitro* system after replacing ubiquitin by ubiquitin R-48. Furthermore, mixing of wild type ubiquitin with ubiquitin R-48 results in partial inhibition of ubiquitin dependent proteolysis *in vitro* (Chau *et al.*, 1989; V.Chau, personal communication).

Here, we demonstrate that ubiquitin R-48 can be used to perturb ubiquitin dependent proteolysis *in vivo* in a higher plant, where it competes with the pool of endogenous wild type ubiquitin (Figure 1). Furthermore, we show that, in higher plants, certain perturbations of the ubiquitin system induce a necrotic response on leaves.

Results

Generation of plant cells expressing a ubiquitin variant with Lys48 replaced by Arg

A gene was constructed which codes for plant polyubiquitin, except that Lys48 is replaced by Arg in each ubiquitin repeat. The poly-structure was chosen to ensure a high level of expression of the proteolysis inhibitor, ubiquitin R-48. The promoter of the cauliflower mosaic virus 35S RNA was modified by duplication of its enhancer sequence (Kay *et al.*, 1987). This promoter was used to express polyubiquitin R-48. In a parallel construction scheme, wild type ubiquitin was assembled into a polyubiquitin gene (Figure 1A).

While these genes, containing 32 ubiquitin units, are stable

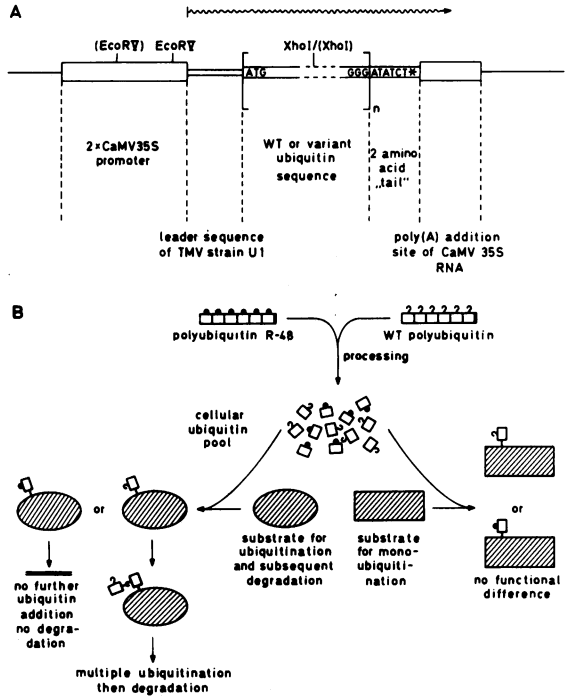

Fig. 1. (**A**) Schematic drawing of transgenes used in this work (for construction, see Materials and methods). A promoter derived from the cauliflower mosaic virus 35S gene by duplication of the enhancer region (positions −415 to −90) is followed by a short part of the leader sequence of tobacco mosaic virus strain U1 to ensure efficient translation initiation. The open reading frame codes for plant polyubiquitin having either wild type sequence, or a Lys to Arg change at codon 48. In the latter case, an internal *Xho*I site was destroyed to allow for easy distinction between the two genes. The termination region is from the cauliflower mosaic virus 35S gene. For further details, see Materials and methods. In the plants investigated, the number of ubiquitin repeats (*n*) is one, two or six (see text). (**B**) Proposed scheme of action of polyubiquitin R-48. The model is based on *in vitro* evidence (Chau *et al.*, 1989) and implies that cellular enzymes do not select against the ubiquitin variant containing Arg at position 48. This change does not allow formation of ubiquitin–ubiquitin isopeptide bonds necessary for protein degradation. WT polyubiquitin, wild type polyubiquitin.

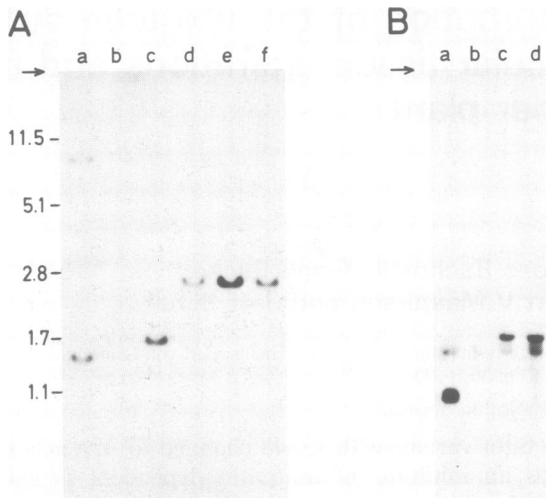

Fig. 2. (**A**) Southern hybridization using DNA from transgenic tobacco leaves. A fragment containing the promoter, but no ubiquitin coding sequence, was used as a probe. All lanes contain 10 μg of tobacco DNA digested with *Eco*RI and *Kpn*I, which release fragments containing the promoter and the open reading frame of the transgenes. For molecular weight, see position of size markers on the left margin (size in kbp). Lane a, DNA from untransformed tobacco was mixed with *Eco*RI/*Kpn*I digests of the plasmids pΔRV32 and pΔRV1 corresponding to 5–10 copies per haploid genome of a polyubiquitin gene with 32 repeats and a monoubiquitin gene, respectively. Lane b, DNA from tobacco containing an integrated diubiquitin gene visible only upon longer exposure. Lane c, same as lane b, but multiple copies integrated. Lane d, DNA from tobacco with multiple integrations of polyubiquitin containing six repeat units. For phenotype of such plants, see Figure 4k, left plant, and text. Lane e, DNA from tobacco containing several copies of hexaubiquitin R-48. For phenotype, see Figure 4k, right plant, Figure 4i and text. Lane f, same as lane d, but different transformant. (**B**) Northern analysis of selected plants. The probe used consists of ubiquitin coding sequence, so that endogenous ubiquitin transcripts are weakly cross-hybridizing. 12 μg of total RNA were used in each lane. Lane a, diubiquitin mRNA from plants (Southern hybridization shown in panel A, lane b). Lane b, RNA from untransformed tobacco. Lane c, RNA from plant expressing hexaubiquitin (Southern hybridization shown in panel A, lane d). Lane d, RNA from a plant expressing hexaubiquitin R-48 (Southern hybridization shown in panel A, lane e).

in *Escherichia coli* recA$^-$ strains, we observed specific recombination events upon introduction of binary T-DNA vectors into *Agrobacterium tumefaciens*. Recombination occurs exclusively in the ubiquitin repeats, and other parts of the vector, such as the CaMV 35S-derived promoter, are not altered. For instance, prolonged growth of these plasmids in *Agrobacterium* leads to precise deletion of all but one repeat unit, which was confirmed by sequence analysis.

As a result, the genes integrated into the tobacco genome contain a smaller number of repeats (Figure 2) and were in the size range of natural ubiquitin genes which usually have between five and eight repeat units. Figure 2 shows that multiple copies of polyubiquitin genes of the same size integrated into the plant genome, again suggesting that recombination did occur in *Agrobacterium* prior to T-DNA transfer, and not in the plant. In separate experiments, we also introduced monoubiquitin and monoubiquitin R-48 into tobacco.

Figure 1B shows schematically the rationale of these experiments. Expression of ubiquitin R-48 is expected to lead to partial inhibition of ubiquitin dependent protein degradation without perturbing other ubiquitin dependent processes which involve addition of single ubiquitin moieties.

Expression of variant ubiquitin causes increased sensitivity to an amino acid analogue

Transgenic calli resistant to the selectable marker kanamycin were investigated by placing them onto agar medium containing L-canavanine. Canavanyl peptides are subject to rapid proteolytic destruction by the ubiquitin system (Ciechanover *et al.*, 1984; Finley *et al.*, 1984). Figure 4b (right plate with L-canavanine) shows that calli expressing polyubiquitin R-48 are inhibited in their growth by L-canavanine, while calli expressing either wild type polyubiquitin (Figure 4a), or *E.coli* β-glucuronidase (Figure 4c), are not. In contrast, without addition of L-canavanine, calli expressing polyubiquitin R-48 grow as well as, or even better than, the control calli (Figure 4a–c, left plates). The observed sensitivity to L-canavanine is consistent with a specific inhibition of ubiquitin dependent protein degradation, and yeast mutants in ubiquitin dependent proteolysis share this sensitivity to amino acid analogues (Finley *et al.*, 1987; Seufert and Jentsch, 1990).

The level of free ubiquitin is not significantly altered in transgenic plant cells

Analysis of the ubiquitin content by Western blotting (Figure 3) shows that expression of additional ubiquitin genes

does not significantly alter the level of free ubiquitin in leaf cells. High levels of transgene mRNA do not lead to poor transcription of endogenous ubiquitin genes (see Figures 2B and 5B). Therefore, negative interference between newly introduced and endogenous mRNA, which has been observed for chalcone synthase (Napoli *et al.*, 1990; van der Krol *et al.*, 1990), is not operating in our case. Furthermore, the transgenes are clearly biologically active, as judged by their effect on plant shape (see below) and the increased sensitivity to an amino acid analogue.

Western analysis of higher molecular weight proteins does not show appearance of prominent new bands, thus ruling out major accumulation of ubiquitin conjugates or unprocessed polyubiquitin (data not shown). However, there may be less prominent, but physiologically relevant changes which cannot be detected by our current Western protocol.

We conclude that plant cells maintain roughly constant levels of free ubiquitin, and the ubiquitin variant forms one common pool with its wild type counterpart. Most likely, an increased synthesis of ubiquitin in the transgenic plants leads to more rapid turnover of the ubiquitin pool.

Expression of the monoubiquitin variant in plants leads to decreased internode growth

Many of the calli obtained gave rise to shoots capable of forming roots. We chose plants derived from nine of the calli expressing monoubiquitin R-48 to observe the effect of a ubiquitin R-48 transgene on plant growth and development. Staining of leaf sections which express the marker gene β-glucuronidase driven by our CaMV 35S-derived promoter shows that this promoter ensures a high level of gene expression in leaf mesophyll cells and in the part of the midrib which contains the vascular tissue (Figure 4d). Therefore, we expected that possible alterations caused by our transgenes could be observed particularly well in these tissues.

Figure 4e shows a comparison of a plant expressing monoubiquitin R-48 (right) with a plant expressing wild type ubiquitin. The white bars indicate the length of one internode. The smaller size of ubiquitin R-48 expressing plants appears to be due to decreased growth of internodes. Such plants do not seem to have thinner stems or a reduced leaf area. In contrast, plants expressing β-glucuronidase, or wild type ubiquitin, are normal in size.

One interpretation of this result is that plants expressing the proteolysis inhibitor ubiquitin R-48 have difficulties in forming vascular tissue, a major component of the stem and a type of tissue which requires extensive degradation of intracellular proteins during differentiation (for review, see Woolhouse, 1984). To observe possibly more pronounced phenotypic alterations, we decided to investigate plants expressing polyubiquitin R-48.

Plants expressing polyubiquitin R-48 show leaf curling, stunting and develop necrotic lesions

For analysis, we chose plants derived from six independent calli transformed with polyubiquitin R-48, and one transformed with wild type polyubiquitin (for Southern and Northern data of some of these plants, see Figure 2).

The most obvious phenotypic difference between plants expressing polyubiquitin R-48 and those expressing wild type polyubiquitin is a distorted growth of leaves in the former plants. Figure 4(f and i) shows that, in many cases, leaves

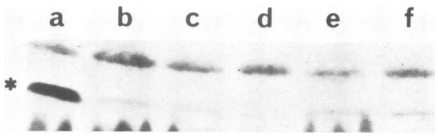

Fig. 3. The levels of free ubiquitin are not significantly altered in the transgenic plants. Western analysis using anti-ubiquitin antibodies was carried out as described in Materials and methods. The asterisk denotes the band of free ubiquitin. (**a**) Cell extract from leaf cells of a plant expressing β-glucuronidase, plus human ubiquitin as a marker. (**b**) Same as (a), but without exogenously added ubiquitin. (**c**) Extract derived from leaf cells of a plant expressing a diubiquitin transgene (for Southern analysis, see Figure 2A, lane c; for phenotype, see Figure 4e, left plant). (**d**) Same as (c), but plant expresses a hexaubiquitin transgene (for Southern and Northern analysis, see Figure 2A, lane d and Figure 2B, lane c, respectively; for phenotype, see Figure 4k, left plant, and text). (**e**) Same as (c), but plant expresses hexaubiquitin R-48 (for Southern analysis, see Figure 2A, lane f). (**f**) Same as (c), but plant expresses monoubiquitin R-48 (for phenotype, see Figure 4e, right plant).

roll downwards to form almost a tube. In all these cases, growth of the midrib and side veins is apparently inhibited compared with that of mesophyll cells. This growth imbalance seems to be the primary reason for the distorted leaf shape.

In order to observe possible alterations in vascular tissue, we looked at sections of leaf midribs. Figure 4j compares sections through the midrib of a plant transformed with polyubiquitin R-48 (top) with those from a plant transformed with β-glucuronidase (bottom) which is indistinguishable from our wild type polyubiquitin control. The larger fraction of the cross-sectional area occupied by vascular tissue in the polyubiquitin R-48 plant is consistent with our interpretation that changes in vascular tissue are the primary cause for the altered leaf shape. For instance, the plant might partially compensate suboptimal formation or functioning of vascular tissue by committing more cells to this differentiation pathway. Furthermore, the brown colour visible on the abnormal vascular tissue, which develops upon exposure of sections to the air, is indicative of the increased presence of phenolic compounds.

We also see an effect on internode length. Figure 4g shows a comparison of a plant expressing β-glucuronidase (left side) with one expressing polyubiquitin R-48 (right side). A very drastic case of slow internode growth is shown in Figure 4h.

In addition to changes that can be attributed to altered characteristics of vascular tissue, we see another phenotype in plants expressing polyubiquitin R-48: after some time of growth in sterile glass jars, plants expressing polyubiquitin R-48 develop discrete necrotic lesions on their leaves (Figure 4l and m). In more severe cases, the lesions coalesce later on and destroy the entire leaf (Figure 4k, right plant). Such plants do not survive in the greenhouse. The severity of this phenotype is dependent on the expression level of the transgene. Different plants derived from the same callus show an almost identical severity of necrosis (data not shown). Under the high humidity conditions of sterile culture, the necrotic spots usually have a completely round shape (Figure 4l and m), whereas those observed in the greenhouse frequently develop around minor leaf veins which gives them a somewhat irregular appearance (Figure 4n). This trait (as well as the one described before) is inheritable and cosegregates with the kanamycin resistance gene of the T-DNA vector (data not shown).

Fig. 4. (**a**) Calli transformed with a polyubiquitin gene. Left plate: growth on standard medium, right plate: growth on medium containing 3.3 mg/l L-canavanine. (**b**) Same as panel (a), but calli transformed with a polyubiquitin R-48 gene. (**c**) Same as panel (a), but calli transformed with a bacterial β-glucuronidase gene. Note the brown colour and poor growth of most of the calli on the right plate of panel b. (**d**) Tissue section of a leaf

Figure 4k shows a plant expressing wild type polyubiquitin on the left side, and a polyubiquitin R-48 expressing plant on the right side (for Southern and Northern data, see Figure 2A, lanes d and e, and Figure 2B, lanes c and d). Interestingly, while plants expressing a wild type polyubiquitin transgene do have normal vascular tissue and are growing normally in the greenhouse, they have a slight tendency to develop necrotic lesions when grown in glass jars for a prolonged period of time. The simplest interpretation of this finding is that several different perturbations of the ubiquitin system result in a program of induced necrosis. For further discussion of this striking result, see below.

Increased synthesis of ubiquitin suppresses the leaf curling caused by polyubiquitin R-48

The model shown in Figure 1B implies that expression of additional wild type ubiquitin genes should mitigate or even suppress alterations that occur via proteolysis inhibition. Therefore, we crossed a plant carrying a polyubiquitin R-48 gene with a plant which expresses a wild type diubiquitin transgene. The result of this experiment is shown in Figure 5A. The curved leaves are from plants expressing polyubiquitin R-48 only (for representative Northern data, see Figure 5B, lane a). The two flat leaves are from plants expressing both a polyubiquitin R-48, and a wild type diubiquitin gene (Northern data shown in Figure 5B, lane b). This result is consistent with the interpretation that leaf curling is a specific consequence of the Lys to Arg change in the ubiquitin variant.

Discussion

In this work, we looked at developmental abnormalities caused by perturbation of the ubiquitin system in the plant *Nicotiana tabacum*. The ubiquitin system has been shown to be essential for eukaryotic cells in general and is known to fulfill a set of housekeeping functions (Ciechanover *et al.*, 1984; Finley *et al.*, 1987, 1989; Jentsch *et al.*, 1987; Goebl *et al.*, 1988; Redman and Rechsteiner, 1989; Seufert and Jentsch, 1990).

We were particulary interested in ubiquitin dependent proteolysis which is involved in degradation of abnormal and naturally short-lived proteins in every cell. Protein turnover, however, also plays a very specific role during some differentiation processes which require extensive changes in cellular protein composition. In plants, for instance, formation of vascular tissue involves degradation of organelles and other cell components. Xylem differentiation leads to formation of structures derived from cell walls and devoid of any intracellular material (for reviews, see Woolhouse, 1984; Aloni, 1987).

To assess a possible contribution of ubiquitin dependent proteolysis to differentiation events, we expressed *in vivo* a ubiquitin variant in which Lys at position 48 was replaced by Arg. This protein has been shown previously to act as an inhibitor of ubiquitin dependent protein degradation in an *in vitro* system (Chau *et al.*, 1989). We see differences between the vascular tissue of plants expressing the ubiquitin variant, and control plants (see Figure 4j). In addition, plants expressing the inhibitor have an abnormal leaf shape and decreased internode growth.

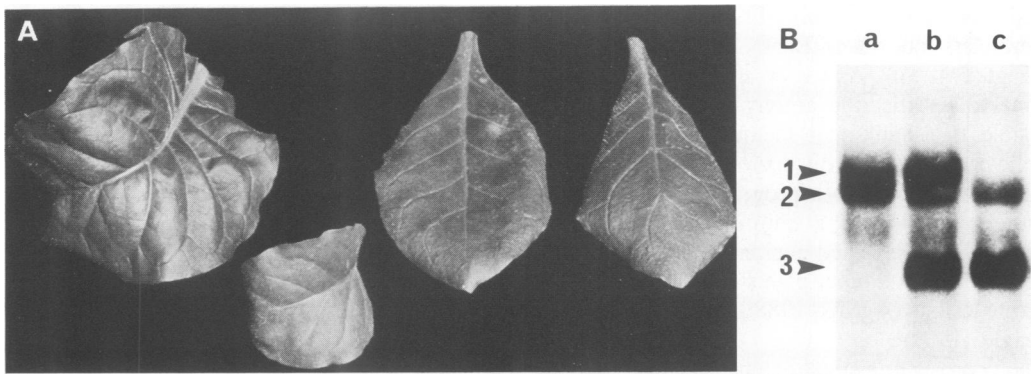

Fig. 5. (**A**) Comparison of leaves from plants expressing hexaubiquitin R-48 (the two curved leaves on the left side) with those expressing both hexaubiquitin R-48 and an additional wild type diubiquitin gene (the two flat leaves on the right side). For interpretation of this result, see text. (**B**) Representative Northern data of plants expressing a hexaubiquitin R-48 transgene (lane **a**), both hexaubiquitin R-48 and wild type diubiquitin (lane **b**), and a wild type diubiquitin transgene (lane **c**). Band 1 is hexaubiquitin R-48 transgene mRNA, band 2 is the major crossreacting endogenous ubiquitin mRNA, and band 3 is diubiquitin transgene mRNA (see also Figure 2B).

expressing β-glucuronidase after staining with X-gluc. (**e**) Comparison of a plant expressing high levels of wild type ubiquitin (left) and a plant expressing monoubiquitin R-48 (right). Note the difference in internodal distances between the two plants, marked by the white bars. (**f**) Leaf of a plant expressing polyubiquitin R-48 at an early stage of growth in a sterile beaker, showing the curling of the leaf blade. (**g**) Comparison of a plant expressing β-glucuronidase (left) with a plant expressing polyubiquitin R-48 (right). Note the bending of the leaves and the decreased size. (**h**) Plant expressing high levels of polyubiquitin R-48. Note stunting and altered leaf shape, with the leaf blade curving up between the side veins. (**i**) Same as panel f, but plant derived from a different callus. (**j**) Tissue section through the midribs of a curved leaf from a plant expressing polyubiquitin R-48 (top) and a leaf expressing β-glucuronidase (bottom). The sections were made through a part close to the stem (left side), and near the leaf tip (right). Note the large fraction of the cross-sectional area occupied by vascular tissue in the top sections as compared with the bottom sections. (**k**) Comparison of a plant expressing polyubiquitin (left; for Southern and Northern data, cf. Figure 2A, lane d and Figure 2B, lane c, respectively) with a plant expressing polyubiquitin R-48 (right; for Southern and Northern data, cf. Figure 2A, lane e and Figure 2B, lane d, respectively). (**l**) Leaf of a plant expressing polyubiquitin R-48, grown in a sterile beaker. (**m**) Same as (l) leaf still attached to the plant. (**n**) Grafting of a W38 tobacco shoot expressing polyubiquitin R-48 onto a SR1 wild type plant. Arrowhead: junction between graft and host. SR1 leaves are lanceolate (see left branch emerging from host plant), whereas W38 leaves are normally broad and flat (see panel g, left plant). Note the curling and the necrotic areas on the graft, but not on the host.

Decreased internodal distances have also been observed in plants expressing a high level of the light regulator protein phytochrome (Keller *et al.*, 1989). Because phytochrome turnover is probably a ubiquitin dependent process (Jabben *et al.*, 1989), altered levels of active form of phytochrome (Pfr) might be present in our plants. This could influence internode length (Ballaré *et al.*, 1990). However, our plants do not have the abnormally dark leaf colour seen on phytochrome overexpressing plants (Keller *et al.*, 1989), and we observe additional phenotypic changes (see above).

We think that another effect is largely responsible for the alterations we observe. Partial inhibition of ubiquitin dependent protein degradation might lead to difficulties in the formation or maintenance of a tissue that depends on this process to a particularly great extent, while other cell types not as sensitive are largely unaffected. Thus, because of decreased proteolytic capacity, our transgenic plants cannot form vascular tissue with wild type efficiency. This leads to slower growth of plant parts containing vascular tissue such as stem and leaf midrib. Consistent with this interpretation, we find that increased expression of wild type ubiquitin can suppress the leaf curling caused by a polyubiquitin R-48 transgene (see Figure 5). This result is also in accordance with the model shown in Figure 1B.

As a second phenotype, we find that high-level expression of variant or, to a much lesser extent, wild type polyubiquitin, induces formation of necrotic lesions. One of the surprising aspects is that the plants affected show a discontinuous reaction in response to a continuous perturbation. All the available data concerning CaMV 35S-derived promoters (cf. Benfey and Chua, 1989; Stanford *et al.*, 1990), as well as our own data, indicate that gene expression from 35S-derived promoters is uniform throughout the leaf mesophyll. Nevertheless, our plants usually develop discrete necrotic spots (Figure 4l–n). As we do not know of any relevant functional diversification among leaf mesophyll cells, it is possible that additional factors such as, for instance, metabolic parameters, or fluctuations in sensitivity, influence the initiation of the necrosis programme which we observe. A discrete reaction to a uniform stimulus is, however, not entirely unprecedented in plants. For instance, ozone damage on tobacco and other plants is manifested as a set of tiny discrete lesions (Agrios, 1988; for reviews, see Rich, 1964; Heagle, 1989).

Because we see necrosis to a small extent with wild type polyubiquitin, as well, the model depicted in Figure 1B may not be applicable to the induced necrosis phenotype. While mechanistic aspects are, at present, unclear, we note that the ubiquitin system is a pivotal component of the eukaryotic cellular stress response (Finley *et al.*, 1984; Bond and Schlesinger, 1985). Therefore, overproduction of polyubiquitin might perturb certain aspects of the cellular stress response. In our experiments, we observe how the plant as a whole copes with this perturbation. Could our results be relevant for other cases of induced necrosis? We believe that some environmental stress factors, or production of deleterious gene products in plants, might induce cellular stress response and cause necrosis via the pathway uncovered by our studies. Further experiments will show whether our transgenic plants, 'pre-stressed' by polyubiquitin overexpression, do react differently to adverse environmental influences or to attack by fungal, bacterial, or viral pathogens.

We observe necrosis on plants expressing polyubiquitin, but not on those expressing monoubiquitin or diubiquitin so that unprocessed polyubiquitin might play a causative role (in the case of polyubiquitin R-48, the phenomenon is more pronounced, but qualitatively similar). However, we cannot rule out that still higher expression of monoubiquitin might lead to a necrosis phenotype as observed on the polyubiquitin expressing plants. In any case, further investigation of the plants obtained might offer insights into the way plants translate intracellular perturbations into a reaction involving the whole organism.

Materials and methods

Strains and plasmids

The plasmid pHUB was a gift from Dr D.Ecker, SK & F, King of Prussia, USA, pRT 103 (Töpfer *et al.*, 1987) and pRT 104 GUS were gifts from Dr R.Töpfer, MPI Köln, pPCV 702 (Koncz *et al.*, 1989) and *A.tumefaciens* GV 3101 pMP90RK (Koncz and Schell, 1986) were obtained from Dr C.Koncz, MPI Köln.

Construction of plasmids

Using synthetic double-stranded oligonucleotides, an artificial human ubiquitin gene (Ecker *et al.*, 1987) was changed by replacing the *Eco*RI–*Bgl*II, the *Xba*I–*Bsm*I, the *Xho*I–*Sal*I and the *Afl*II–*Kpn*I fragments to create the plant ubiquitin reading frame GAATTCTGCG CAG ATC TTC GTC AAG ACG TTA ACC GGT AAA ACC ATA ACT CTA GAG GTT GAG TCT TCA GAC ACC ATC GAC AAC GTT AAG GCT AAA ATC CAG GAC AAG GAA GGC ATT CCA CCT GAT CAA CAA AGA TTG ATC TTT GCC GGT AAG CAG CTC GAG GAC GGT AGA ACT CTT GCT GAC TAC AAT ATT CAG AAG GAG TCG ACC TTA CAT CTT GTC TTA AGA CTT AGA GGT GGG ATA TCT TGAGGTACC. Site-directed mutagenesis (Bio-Rad Kit) was used to change codon 48 (AAG) to AGA and codon 50 (CTC) to CTT (a silent change that destroys the *Xho*I site and thus allows for easy distinction between wild type and R-48 ubiquitin). The reading frame contains an *Fsp*I site at the beginning and an *Eco*RV site at the end. Ligation of an *Fsp*I–*Kpn*I fragment into an *Eco*RV–*Kpn*I digested pUC based plasmid containing the above insert results in tandem duplication of the ubiquitin reading frame. Analogously, two dimers can be assembled into a tetramer etc. A polyprotein gene with 32 ubiquitin repeats, and one with 32 ubiquitin R-48 repeats, was constructed. Together with oligonucleotide

T CGAGCTGCAGAATTACTATTTACAATTACAAT
CGACGTCTTAATGATAAATGTTAATGTTA

fragments with 32 repeat units, excised from the vector using *Fsp*I and *Kpn*I, were ligated into pRT 103 digested with *Xho*I and *Kpn*I to give plasmids pRTUB32 and pRTRV32, respectively. The oligonucleotide contains the 20 nucleotides in front of the start codon of tobacco mosaic virus strain U1 (Gallie *et al.*, 1987). Vector pPCV 702 was modified by replacing the part between the *Hin*dIII site and the *Eco*RV site in the CaMV 35S promoter by a *Hin*dIII linker. After digestion of this vector (pΔHV) with *Hin*dIII, *Hin*dIII fragments from pRTUB32 and pRTRV32 were inserted such that the CaMV enhancer of the vector is adjacent to the CaMV 35S promoter of the insert. This arrangement has been shown to ensure high expression levels of transcripts (Kay *et al.*, 1987; Fang *et al.*, 1989). These vectors were called pΔUB32 and pΔRV32, respectively. Analogously, a *Hin*dIII fragment from pRT 104 GUS containing the *E.coli* β-glucuronidase gene from pRAJ 275 (Jefferson, 1987) inserted between the *Nco*I and the *Eco*RI sites of pRT 104 (Töpfer *et al.*, 1987) was ligated into vector pΔHV. pΔRV1, a plasmid analogous to pΔRV32, but with only one repeat, was made by propagation of pΔRV32 in *A.tumefaciens*. Subsequent restriction analysis and sequencing of the remaining ubiquitin R-48 reading frame confirmed the precise deletion of all but one repeat unit.

All cloning steps involving oligonucleotides were confirmed by subsequent sequencing of the region encompassing the oligonucleotide (Pharmacia T7 sequencing kit; Ausubel *et al.*, 1987).

Plant transformation

Plasmids pΔUB32, pΔRV32, pΔGUS and pΔRV1 were transferred to *A.tumefaciens* GV 3101 pMP90RK via transformation by a freeze–thaw procedure (Holsters *et al.*, 1978). In the case of pΔUB32 and pΔRV32, colonies were scraped off the plates after 3 days and directly used for leaf disc transformation.

Leaf pieces from *N.tabacum* cv. W 38, grown in sterile glass beakers on agar medium (4.6 g/l Murashige and Skoog salts, 30 g/l sucrose, 9 g/l agar, 0.1 g/l myoinositol, 0.5 g/l MES, 10 mg/l thiamine, 1 mg/l nicotinic acid, 1 mg/l pyridoxin and 1 mg/l biotin pH 5.7) were used for transformation as described by Horsch *et al.* (1985), with minor modifications.

Callus propagation

Calli were grown on agar medium (see above) plus 1 mg/l naphthylacetic acid, 0.2 mg/l kinetin, 50 mg/l kanamycin and 200 mg/l claforan. For experiments involving L-canavanine, 3.3 mg/l L-canavanine were added to the above medium.

Hybridizations

Southern and Northern experiments were carried out using Nylon membranes (Amersham Hybond) according to Sambrook *et al.* (1989). Probe DNA was prepared by random prime labelling (Boehringer Mannheim Random Primed DNA Labeling Kit). RNA was prepared by a guanidium thiocyanate/CsCl cushion centrifugation method (Sambrook *et al.*, 1989) using leaf material pulverized in liquid nitrogen. DNA was prepared according to Draper *et al.* (1988).

Tissue staining

Staining of tissue sections to detect β-glucuronidase activity was done according to Jefferson (1987).

Western blotting

Sample preparation was carried out as described (Ferguson *et al.*, 1990) using green leaf tissue. Samples were analysed on a gradient gel (10–18%). A strip containing the region around 8 kd was equilibrated in transfer buffer and transferred onto a PVDF membrane (Millipore Immobilon P) using a semi-dry procedure according to the manufacturer's recommendations (Bio-Rad Trans-Blot SD). After transfer (20 V, 20 min), the membrane was blocked in 20% newborn calf serum (Flow Laboratories) for 1 h. Incubation with rabbit anti-ubiquitin antibody (1 to 100 dilution) was carried out overnight at 4°C. Incubation with secondary antibody (sheep anti-rabbit, ^{35}S-labelled; Amersham plc.) was carried out at room temperature for 5 h. Between the steps, the filter was incubated with buffer (150 mM NaCl, 50 mM Tris pH 8, 0.05% Tween 20) for 40 min (three buffer changes).

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