Synthetic Biology

DOI: 10.1002/anie.201303477

Expanding the Genetic Code for Photoclick Chemistry in *E. coli*, Mammalian Cells, and *A. thaliana***

*Fahui Li, Hua Zhang, Yun Sun, Yanchao Pan, Juanzuo Zhou, and Jiangyun Wang**

Bioorthogonal chemical reactions together with techniques to expand the genetic code have provided exciting new means for protein labeling and visualization in living systems,[1] as well as for optimizing the efficacy of therapeutic proteins.[2] Toward these goals, amino acids with small bioorthogonal functional groups, such as azide, alkyne, or cyclopropene moieties,[3] as well as larger reactive bioorthogonal groups,[4] such as cyclooctyne, norbornene, *trans*-cyclooctene, aryltetrazole, or aryltetrazine, have been site-specifically incorporated into proteins, allowing for selective conjugation of biophysical probes through azide–alkyne click chemistry (AAC), tetrazole–alkene photoclick chemistry (TAP), and reverse-electron demand Diels–Alder reactions.[5] The main advantages of the photoclick reaction (Supporting Information, Scheme S1) are: 1) its fast rate (up to $50\,M^{-1}s^{-1}$); 2) that spatiotemporal control is initiated by a photo-induced reaction; 3) that the photoclick reaction is fluorogenic, allowing for high-contrast fluorescence imaging without tedious washing steps. In previous studies, we reported the site-specific incorporation of *p*-(2-tetrazole)phenylalanine (*p*-Tpa)[4a] and *N*-ε-(1-methylcycloprop-2-enecarboxamido)-lysine (CpK)[3c] in *E. coli* and mammalian cells. Subsequent photoirradiation of labeled proteins with UV light facilitates selective conjugation with dimethyl fumarate or diaryltetrazole, respectively.

By expanding the genetic code and introducing photoclick chemistry to plants, important problems in plant chemical biology can be addressed,[6] such as photosynthesis and stress response, which can only be studied at the organismal level. Recently, expansion of the genetic code has been used to optimize therapeutic proteins produced in bacteria and mammalian cells.[2] Because plants offer an attractive alternative to microbial fermentation and animal cell cultures for high-yield production of recombinant proteins on an agricultural scale,[8] expanding the genetic code in plants would be useful for producing recombinant therapeutic proteins and enzymes with enhanced properties, better safety, and lower costs.[8]

To fully realize the potential of photoclick reaction for tracking fast cellular processes, it is desirable that the unnatural amino acid (UAA) used has a small functional group such that there is minimal perturbation of the target protein, and a very brief exposure to long-wavelength UV light or violet-blue light is used to drive the photoclick reaction to minimize damage to cells and plants. Herein, we addressed these issues by genetically incorporating *N*-ε-acryllysine (AcrK, Figure 1 A), in response to an amber stop codon (TAG) in bacterial cells, mammalian cells, and plants. This new strategy was then used to efficiently label proteins both in vitro and in vivo. In comparison to lysine, AcrK has only four extra non-hydrogen atoms, which is significantly less than other UAAs.[3,4] Replacing one lysine residue with AcrK should cause only minimal perturbation to the target protein. In addition, the electron-withdrawing amido group should activate the terminal alkene group to achieve a higher photoclick reaction rate. Indeed, the photoclick reaction between tetrazole and acrylamide was found to proceed nearly one hundred times faster than that of allylphenylether[9] and 1.5 times faster than that of cyclopropene.[3c] AcrK was synthesized by reacting *N*-α-Boc-lysine with acryloyl chloride in a basic ethyl acetate/water solution at 0 °C (Figure 1 A), followed by deprotection with HCl gas with an overall yield of 72%, without the need for metal catalysts. AcrK was found to be relatively stable in the presence of glutathione, an abundant biomolecule both amine and thiol groups; greater than 95% of AcrK remained following incubation with 5 mM reduced glutathione in a buffer at pH 7 for 24 hours (Figure S1). At neutral pH, most primary amines are protonated and most thiols are neutral. Therefore, glutathione could react slowly with the acrylamido group through a Michael addition reaction. While AcrK has comparable stability to CpK under physiological conditions, the synthetic route for CpK requires six steps, expensive heavy metal catalysis, with an overall yield of 15%.[3c] Because a large amount of the UAA is required for modifying plant proteins on an agricultural scale, it is essential that the UAA is synthesized in an economically, without the use of toxic heavy-metal catalysts.

We chose diaryltetrazole **2** (Figure 1 B) because it is highly reactive for photoclick reactions[9] and is soluble in water. Because the molar extinction coefficients of **2** at 365 nm and 405 nm are around $100\,M^{-1}cm^{-1}$ (Figure S2) and the quantum yields for the photolysis of diaryltetrazoles are very high (0.5–0.9),[5f] **2** should be efficiently activated by long-wavelength UV light or violet light. An amine functional group provides a convenient handle for further derivatization. Upon irradi-

[*] F. H. Li,[+] H. Zhang,[+] Y. Sun,[+] Y. C. Pan,[+] J. Z. Zhou, Prof. Dr. J. Y. Wang
Laboratory of Non-coding RNA, Institute of Biophysics
Chinese Academy of Sciences
15 Datun Road, Chaoyang District, Beijing, 100101 (China)
E-mail: jwang@ibp.ac.cn

[+] These authors contributed equally to this work.

[**] We gratefully acknowledge the Major State Basic Research Program of China (2010CB912301, 2009CB825505), the National Science Foundation of China (90913022, 31000364), and CAS grant (KSZD-EW-Z-003). We thank Prof. Peter Schultz for providing the pEVOL vector and Dr. Xiang Ding for help with mass spectrometry.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201303477.

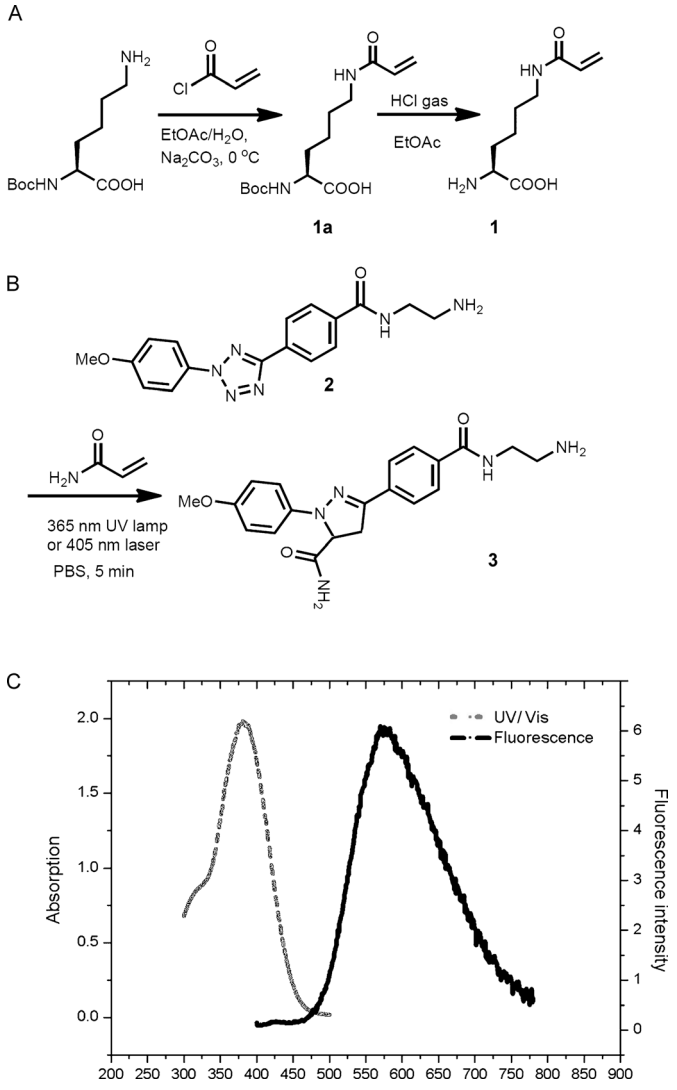

Figure 1. A) Synthesis of AcrK **1**. B) Photoclick reaction of diaryltetrazole **2** with acrylamide yields the fluorescent pyrazoline product **3** under 365 nm UV irradiation in PBS buffer. C) Absorption and emission spectra of pyrzoline **3** (pH 7.4, 100 mM sodium phosphate buffer). Extinction coefficient = 15 000 at 380 nm with a quantum yield of 0.27.

ation with 365 nm UV light (lamp: UVP TFML-20, 36 W), **2** reacted with acrylamide to generate a fluorescent pyrazoline product **3** with absorption maxima at 360 nm and a broad emission band at 400–700 nm (Figure 1C), which matches well with the DAPI filter setting ($\lambda_{ex} = 365$ nm, $\lambda_{em} = 445 \pm 25$ nm).

To evolve an orthogonal tRNA/aminoacyl-tRNA synthetase pair that selectively charges AcrK in response to a TAG amber codon, we performed three rounds of positive and two rounds of negative selections with an MbPylRS library, as previously described.[3c] An AcrK-specific PylRS mutant, termed AcrKRS, was identified. Sequencing of this clone revealed the following five mutations: D76G, L266M, L270I, Y271F, L274A, and C313F. To test the efficiency and selectively of AcrKRS, we first ligated AcrKRS sequentially into SalI/BglII and Pst1/Nde1 restriction sites into the pEVOL vector[10] and $MbtRNA_{CUA}^{Pyl}$ (pylT) into an ApaL1/Xho1 restriction site to generate the pEVOL-AcrK vector (Figure S3). An amber stop codon was substituted for Tyr151 in green fluorescent protein (GFP) that contained a C-terminal six-histidine tag, affording the pET22b-GFP151TAG vector. GFP expression was then carried out in the presence of AcrKRS and $MbtRNA_{CUA}^{Pyl}$ in *E. coli* grown in LB media supplemented with 1 mM AcrK. As a negative control, protein was also expressed in the absence of AcrK. Analysis of the purified GFP by SDS-PAGE showed that full length GFP was expressed only in the presence of AcrK (Figure 2A). The yield of the mutant GFP was 20 mg L^{-1}. In comparison, the yield of wild-type GFP under similar conditions was 50 mg L^{-1}. To the best of our knowledge, this is the highest UAG codon suppression efficiency (40%) reported for UAA incorporation using the PylRS/$MbtRNA_{CUA}^{Pyl}$ pair. ESI-MS analysis of the mutant GFP showed an average mass of 27 728 Da, consistent with the calculated mass of 27 728 Da (Figure 1B). No peaks corresponding to incorporation of any of the 20 natural amino acids into the Tyr151 site of GFP were observed. A small peak at 28 035 Da was also found, consistent with the formation of a Michael addition adduct with glutathione (molecular weight = 307 Da). The relative signal intensities of the 28 035 Da peak versus the 27 728 Da peak indicates that less than 5% of the modified GFP reacted with glutathione. To assess whether AcrK can serve as a bioorthogonal reporter for protein labeling, we incubated myoglobin-4-AcrK with tetrazole **3** in PBS buffer and irradiated the sample at 365 nm for ten minutes. MS-MS analysis of the product mixture revealed that the AcrK at the fourth position was selectively and completely converted to the pyrazoline product (Figures S4, S5).

To confirm whether AcrK can be used as a reactive bioorthogonal group in living cells, we used a tubulin-like bacterial cytoskeleton protein FtsZ as a model. Assembly of FtsZ into ring structures is required for bacterial division and small-molecule inhibitors of FtsZ have been shown to be promising antibiotics.[11,12] We chose to incorporate **1** near the N-terminus of FtsZ, at the third position, because this was likely to cause the minimum perturbation to FtsZ assembly and function.[12] An amber codon was substituted for the third position of FtsZ, which also contained a C-terminal six-histidine tag, affording the pET22b-FtsZ3TAG vector. Using this and the pEVOL-AcrK vector, full length mutant FtsZ protein with AcrK site-specifically incorporated at the third position (FtsZ-3-AcrK) was expressed with a yield of 20 mg mL^{-1}. As a control, we also expressed wild-type (wt) FtsZ, which yielded 50 mg mL^{-1}. Both wt FtsZ and FtsZ-3-AcrK were then purified using Ni-NTA chromatography. Wt FtsZ or FtsZ-3-AcrK (10 μM) were then incubated with 100 μM diaryltetrazole **2** in PBS buffer and irradiated at 365 nm for five minutes. Because the pyrazoline product is fluorescent, we could easily follow this reaction using in-gel fluorescence. A fluorescent FtsZ band was observed for FtsZ-3-AcrK but not for wt FtsZ, and fluorescence was observed for FtsZ-3-AcrK only after UV irradiation (Figure 2C). Therefore, the photoclick reaction only occurred between tetrazole **2** and AcrK but not the natural amino acids. Photoclick reactions were then run under similar conditions,

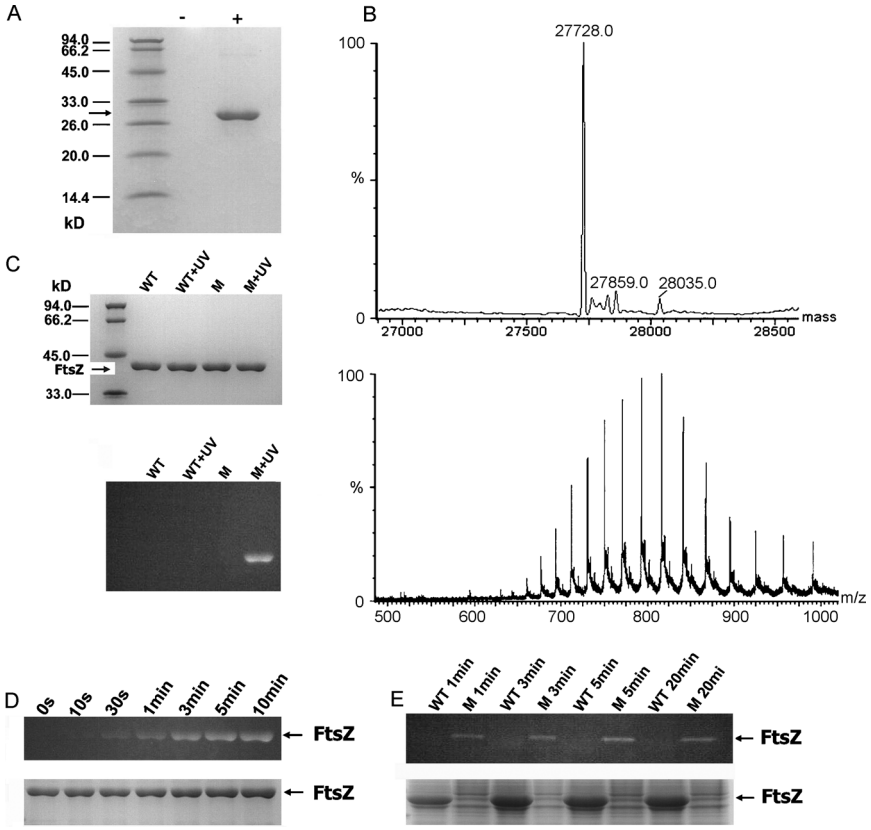

Figure 2. A) Coomassie Blue-stained SDS-PAGE of GFP-151-AcrK (arrow) expressed in the presence (+) and absence (−) of 1 mM AcrK. B) ESI-MS spectra of GFP-151-AcrK. Top: deconvoluted spectrum. Expected mass = 27 728 Da, found 27 728 Da. Bottom: orginal mass spectra. C) Coomassie Blue-stained SDS-PAGE (top) and in-gel fluorescence image (bottom) of purified wt FtsZ (WT) and FtsZ-3-AcrK (M), with or withoug 365 nm irradiation for 5 min in the presence of 100 mM **2**. D) In-gel fluorescence image (top) and SDS-PAGE (bottom) of FtsZ-3-AcrK following 365 nm irradiation for 0–10 min in the presence of 100 mM **2**. E) In-gel fluorescence image (top) and SDS-PAGE (bottom) of crude cell lysates (WT = cells expressing wt FtsZ; M = cells expressing FtsZ-3-AcrK), following 365 nm irradiation for 1–20 min in the presence of 100 mM **2**.

with 365 nm or 405 nm UV irradiation for 0–10 minutes. Only 30 seconds after UV irradiation, significant labeled FtsZ has already formed as shown by in-gel fluorescence (Figure 2D), and the reaction was complete within five minutes. Using 405 nm laser irradiation, the reaction was also complete within five minutes (Figure S6).

To further test the utility of the photoclick reaction for protein labeling inside living cells, we incubated *E. coli* cells expressing either wt FtsZ or FtsZ-3-AcrK with 100 μM tetrazole **2**, followed by irradiation at 365 nm for 1–20 minutes. The cells were then lysed and proteins were analyzed by both SDS-PAGE and in-gel fluorescence. UV irradiation for one minute was sufficient to observe the photoclick labeling of FtsZ in vivo (Figure 2E). As expected, the photoclick reaction occurred selectively only in cells expressing FtsZ-3-AcrK, but not wt FtsZ (Figure 2E). This indicates that there was no mis-incorporation of AcrK into wild-type FtsZ or other cellular proteins in *E. coli*.

We then tested if this new method could be applied to live cells for fluorescent imaging. BL21 (DE3) cells expressing either wt FtsZ or FtsZ-3-AcrK were incubated with 100 μM diaryltetrazole **2** in PBS buffer for 30 minutes at 37 °C and then irradiated at 365 nm for one minute before fluorescence imaging using a DAPI channel. The fluorescent signal was only detected in cells expressing FtsZ-3-AcrK, but not wt FtsZ (Figure S7). Thus, our strategy to genetically incorporate **1** into proteins followed by a photoclick reaction was found to be suitable for imaging in live cells. Because this method requires the mutation of only one amino acid and diaryltetrazole **2** has a molecular weight of only 338 Da, this method has minimal perturbation on the native proteins for visualization in living cells.

Because the pyrrolysyl tRNA synthetase/tRNA pair is orthogonal in both bacterial and mammalian cells,[1] we tested if the AcrKRS and $MbtRNA_{CUA}^{Pyl}$ pair could facilitate the genetic incorporation of **1** into mammalian cells. We cloned AcrKRS into the pCMV-NBK-1 vector,[4f] generating a pCMV-AcrKRS vector, where the expression of AcrKRS is driven by the CMV promoter and the transcription of $MbtRNA_{CUA}^{Pyl}$ is under the control of a human U6 promoter. The plasmids pCMV-AcrKRS and pSwan-EGFP37-TAG were cotransfected into Chinese hamster ovary (CHO) cells. The cells were then grown in the presence and absence of 1 mM **1** for 36 hours. EGFP fluorescence was observed only for cells grown in the presence of **1**, but not in its absence (Figure S8), indicating that **1** was introduced into the 37th position of EGFP through amber codon suppression in mammalian cells using the AcrKRS/$MbtRNA_{CUA}^{Pyl}$ pair.

Because many important plant processes, such as photosynthesis, light sensing, and growth control, can only be studied at the organismal level, we tested whether unnatural amino acids could be introduced into the genetic code of the plant model organism *Arabidopsis thaliana*. We chose *A. thaliana* because it is the first plant species to have its genome sequenced, transformation of DNA into *A. thaliana* using *Agrobacterium* is routine, high accumulation of therapeutic antibodies (35% of the total soluble protein) in *A. thaliana* seeds has been achieved,[8] and it is a model organism for plant biology much like *Caenorhabditis elegans* and *Drosophila melanogaster* are for animal biology (expansion of the genetic code in these two species was recently reported)[13].

We first constructed a shuttle vector pARABZH-AcrKRS that can replicate in *Agrobacterium* and deliver genes into plant hosts. In this vector, AcrKRS expression was driven by the CaMV 35S promoter, $MbtRNA_{CUA}^{Pyl}$ transcription was driven by the 7L4 promoter, followed by the

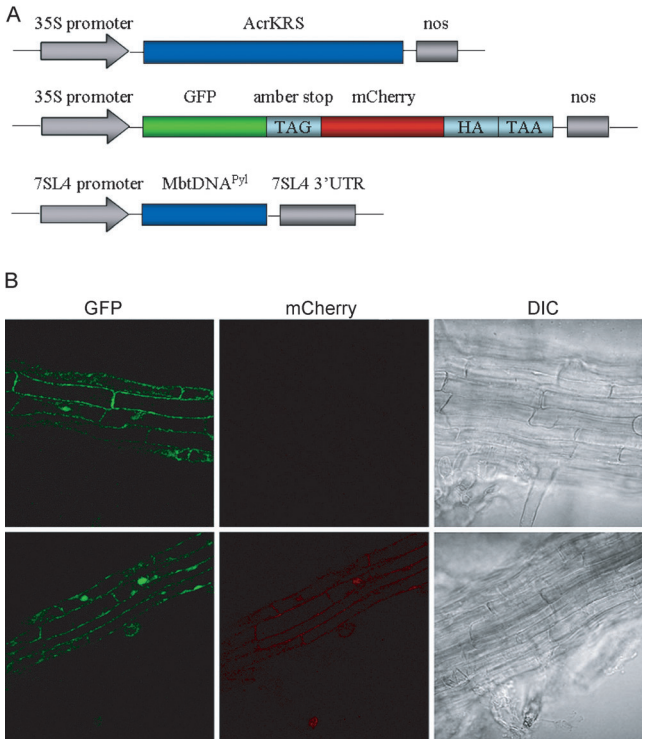

Figure 3. A) DNA constructs for expanding the genetic code of *A. thaliana*. B) Fluorescence images of *A. thaliana* expressing $MbtRNA_{CUA}^{Pyl}$, AcrKRS, and GFP-TAG-mCherry-HA in the absence (top) or presence (bottom) of 5 mM AcrK. DIC = differential interference contrast microscopy.

7L4-3′UTR,[14] expression of the reporter GFP-TAG-mCherry-HA was driven by the CaMV 35S promoter (Figure 3 A). The 35S promoter drives strong expression of AcrKRS and GFP in the plant, the HA tag allows for convenient detection of fusion protein by anti-HA antibodies. We chose the 7L4 promoter to drive $MbtRNA_{CUA}^{Pyl}$ expression, because it is highly expressed in plants.[14] If the TAG amber codon is efficiently suppressed, then full-length GFP-mCherry fusion protein should be expressed. For efficient amber codon read-through, it is necessary to minimize nonsense-mediated mRNA decay (NMD), a surveillance mechanism which eliminates mRNAs which contain premature termination codons (PTC). Previous studies showed that the UP-Frameshift (UPF) protein UPF1 is essential for normal NMD function in *A. thaliana*,[15] therefore we chose a *upf1-5* mutant lacking the UPF1 gene for these experiments.

The plasmid pARABZH-AcrKRS was first transformed into *Agrobacterium tumefaciens* GV3101 competent cells using electroporation. *A. thaliana upf1-5* mutants were then transformed by dipping their flowers into a broth of *Agrobacterium* carrying the pARABZH-AcrKRS plasmid. The T2 generation positive transgenic plants were screened on solid MS medium containing 50 $\mu g\,mL^{-1}$ hygromycin B, and one line (*upf1-5-4*) was chosen for further investigation. The roots of two-week *upf1-5-4* seedlings were used for GFP and mCherry fluorescence visualization under a confocal microscope. In the absence of AcrK, green fluorescence, but not red fluorescence, was observed (Figure 3 B), indicating that full-length GFP-mCherry-HA fusion protein was not expressed. No full-length GFP-mCherry-HA fusion protein was expressed in the absence of AcrKRS or $MbtRNA_{CUA}^{Pyl}$ (Figure S10). These results indicate that no endogenous tRNA synthetase can charge $MbtRNA_{CUA}^{Pyl}$ with any amino acids. By contrast, red fluorescence was observed in the presence of 5 mM AcrK, indicating that the TAG amber codon was efficiently suppressed, resulting in GFP-mCherry-HA fusion protein expression (Figure 3 B).

To further demonstrate that AcrK was selectively incorporated in response to the amber codon and its ability to direct the photoclick reaction to a specific site in a protein, we lysed seedlings grown in the presence or absence of 5 mM AcrK, extracted the total protein, and performed western-blots. Anti-HA western blots confirmed AcrK-dependent production of GFP-mCherry-HA in seedlings (Figure S9). The cell lysates were then reacted with 100 μM biotin-tetrazole under 365 nm UV irradiation for ten minutes. The products were then probed with streptavidin. Our results show that the GFP-mCherry-HA fusion protein purified from seedlings grown in the presence of AcrK was selectively labeled with biotin-tetrazole through light-induced photoclick reaction (Figure S9). No biotin labeling was observed in the absence of AcrK. As a control, we also expressed GFP-mCherry-HA using a vector where the TAG amber codon was mutated back to a sense codon, TCG, in the presence of AcrK. We found that GFP-mCherry-HA expressed under these conditions did not react with biotin-tetrazole and therefore did not produce a positive streptavidin signal (Figure S9). These results indicate that AcrK was not incorporated into protein in response to a sense codon. Taken together, the fluorescence imaging and western blot results demonstrate that AcrKRS and $MbtRNA_{CUA}^{Pyl}$ direct the site-specific incorporation of AcrK into proteins in response to an UAG codon in *A. thaliana*.

In summary, we have demonstrated the site-specific incorporation of AcrK into proteins in bacterial cells, mammalian cells, and plants and the use of AcrK as a bioorthogonal handle for directing rapid fluorescent labeling and ligation of chemical probes to the target protein. The site-specific, photoclick protein labeling method described herein has the following advantages: 1) it is spatiotemporally controllable; 2) it causes less cellular damage through the use of 365 nm UV or 405 nm violet light; 3) AcrK introduces minimal perturbation to the target proteins; 4) its fast rate allows for efficient bioconjugation; 5) AcrK can be easily synthesized in two steps in bulk quantities using cheap starting materials, without the need for metal catalysts. This new method has great potential for investigating proteins that assemble into large complexes, such as the cytoskeleton or nucleosome, in which the introduction of a GFP fusion is likely to perturb their functions. Importantly, because *Agrobacterium* is broadly applicable for plant genetic engineering and crop improvement, our new method should be useful for producing therapeutic proteins or enzymes with unnatural amino acids at a specific site in well-establish plant hosts such as tobacco, on an agricultural scale using only simple nutrients, an inexpensive unnatural amino acid, water, and sunlight.[7,8] Subsequent bioorthogonal chemical ligations

would allow for the large-scale production of proteins with enhanced properties.

Received: April 24, 2013
Revised: June 24, 2013
Published online: July 19, 2013

Keywords: *Arabidopsis thaliana* · click chemistry · bioorthogonal groups · protein expression · unnatural amino acids

[1] a) L. Wang, A. Brock, B. Herberich, P. G. Schultz, *Science* **2001**, *292*, 498 – 500; b) C. C. Liu, P. G. Schultz, *Annu. Rev. Biochem.* **2010**, *79*, 413 – 444; c) L. Davis, J. W. Chin, *Nat. Rev. Mol. Cell Biol.* **2012**, *13*, 168 – 182; d) E. M. Sletten, C. R. Bertozzi, *Angew. Chem.* **2009**, *121*, 7108 – 7133; *Angew. Chem. Int. Ed.* **2009**, *48*, 6974 – 6998.

[2] a) J. Y. Axup, K. M. Bajjuri, M. Ritland, B. M. Hutchins, C. H. Kim, S. A. Kazane, R. Halder, J. S. Forsyth, A. F. Santidrian, K. Stafin, Y. C. Lu, H. Tran, A. J. Seller, S. L. Biroce, A. Szydlik, J. K. Pinkstaff, F. Tian, S. C. Sinha, B. Felding-Habermann, V. V. Smider, P. G. Schultz, *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 16101 – 16106; b) H. Cho, T. Daniel, Y. J. Buechler, D. C. Litzinger, Z. W. Maio, A. M. H. Putnam, V. S. Kraynov, B. C. Sim, S. Bussell, T. Javahishvili, S. Kaphle, G. Viramontes, M. Ong, S. Chu, G. C. Becky, R. Lieu, N. Knudsen, P. Castiglioni, T. C. Norman, D. W. Axelrod, A. R. Hoffman, P. G. Schultz, R. D. DiMarchi, B. E. Kimmel, *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 9060 – 9065.

[3] a) D. P. Nguyen, H. Lusic, H. Neumann, P. B. Kapadnis, A. Deiters, J. W. Chin, *J. Am. Chem. Soc.* **2009**, *131*, 8720 – 8721; b) Z. Y. Hao, Y. Q. Song, S. X. Lin, M. Y. Yang, Y. J. Liang, J. Wang, P. R. Chen, *Chem. Commun.* **2011**, *47*, 4502 – 4504; c) Z. P. Yu, Y. C. Pan, Z. Y. Wang, J. Y. Wang, Q. Lin, *Angew. Chem.* **2012**, *124*, 10752 – 10756; *Angew. Chem. Int. Ed.* **2012**, *51*, 10600 – 10604.

[4] a) J. Y. Wang, W. Zhang, W. J. Song, Y. Z. Wang, Z. P. Yu, J. S. Li, M. H. Wu, L. Wang, J. Y. Zang, Q. Lin, *J. Am. Chem. Soc.* **2010**, *132*, 14812 – 14818; b) T. Plass, S. Milles, C. Koehler, C. Schultz, E. A. Lemke, *Angew. Chem.* **2011**, *123*, 3964 – 3967; *Angew. Chem. Int. Ed.* **2011**, *50*, 3878 – 3881; c) T. Plass, S. Milles, C. Koehler, J. Szymanski, R. Mueller, M. Wiessler, C. Schultz, E. A. Lemke, *Angew. Chem.* **2012**, *124*, 4242 – 4246; *Angew. Chem. Int. Ed.* **2012**, *51*, 4166 – 4170; d) S. Milles, S. Tyagi, N. Banterle, C. Koehler, V. VanDelinder, T. Plass, A. P. Neal, E. A. Lemke, *J. Am. Chem. Soc.* **2012**, *134*, 5187 – 5195; e) K. Lang, L. Davis, S. Wallace, M. Mahesh, D. J. Cox, M. L. Blackman, J. M. Fox, J. W. Chin, *J. Am. Chem. Soc.* **2012**, *134*, 10317 – 10320; f) K. Lang, L. Davis, J. Torres-Kolbus, C. J. Chou, A. Deiters, J. W. Chin, *Nat. Chem.* **2012**, *4*, 298 – 304; g) E. Kaya, M. Vrabel, C. Deiml, S. Prill, V. S. Fluxa, T. Carell, *Angew. Chem.* **2012**, *124*, 4542 – 4545; *Angew. Chem. Int. Ed.* **2012**, *51*, 4466 – 4469; h) J. L. Seitchik, J. C. Peeler, M. T. Taylor, M. L. Blackman, T. W. Rhoads, R. B. Cooley, C. Refakis, J. M. Fox, R. A. Mehl, *J. Am. Chem. Soc.* **2012**, *134*, 2898 – 2901.

[5] a) R. K. V. Lim, Q. Lin, *Acc. Chem. Res.* **2011**, *44*, 828 – 840; b) J. C. Jewett, C. R. Bertozzi, *Chem. Soc. Rev.* **2010**, *39*, 1272 – 1279; c) Y. X. Chen, G. Triola, H. Waldmann, *Acc. Chem. Res.* **2011**, *44*, 762 – 773; d) M. F. Debets, S. S. Van Berkel, J. Dommerholt, A. J. Dirks, F. Rutjes, F. L. Van Delft, *Acc. Chem. Res.* **2011**, *44*, 805 – 815; e) D. S. Liu, A. Tangpeerachaikul, R. Selvaraj, M. T. Taylor, J. M. Fox, A. Y. Ting, *J. Am. Chem. Soc.* **2012**, *134*, 792 – 795; f) Y. Z. Wang, W. J. Wu, W. J. Song, R. K. V. Lim, Q. Lin, *Org. Lett.* **2008**, *10*, 3725 – 3728.

[6] G. R. Hicks, N. V. Raikhel, *Nat. Chem. Biol.* **2009**, *5*, 268 – 272.

[7] R. Fischer, E. Stoger, S. Schillberg, P. Christou, R. M. Twyman, *Curr. Opin. Plant Biol.* **2004**, *7*, 152 – 158.

[8] a) G. De Jaeger, S. J. Scheffer, A. M. Zambre, O. Zobell, A. Goossens, A. Depicker, G. Angenon, *Nat. Biotechnol.* **2002**, *20*, 1265 – 1268; b) H. G. Goojani, M. J. Javaran, J. Nasiri, E. G. Goojani, H. Alizadeh, *Appl. Biochem. Biotechnol.* **2013**, *169*, 1940 – 1951; c) J. F. Xu, M. C. Dolan, G. Medrano, C. L. Cramer, P. J. Weathers, *Biotechnol. Adv.* **2012**, *30*, 1171 – 1184.

[9] Y. Z. Wang, W. J. Song, W. J. Hu, Q. Lin, *Angew. Chem.* **2009**, *121*, 5434 – 5437; *Angew. Chem. Int. Ed.* **2009**, *48*, 5330 – 5333.

[10] T. S. Young, I. Ahmad, J. A. Yin, P. G. Schultz, *J. Mol. Biol.* **2010**, *395*, 361 – 374.

[11] D. J. Haydon, N. R. Stokes, R. Ure, G. Galbraith, J. M. Bennett, D. R. Brown, P. J. Baker, V. V. Barynin, D. W. Rice, S. E. Sedelnikova, J. R. Heal, J. M. Sheridan, S. T. Aiwale, P. K. Chauhan, A. Srivastava, A. Taneja, I. Collins, J. Errington, L. G. Czaplewski, *Science* **2008**, *321*, 1673 – 1675.

[12] W. Margolin, *Nat. Rev. Mol. Cell Biol.* **2005**, *6*, 862 – 871.

[13] a) S. Greiss, J. W. Chin, *J. Am. Chem. Soc.* **2011**, *133*, 14196 – 14199; b) A. Bianco, F. M. Townsley, S. Greiss, K. Lang, J. W. Chin, *Nat. Chem. Biol.* **2012**, *8*, 748 – 750.

[14] S. Lu, R. Shi, C. C. Tsao, X. Yi, L. Li, V. L. Chiang, *Nucleic Acids Res.* **2004**, *32*, e171, 1 – 7.

[15] a) Y. Kurihara, A. Matsui, K. Hanada, M. Kawashima, J. Ishida, T. Morosawa, M. Tanaka, E. Kaminuma, Y. Mochizuki, A. Matsushima, T. Toyoda, K. Shinozaki, M. Seki, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 2453 – 2458; b) L. Arciga-Reyes, L. Wootton, M. Kieffer, B. Davies, *Plant J.* **2006**, *47*, 480 – 489.

